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# ProteoSOL™ Tissue Extraction System

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For the extraction of proteins and peptides from formalin-fixed tissue for immunodetection analysis.

**Catalog No. 554-00-02**

**10 Applications**

**Store at -20°C**



This product is manufactured and sold under a license from Expression Pathology, Inc., Gaithersburg, MD, and incorporates Expression Pathology's proprietary *Liquid Tissue*® reagents for extraction of biomolecules from formalin-fixed paraffin-embedded tissue.

Limited Use Restrictions:

FOR RESEARCH USE ONLY – Not intended for animal or human therapeutic use.

Other uses may be restricted under the terms of sale. Refer to the Limited Use Statement on the following page.



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### **Patents**

The process for making *Liquid Tissue*<sup>®</sup> preparations is patent pending in the United States and the rest of the world.

### **Trademarks**

Liquid Tissue is a registered trademark of Expression Pathology, Inc. ProteoSOL and Protein Detector are trademarks of KPL.

### **Disclaimer**

The recommendations of this bulletin are provided solely for the benefit of users who need practical guidance on the extraction of proteins and peptides from formalin fixed tissue for immunodetection analysis. Because experimental conditions for the use of the suggested products are beyond the control of KPL, it is impossible for KPL to implicitly guarantee the performance of the mentioned products for any and all assay procedures. Users who need additional information or technical support should call Technical Services at 800/638-3167 or 301/948-7755 for assistance.

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## Introduction/Background

The ProteoSOL™ Tissue Extraction System uses a patent-pending process owned by Expression Pathology, Inc., Gaithersburg, MD, to extract proteins directly from formalin-fixed paraffin-embedded (FFPE) tissue samples, using *Liquid Tissue*® reagents. For the first time, this technology enables relative quantitative analysis of FFPE tissue samples in membrane-based immunoassays.

While it is difficult to measure individual proteins because of the proteomic complexity of cells, the ability to measure protein expression levels is inherently valuable because of the direct relationship between protein expression and biological function. Both fresh frozen tissue and formalin fixed archival tissue can be used for proteomic analysis. While fresh frozen tissues are difficult to obtain in large numbers, expensive to store, and difficult to process, there are large collections of well-defined formalin fixed archival tissues. Until now, routine analysis of proteins in FFPE was limited to immunohistochemistry (IHC) techniques because the chemical cross-links formed during formalin fixation made it impossible to extract usable soluble proteins. Samples prepared with *Liquid Tissue*® reagents overcome this problem and enable the proteomic analysis of proteins from formalin fixed tissue.

The ProteoSOL™ Tissue Extraction System has been optimized for FFPE tissue to be analyzed by membrane-based immunodetection methods. There are many advantages to using this method for scientific studies. Frequently, FFPE tissues have a good history with documented pathology and clinical outcomes, including disease progression and drug response. This technology combines the strength of histology, including visualization of normal and pathologic tissue morphology, with the ease and relative quantitation abilities of membrane-based technology. Samples processed with this system are arrayed and detected on a membrane with high-throughput detection methods. Immunodetection with specific antibodies allows the direct comparison of a specific protein across multiple samples in a single array. Finally, *Liquid Tissue*® preparations are large enough to make multiple arrays for separately analyzing multiple proteins from the same tissue sample for protein profiling.

## Kit Information

Recommended Storage Conditions: -20°C

<b>Chemical Hazard</b>	Handle with care, always wear gloves and follow standard safety precautions.		
<b>Kit Contents</b>	The solutions in this kit should be clear. They should not be used when precipitates are present. The components must be stored at -20°C when not in use. Buffer A is stable at room temperature but can also be stored at -20°C. Buffer B should be placed on ice when being used.		
	<b>Component</b>	<b>Quantity</b>	<b>Quantity</b>
	ProteoSOL™ Buffer A	1 tube	200 µL
	ProteoSOL™ Buffer B	1 tube	*Reconstitute to 20 µL
	Sample Preparation Tube, 1.5 mL low binding	10 tubes	
	Product Manual	1	

**\*Please read the special handling instructions for the reconstitution of Buffer B on page 14 (*Liquid Tissue*® Preparation section) prior to use.**

## **Additional Equipment and Materials Required**

Oven, 58°C to 60°C  
Heating Block, 95°C  
Waterbath, 37°C  
Microcentrifuge  
Vortex Mixer  
Microtome  
200 µL Pipettor  
10 µL Pipettor  
Disposal gloves  
Plastic Slide Jars  
Forceps  
Timer  
Xylene or Xylene Substitute  
100% Ethanol  
Water, double distilled or other high purity grade  
Dilute Alkaline Solution  
Mayer's Hematoxylin Solution  
Eosin  
Microscope Slides and Coverslips  
Permanent Mounting Medium  
30 Gauge Needle  
Syringe  
Dissecting Microscope or Magnifying Glass  
Microarrayer Apparatus  
Nitrocellulose membranes for detection (0.1 – 0.2 µm pore size)  
0.02% Bromophenol blue in 20 mM Tris-HCl, pH 7.8  
TBS, pH 9.5  
Reagents for immunodetection

## Procedures

### Note: 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 standard glass 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 aims 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 processing tissue into *Liquid Tissue*<sup>®</sup> preparations, 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.

