

Protein Microarray Detection of Prostate Cancer Markers in FFPE Tissue Prepared with the Proteosol™ Tissue Extraction System

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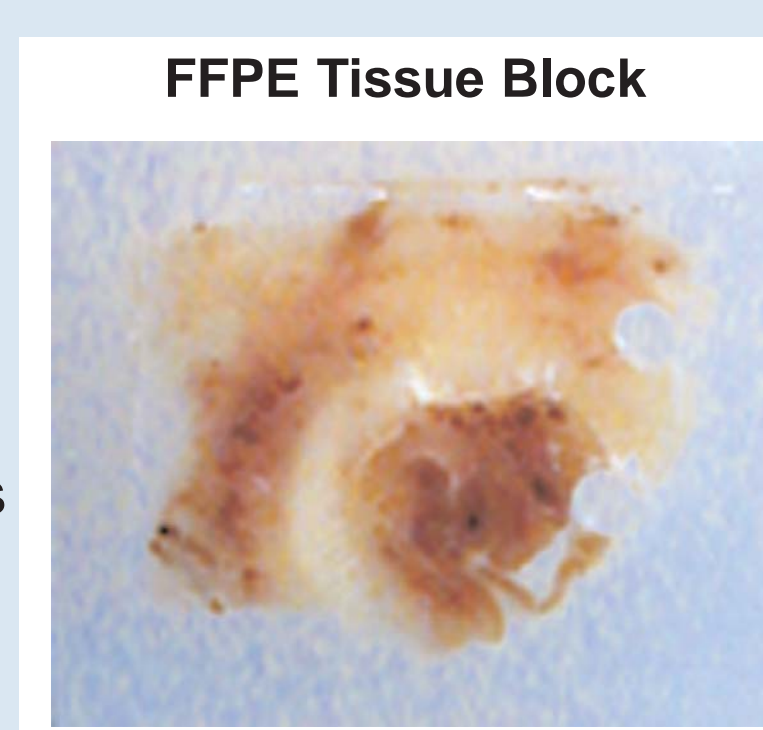
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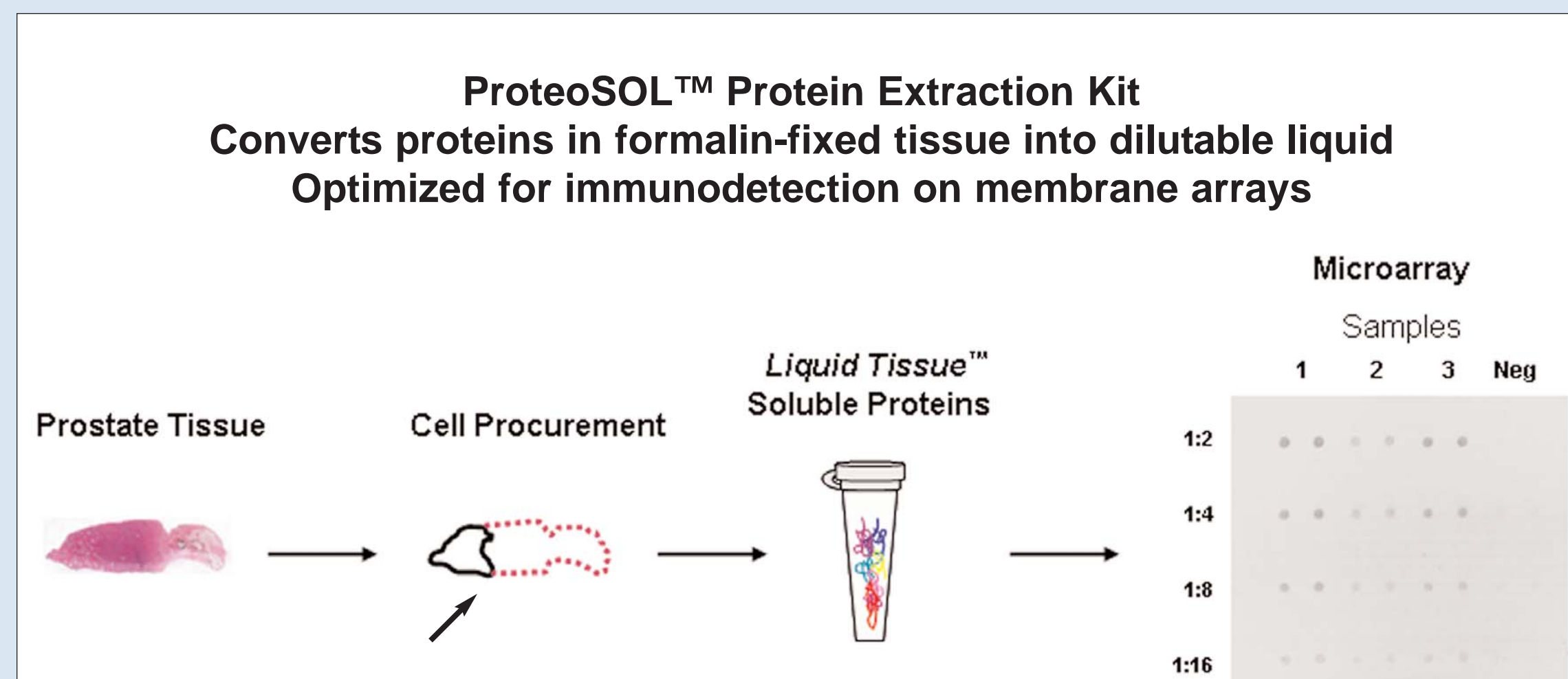
INTRODUCTION

Classically, the only immunoassay methods suitable for formalin fixed paraffin embedded (FFPE) tissue have been immunohistochemistry techniques. Formalin fixation crosslinks the proteins in the tissue, making extraction and solubilization of proteins for high throughput proteomic methods very difficult. The ProteoSol™ Tissue Extraction System provides a very enabling method for preparing protein from FFPE tissue samples for use in membrane based immunodetection methods for proteomic analysis.