### Tissue Evaluation

It is important to evaluate tissues by microscopic analysis 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 mL of solution can be processed before the solutions should be changed.

#### 1. 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 a standard glass microscope slide.
- b. Label 13 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 fresh solutions.
- d. Place the slide(s) to be processed in a 58° to 60°C oven for 30 to 60 minutes to melt the paraffin and allow the tissue to physically contact the slide.
- e. Deparaffinization and Rehydration
  - 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 slide(s) in the plastic slide jar containing Xylene #1 for 5 minutes.
  - (2) Transfer the slide(s) to the plastic slide jar containing Xylene #2 for 5 minutes.
  - (3) Transfer the slide(s) to the plastic slide jar containing 100% Ethanol #1 for 5 minutes.
  - (4) Transfer the slide(s) to the plastic slide containing 100% Ethanol #2 for 5 minutes.
  - (5) Transfer the slide(s) to the plastic slide jar containing 85% Ethanol for 1 minute.
  - (6) Transfer the slide(s) to the plastic slide jar containing 70% Ethanol for 1 minute.
  - (7) Transfer the slide(s) to the plastic slide jar containing Distilled Water for 1 minute.

f. Hematoxylin Stain

- (1) 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 minutes. Staining time may be varied for individual color preference.
- (2) Rinse the slide in Distilled Water for 30 seconds.
- (3) 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).
- (4) Rinse the slide in fresh Distilled Water for 30 seconds.
- (5) Rinse the slide in 95% Ethanol #1 for 30 seconds.

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

h. Evaluate the tissue section on a microscope to locate the cells to be collected for processing. The cells can be identified for reference by circling the area with a marker on the slide.

i. A standard *Liquid Tissue*<sup>®</sup> preparation has been optimized for approximately 60,000 cells. There are approximately 382,000 cells per cubic millimeter of tissue and approximately 60,000 cells are contained in a volume of 0.16 cubic millimeters.

j. 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 following chart to determine the approximate number of cells being collected.

Chart of Areas

<b>Section Thickness</b>	<b>Area Containing Approximately 60,000 cells</b>	<b>Perimeter Containing Approximately 60,000 cells</b>	<b>Circle Diameter Containing Approximately 60,000 cells</b>
<b>5 microns</b>	<b>32 mm<sup>2</sup></b>	<b>6.0 x 5.5 mm</b>	<b>6.4 mm</b>
<b>7 microns</b>	<b>24 mm<sup>2</sup></b>	<b>6.0 x 4.0 mm</b>	<b>5.5 mm</b>
<b>10 microns</b>	<b>16 mm<sup>2</sup></b>	<b>4.0 x 4.0 mm</b>	<b>4.5 mm</b>
<b>15 microns</b>	<b>10.5 mm<sup>2</sup></b>	<b>3.5 x 3.0 mm</b>	<b>3.6 mm</b>

### **Cell Procurement**

- 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.
- After H&E staining, cut a section(s) of the formalin fixed, paraffin embedded tissue on a microtome at 5 to 15 microns in thickness and place on untreated, standard glass microscope slides.
- The appropriate subset of cells can be isolated by needle dissection, laser capture microdissection (LCM) or PALM<sup>®</sup> laser microdissection (see individual protocols below).
- There may be some cases where the tissue is not dissected but the entire section is taken for processing if all of the cells are appropriate for collecting.

#### **1. Needle Dissection Protocol**

- This procedure should be practiced to become familiar with the technique before it is attempted with precious samples.
- A tissue section from the same tissue block, previously stained with H&E, can be used as a guide to align the unstained section when selecting which cells to collect.

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 minutes 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 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 minutes.

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

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

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

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

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

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

e. Sample Collection

- (1) For each 60,000 cells collected, 20  $\mu$ l of ProteoSOL™ Buffer A is required. Thaw Buffer A and mix briefly to ensure the solution is uniform. Pipet the required amount into a 1.5 ml low-binding Sample Preparation Tube and close the tube.
- (2) Place a new 30 gauge needle on a syringe.
- (3) Remove the slide from the distilled water container and use a Kimwipe 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.
- (4) Place the slide on a dissecting microscope or visualize under a magnifying glass. Use the areas on the H&E stained slide to direct the location of the cells for needle dissection.
- (5) Gently scrape the areas of the tissue to be removed with the needle.
- (6) 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.
- (7) Collection of an initial tissue fragment on the tip of the needle will assist in collecting more tissue. The tip of the needle with the clumped tissue fragments should be carefully placed into the appropriately labeled Sample Preparation Tube containing Buffer A.
- (8) Gently shaking the needle will ensure the tissue detaches from the tip of the needle into the buffer.
- (9) DO NOT aspirate the sample into the needle to avoid losing a portion of it.
- (10) Microcentrifuge the tube(s) containing the cells in Buffer A at 10,000 rpm for 1 minute.

## 2. Laser Capture Microdissection (LCM) Protocol

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

b. Microdissection

(1) 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

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

(3) Place the slides to be processed in a 58–60°C oven for 30 to 60 minutes to melt the paraffin.

(4) 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 a 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.

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

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

- (c) Transfer the slides to the plastic slide jar containing 100% Ethanol #1 for 5 minutes.
  - (d) Transfer the slides to the plastic slide jar containing 85% Ethanol #1 for 1 minute.
  - (e) Transfer the slides to the plastic slide jar containing 70% Ethanol #1 for 1 minute.
  - (f) Transfer the slides to the plastic slide jar containing water for 1 minute.
  - (g) Apply Mayer's Hematoxylin Solution to the tissue section so that it covers the entire section.
  - (h) Stain for 1 minute to 5 minutes at room temperature. Staining time may vary depending on tissue type.
  - (i) Tap off excess stain before proceeding with the following steps.
  - (j) Rinse slide in double distilled or other high purity grade water for 30 seconds.
  - (k) Place slide in fresh double distilled or other high purity grade water for 30 seconds.
  - (l) Transfer the slides to plastic slide jar containing 70% Ethanol #2 for 1 minute.
  - (m) Transfer the slides to plastic slide jar containing 85% Ethanol #2 for 1 minute.
  - (n) Transfer the slides to plastic slide jar containing 100% Ethanol #2 for 1 minute.
  - (o) Transfer the slides to plastic slide jar containing Xylene #3 for 1 minute.
  - (p) Air dry.
- (5) Sample Collection

- (a) Perform laser capture microdissection. Consult the User Guide for the instrument you will use for detailed instructions.
- (b) For each 60,000 cells collected, 20  $\mu$ l of Buffer A is required. Thaw Buffer A and mix briefly to ensure the solution is uniform. Pipet the required amount into a 1.5 ml low-binding Sample Preparation Tube and close the tube.
- (c) Transfer the LCM captured cells to a Sample Preparation Tube as follows:
  - i. Wearing gloves and working in a clean area, place the LCM cap with the cells up on a flat surface.
  - 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 Sample Preparation Tube.
  - iii. Carefully transfer the film into the appropriately labeled Sample Preparation Tube containing ProteoSOL™ Buffer A 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 ProteoSOL™ Buffer A at 10,000 rpm for 1 minute.

### ***Liquid Tissue***® Preparation

**Important: There are special handling instructions for the reconstitution of Buffer B. Please read prior to the use of Buffer B:**

**(A). Tap or spin down the pellet in the Buffer B vial.**

**(B). Handle the vial carefully to avoid disrupting the pellet. Peel off the label for better visualization and to verify that the pellet is located at the bottom of the vial. The pellet is semi-transparent.**

**(C). In order to avoid losing the pellet, open the seal VERY SLOWLY to release the pressure. Please confirm that your pellet is still at the bottom of the vial.**

**(D). Add 20  $\mu$ l of water to reconstitute Buffer B. Mix the solution gently to dissolve the pellet completely. Store at -20°C. Place the vial on ice during use.**

1. Heat the Sample Preparation Tube(s) containing the procured cells in the Buffer A solution in a heating block at 95°C for 90 minutes. Make sure that the cap is on securely to help prevent evaporation.
2. Every 20 minutes, 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 seconds at 10,000 rpm. 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 minutes at 95°C, microcentrifuge the tube(s) at 10,000 rpm for 1 minute.
  - If the sample was isolated by LCM, 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 minutes.
5. Thaw Buffer B. Mix the vial briefly to ensure the solution is uniform. Place vial on ice. The solution is stable for at least 5 freeze thaw cycles.
6. Carefully open each Sample Preparation Tube and add 1.0  $\mu$ l of Buffer B to each reaction tube (20  $\mu$ l volume). Mix and briefly microcentrifuge to collect the solution at the bottom of the Sample Preparation Tube.
7. Incubate the Sample Preparation Tube(s) in a waterbath at 37°C for one hour. Every 20 minutes, remove the tubes, and vortex

vigorously for 10 to 15 seconds. 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. At the end of the incubation, microcentrifuge the tube(s) at 10,000 rpm for 1 minute. A small pellet may be visible; this depends on the nature of your sample.
9. Heat the reaction tube(s) at 95°C for 5 minutes. Ensure that the cap is closed tightly to prevent evaporation.
10. Microcentrifuge the tube(s) at 10,000 rpm for 1 minute. Note the presence and size of any pellet.
11. Store the *Liquid Tissue*<sup>®</sup> preparation at -20°C until ready for analysis.

### **Membrane-based Immunodetection**

This protocol is designed to spot three separately prepared FFPE samples onto nitrocellulose membrane using a microarrayer. It includes the appropriate experimental controls and describes the detection of a specific biomarker.

#### **1. Preparation and Spotting of the *Liquid Tissue*<sup>®</sup> Preparations**

- a. Process the FFPE tissues using the ProteoSOL Tissue Extraction System reagents to generate *Liquid Tissue*<sup>®</sup> preparations as described in the previous sections.
- b. Dilute each sample with an equal volume of 0.02% bromophenol blue (in 20 mM Tris-HCl, pH 7.8) solution to obtain a 2-fold diluted sample. Bromophenol blue is used to visualize the samples during the spotting procedure. For example, 7.5 µl of sample plus 7.5 µl of 0.02% bromophenol blue would yield 15 µl of a 2-fold diluted sample.
- c. A commercially available purified biomarker antigen may be used as a positive control. Dilute the antigen to 0.01 mg/ml in the bromophenol blue solution. For a negative control, a 0.5 mg/ml dilution of BSA in bromophenol blue solution can be used.

d. Perform a serial dilution of the *Liquid Tissue*<sup>®</sup> sample. One example of serially diluting the *Liquid Tissue*<sup>®</sup> is described as follows:

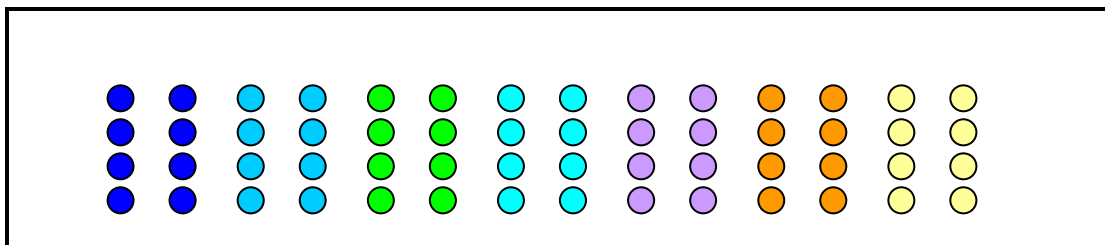
- (1). Pipet 15 µl of the 2-fold diluted *Liquid Tissue*<sup>®</sup> preparation 1 (prepared as described in step 1b) to cell A1 of a 384 well microplate (see diagram below). Repeat the same procedure for *Liquid Tissue*<sup>®</sup> preparation 2, *Liquid Tissue*<sup>®</sup> preparation 3, negative control, BSA and purified biomarker antigen in corresponding wells A2-A6.
- (2). Add 7.5 µl of bromophenol blue buffer to wells A7, B1-B7, C1-C7 and D1-D7.
- (3). Aliquot 7.5 µl of preparation 1 from well A1 to well B1 to create a 1:4 dilution of *Liquid Tissue*<sup>®</sup> preparation 1 in well B1. Repeat the same procedure for *Liquid Tissue*<sup>®</sup> preparation 2 (from A2 to B2), *Liquid Tissue*<sup>®</sup> preparation 3 (from A3 to B3), negative control (from A4 to B4), BSA (from A5 to B5) and purified biomarker antigen (from A6 to B6).
- (4). Repeat the same procedure as described above to create 1:8 and 1:16 dilutions in row C and row D of the microplate for all samples and controls.

	1	2	3	4	5	6	7
(1:2) A	●	●	●	●	●	●	●
(1:4) B	●	●	●	●	●	●	●
(1:8) C	●	●	●	●	●	●	●
(1:16) D	●	●	●	●	●	●	●

1	●	<i>Liquid Tissue</i> <sup>®</sup> preparation 1
2	●	<i>Liquid Tissue</i> <sup>®</sup> preparation 2
3	●	<i>Liquid Tissue</i> <sup>®</sup> preparation 3
4	●	<i>Liquid Tissue</i> <sup>®</sup> preparation – no tissue (negative control)
5	●	BSA (negative control)
6	●	Purified biomarker antigen (positive control)
7	●	Bromophenol blue solution (dilution buffer)

- e. Spot the samples onto a nitrocellulose membrane. In this example, the dilutions are spotted in duplicate (using a manual microarrayer from V&P Scientific) onto a 0.1  $\mu\text{m}$  pore size nitrocellulose membrane (Protran – Schleicher and Schuell). The nitrocellulose membrane is 1 $\frac{3}{4}$ " long x 7/8" wide. The approximate volume of one spot is 0.05  $\mu\text{l}$  per dot application. KPL recommends four applications of the samples for a total spot volume of 0.2  $\mu\text{l}$ .

Spotted membrane:



- f. The samples should also be spotted onto a second membrane, which will be used as a negative control for the detection reagents (i.e. no primary antibody).
- g. The samples should be also spotted onto a third membrane, which will be used to determine total protein levels. KPL recommends using the Memcode protocol (Pierce); refer to the manufacturer's instructions for this protocol.

## 2. Immunodetection of the Membranes

### a. Colorimetric Detection (Protein Detector™ Microarray Dot Blot Kit, AP Colorimetric – Cat. #56-11-50)

- (1) Rinse the membranes in TBS, pH 7.5 (with shaking) for approximately 5 minutes to remove the bromophenol blue buffer.
- (2) Prepare 10 ml of Block Solution: 0.5% (w/v) Microarray Blocking Powder (Cat. #56-00-01) in 1X Microarray Blocking Solution (Cat. #56-00-02):
  - i. Measure out 9.5 ml water.

- ii. Add 0.05 g of Microarray Blocking Powder.
  - iii. Vortex and allow it to dissolve
  - iv. Add 500  $\mu$ l of 20X Microarray Blocking Solution to the solution. Vortex to mix (note: Microarray Blocking Powder will dissolve more readily in water than in Microarray Blocking Solution).
- (3) Place membranes in a suitable dedicated container.  
NOTE: KPL recommends using a separate dedicated container for each step—for example, primary antibody incubations should be done in a container designated for the primary antibody. KPL recommends a Petri dish as a conveniently-sized container.
- (4) Incubate each membrane in 2 ml of Block Solution for 30 minutes at room temperature.
- (5) Dilute the primary antibody into fresh Block Solution. The dilution factor for the antibody will depend on the antibody used; follow the manufacturer's recommendations where possible.
- (6) Drain the Block Solution from the membrane and place the membrane in a fresh container for primary antibody incubation. Incubate the membrane in 1 ml of diluted primary antibody for 30 minutes at room temperature. For the second (negative control) membrane, add 1 ml of Block Solution (no primary antibody) in place of the diluted primary antibody. Incubate the negative control membrane in separate container.
- (7) Prepare 1X Wash Solution (need ~60 ml per membrane). Dilute 6 ml of 20X Wash Solution (Cat. #50-63-01) into 114 ml of water.
- (8) Drain the primary antibody or Block solution from the membrane and place the membrane in a dedicated container. Add at least 5 ml of the 1X Wash Solution to the membrane. Swirl the solution briefly over the membrane and decant the solution. Add fresh Wash Solution to the membrane and shake the container for 5 minutes at room temperature. Repeat this step with two additional aliquots of Wash Solution for 5 minutes

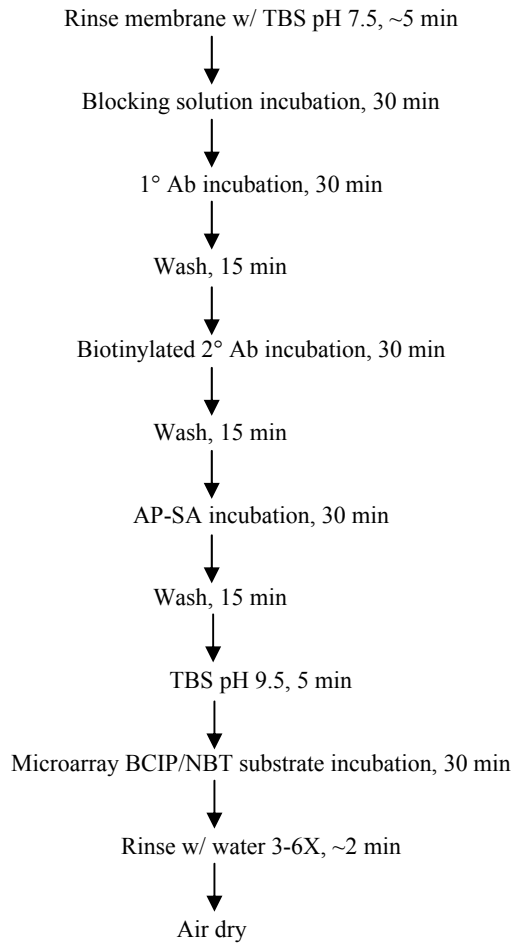
each. (Note: To ensure that the wash solution is free-flowing over the membrane more than 5 ml wash solution can be added.) Wash the negative control membrane in a separate container.

- (9) Reconstitute biotinylated secondary antibody (for example, Biotin, Goat anti-Rabbit IgG (H+L) – Cat. #16-15-16, or Biotin, Goat anti-Mouse IgG (H+L) – Cat. #16-18-06) to 0.5 mg/ml in Reconstitution Buffer (Cat. #50-83-00). Dilute the antibody 1:250 into Block Solution (ex. - need 2 ml—add 8 µl antibody to 1992 µl Block Solution).
- (10) Drain the Wash Solution from the membrane and place the membrane in a fresh dedicated container. Pipette 1 ml of the diluted secondary antibody onto each membrane. Incubate membranes at room temperature for 30 minutes.
- (11) Repeat the wash steps as in step 8.
- (12) Dilute Streptavidin Labeled Alkaline Phosphatase (AP-SA) (Cat. #475-3000) 1:1000 in Block Solution (ex. - add 2 µl AP-SA to 1998 µl Block Solution).
- (13) Prepare the 3-component Microarray BCIP/NBT as follows: Mix 500 µl of Microarray BCIP (Cat. #56-00-03) with 500 µl of Microarray NBT (Cat. #56-00-04) and 5 ml of Tris Buffer (Cat. # 50-81-01). Allow the mixture to warm to **room temperature** (protected from light) before use. Additionally, allow 10 ml of TBS, pH 9.5, to come to **room temperature** as well.
- (14) Remove membranes from wash solution; drain solution from membranes. Place membranes in a dedicated container. Pipette 1 ml of the diluted AP-SA conjugate onto each membrane. Incubate membranes for 30 minutes at room temperature.
- (15) Repeat the wash step as in step 8.
- (16) Remove membranes from wash solution; drain solution from membranes. Place membranes in dedicated rinse container. Add 5 ml TBS pH 9.5 per membrane to a dedicated container (NOTE: pH 9.5 is optimal for AP

- activity). Incubate membranes at room temperature for 5 minutes.
- (17) Remove membranes from the TBS; drain solution from membranes. Place membranes in a dedicated detection container. Pipette 1 ml of Microarray BCIP/NBT substrate mix onto each membrane. Incubate membranes for 30 minutes in the dark at room temperature (note: the reaction may be stopped sooner if background color is observed).
  - (18) Rinse membranes with distilled or deionized water in dedicated water rinse container. Repeat the water rinse 2-5 times.
  - (19) Air dry membranes on filter paper and store protected from light.

**COLORIMETRIC IMMUNODETECTION WITH**  
**STREPTAVIDIN LABELED ALKALINE**  
**PHOSPHATASE**

(Protein Detector™ Microarray Dot Blot Kit, AP Colorimetric, Cat.  
#56-11-50)



Total time: approximately 4 hrs.  
(~30 min. hands on time)

**b. Chemiluminescent Detection (Protein Detector™  
Microarray Dot Blot Kit, AP Chemiluminescent –  
Cat. #56-12-50)**

- (1) Rinse the membranes in TBS pH 7.5 (with shaking) for approximately 5 minutes to remove the bromophenol blue buffer.
- (2) Prepare 10 ml of Block Solution: 1% (w/v) Microarray Blocking Powder (Cat. #56-00-01) in 1X Microarray Blocking Solution (Cat. #56-00-02):
  - i. Measure out 9.5 ml water
  - ii. Add 0.1 g of Microarray Blocking Powder
  - iii. Vortex and allow it to dissolve
  - iv. Add 500 µl of 20X Microarray Blocking Solution to the solution. Vortex to mix (note: Microarray Blocking Powder will dissolve more readily in water than in Microarray Blocking Solution).
- (3) Place membranes in a suitable dedicated container.  
NOTE: KPL recommends using a separate dedicated container for each step—for example, primary antibody incubations should be done in a container designated for the primary antibody. KPL recommends a Petri dish as a conveniently-sized container.
- (4) Incubate each membrane in 2 ml Block Solution for 30 minutes at room temperature.
- (5) Dilute the primary antibody into fresh Block Solution. The dilution factor for the antibody will depend on the antibody used; follow the manufacturer's recommendations where possible.
- (6) Drain the Block Solution from the membrane and place the membrane in a fresh container for primary antibody incubation. Incubate the membrane in 1 ml of diluted primary antibody for 30 minutes at room temperature. For the second (negative control) membrane, add 1 ml of Block Solution (no primary antibody) in place of the diluted primary antibody. Incubate the negative control membrane in separate container.

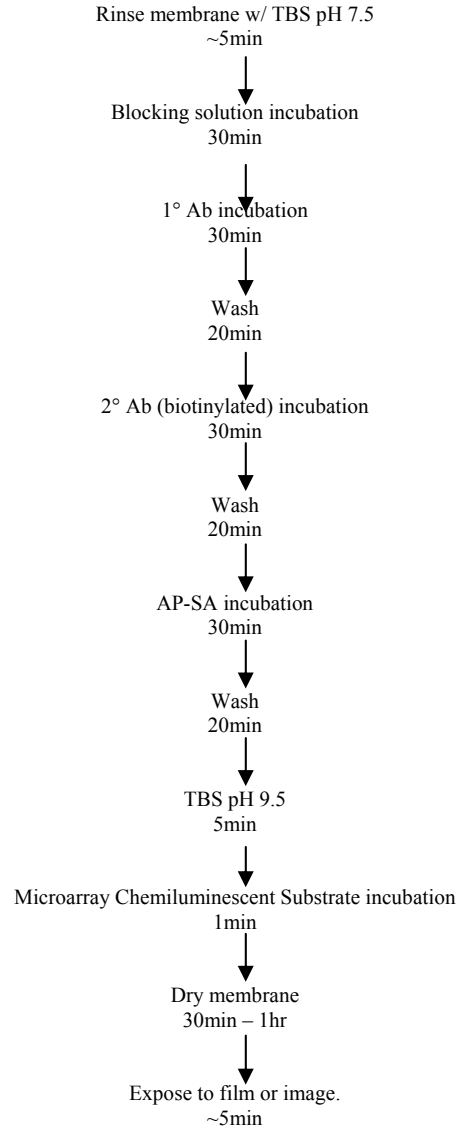
- (7) Prepare 1X Wash Solution (need ~75 ml per membrane). Dilute 10 ml of 20X Wash Solution (Cat. #50-63-01) into 190 ml of water.
- (8) Drain the primary antibody or Block solution from the membrane and place the membrane in a dedicated wash container. Add at least 5 ml of the 1X Wash Solution to the membrane. Swirl the solution briefly over the membrane and decant the solution. Add fresh Wash Solution to the membrane and shake the container for 5 minutes at room temperature, then decant the Wash Solution. Repeat this step with two additional aliquots of Wash Solution for 5 minutes each. (Note: To ensure that the wash solution is free-flowing over the membrane more than 5 ml wash solution can be added.) Wash the negative control membrane in a separate container.
- (9) Perform an additional wash step for 10 minutes with shaking.
- (10) Reconstitute biotinylated secondary antibody (for example, Biotin, Goat anti-Rabbit IgG (H+L) – (Cat. #16-15-16, or Biotin, Goat anti-Mouse IgG (H+L) – Cat. #16-18-06) to 0.5 mg/ml in Reconstitution Buffer (Cat. #50-83-00). Dilute the antibody 1:250 into Block Solution (ex. - need 2 ml - add 8  $\mu$ l antibody to 1992  $\mu$ l Block Solution).
- (11) Drain the Wash Solution from the membrane and place the membrane in a fresh dedicated container. Pipette 1 ml of the diluted secondary antibody onto each membrane. Incubate membranes at room temperature for 30 minutes.
- (12) Repeat the wash step as in step viii and ix.
- (13) Dilute Streptavidin Labeled Alkaline Phosphatase (AP-SA) (Cat # 475-3000) 1:5000 in Block Solution as follows: add 1  $\mu$ l of AP-SA to 2499  $\mu$ l of Block Solution (1:2500). Then, mix 1ml of the 1:2500 dilution into 1ml of Block Solution. This will give an end-dilution of 1:5000.
- (14) Allow 10 ml of TBS pH 9.5 to come to **room temperature** before use. Allow 5 ml of Microarray AP

Chemiluminescent Substrate (Cat. #56-00-05) to come to **room temperature** as well.

- (15) Remove membranes from wash solution; drain solution from membranes. Place membranes in a dedicated container. Pipette 1 ml of the diluted AP-SA conjugate onto each membrane. Incubate membranes for 30 minutes at room temperature.
- (16) Repeat the wash step as in step viii and ix.
- (17) Remove membranes from wash solution; drain solution from membranes. Place membranes in dedicated container. Add 5 ml TBS pH 9.5 per membrane to dedicated container (NOTE: pH 9.5 is optimal for AP activity). Incubate membranes at room temperature for 5 minutes with shaking.
- (18) Remove membranes from the TBS; drain solution from membranes. Place membranes in a dedicated Microarray AP Chemiluminescent Substrate container. Pipette 1ml of substrate onto each membrane. Incubate for 1 minute.
- (19) Remove membranes from the Microarray AP Chemiluminescent Substrate and place on a Kimwipe. Blot the membranes, and allow 30 to 60 minutes for them to completely dry.
- (20) Expose the membranes to x-ray film (start with a 10 second exposure and adjust as needed). Alternatively, the blot can be imaged using a chemiluminescent imager such as Alpha Innotech.

# **CHEMILUMINESCENT IMMUNODETECTION WITH STREPTAVIDIN LABELED ALKALINE PHOSPHATASE**

(Protein Detector™ Microarray Dot Blot Kit, AP Chemiluminescent, Cat. #56-12-50)



Total time ~ 4-4 ½ hours

## Frequently Asked Questions/Troubleshooting

**Question:** Are the extracted proteins full length and can they be analyzed by Western blots?

**Answer:** No. The proteins are not functional and cannot be assayed by using methods for full-length intact proteins due to the fixation and processing of the tissue. However, *Liquid Tissue*<sup>®</sup> sample preparations retain antigenicity and are detectable in other types of immunoassays.

Problem	Answers
1. The paraffin is not completely removed from my tissue.	<ul style="list-style-type: none"> <li>a. To aid in the removal of paraffin, the slides need to be heated in a 60°C oven for 30 to 60 minutes to melt the paraffin just prior to the deparaffinization step.</li> <li>b. 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.</li> </ul>
2. When needle dissecting a tissue, I am unable to visualize the tissue on the end of the needle	<ul style="list-style-type: none"> <li>a. Double-check the dissection method and technique.</li> <li>b. Ensure the calculations are correct for the area being dissected.</li> <li>c. Use a dissection microscope or magnifying glass to visualize the sample.</li> </ul>
3. When performing laser capture microdissection, I am unable to visualize the tissue on the cap.	<ul style="list-style-type: none"> <li>a. Double-check the dissection method and technique.</li> <li>b. Ensure the calculations are correct for the area being dissected.</li> <li>c. Check the User Guide for the LCM instrument being used.</li> <li>d. Make sure that the tissue is completely dry before performing the microdissection.</li> </ul>

<p>4. After preparation of my <i>Liquid Tissue</i><sup>®</sup> preparation, I saw no detection of my proteins.</p>	<p>a. Buffer B pellet was lost. Please ensure that the pellet is at the bottom of the tube before reconstitution.</p> <p>b. Primary antibodies have not been properly optimized.</p> <p>c. Primary antibodies are not optimal for detection of the <i>Liquid Tissue</i><sup>®</sup> preparations.</p>
<p>5. The volume in my <i>Liquid Tissue</i><sup>®</sup> preparation is less at the end of the procedure than when the preparation was started.</p>	<p>a. Make sure the cap of the Sample Preparation Tube is sealed tightly for the incubation steps.</p> <p>b. Only use the Sample Preparation Tubes provided in the kit for preparing <i>Liquid Tissue</i><sup>®</sup> samples.</p>
<p>6. I am not seeing any detection on my membrane.</p>	<p>a. Add 1 <math>\mu\text{L}</math> of labeled streptavidin to 100 <math>\mu\text{L}</math> of colorimetric substrate, to check the efficacy of both the substrate and conjugate.</p> <p>b. To test the chemiluminescent substrate, or to further test the colorimetric substrate, perform a dot blot using the labeled streptavidin.</p> <p>c. Ensure that all reagents have been optimized for each specific reaction.</p>
<p>7. I am seeing diffuse signal on my microarray detection.</p>	<p>a. Reduce spot volume.</p> <p>b. Double-check the pore size of the nitrocellulose membrane. The pore size should be 0.1 – 0.2 <math>\mu\text{m}</math>.</p>

## Related Products

<b>Product</b>	<b>Size</b>	<b>Cat. No.</b>
Protein Detector™ Microarray Dot Blot Kit, AP Colorimetric	200 blots	56-11-50
Protein Detector™ Microarray Dot Blot Kit, AP Chemiluminescent	100 Blots	56-12-50
Biotin, Goat anti-Rabbit IgG (H+L)	0.5 mg	16-15-16
Biotin, Goat anti-Mouse IgG (H+L)	0.5 mg	16-18-06
20X Wash Solution Concentrate	800 mL	50-63-16
AP-labeled Streptavidin	1 mL	475-3000
Microarray BCIP/NBT Substrate	100 mL	50-81-18
Microarray Blocking Powder	10 g	71-02-03



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