The ProteoSol™ Tissue Extraction System was used to solubilize cells from cancerous lesions obtained from sections of FFPE prostate tissue. Serial dilutions of the preparations were spotted onto nitrocellulose membranes in a microarray format and then detected with antibodies against several prostate cancer biomarkers. The results show the relative quantitation of these biomarkers in different FFPE tissue samples.



FFPE Tissue Block



The advantage of this method is that it allows for the extraction and analysis of proteins directly from FFPE tissue. A single FFPE sample can be analyzed for multiple biomarkers or a single biomarker can be compared across a variety of samples in a fast and easy to use microarray format for comparison of relative levels of protein expression.

These results show a breakthrough technique for preparing FFPE tissue samples for the relative quantitation of proteins from specific cells.

Liquid Tissue™ Preparation

The dissected cells were transferred to a reaction tube containing the appropriate amount of Proteosol Reaction Buffer A and heated at 95°C for 90 minutes with agitation every 20 minutes. The samples were microcentrifuged, cooled on ice for 1 to 2 minutes, and then 1.0 ul of Proteosol Reaction Buffer B was added for each 20 ul of reaction volume. The samples were incubated for 60 minutes at 37°C with vortexing every 20 minutes. Finally, the samples were microcentrifuged at 10,000 rpm for 1 minute, heated to 95°C for 5 minutes, and then microcentrifuged again. The preparations were stored at -20°C until use.

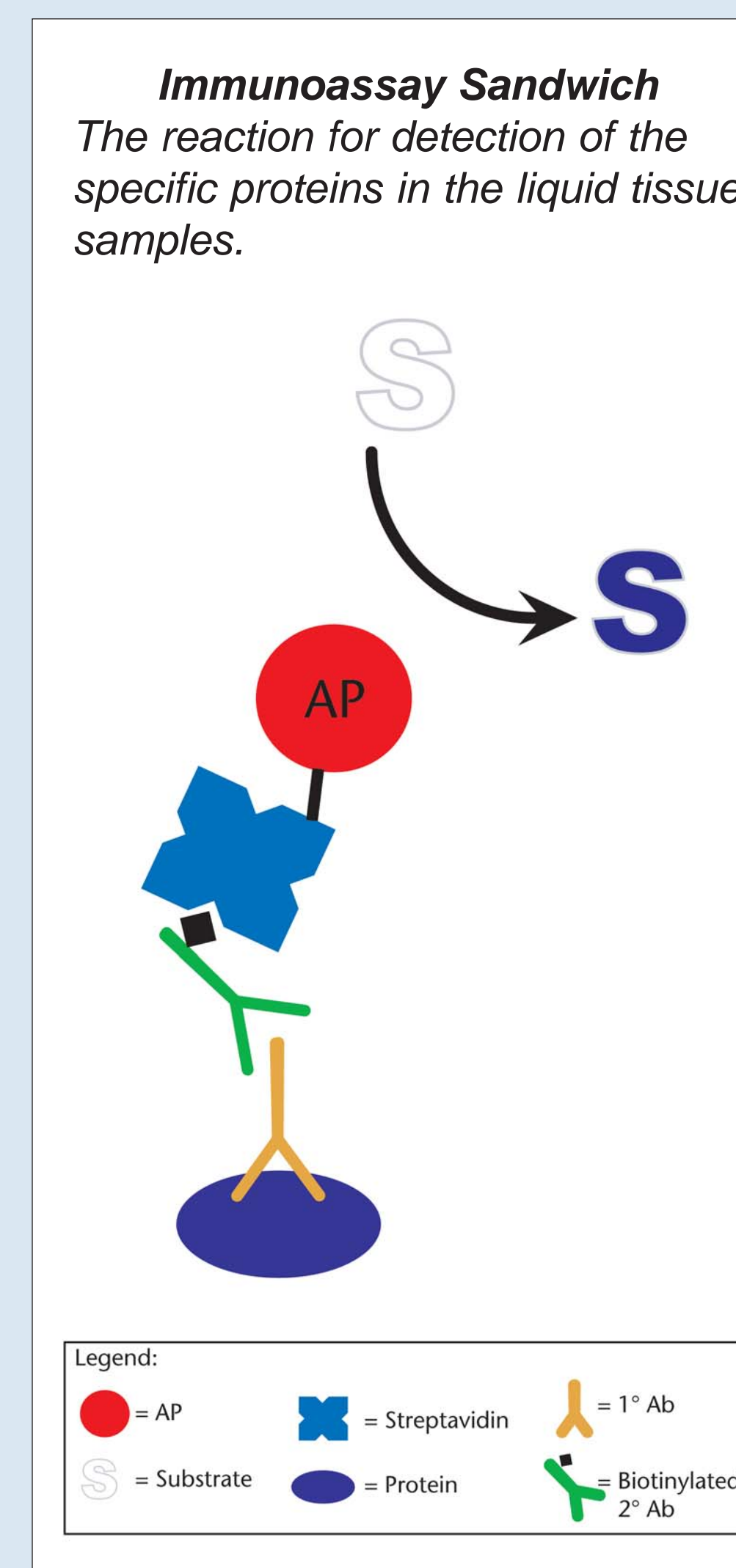
Microarray Spotting

Controls are very important for properly interpreting the results of the Liquid Tissue microarrays. Negative controls spotted on the membrane included a Liquid Tissue preparation processed with reagents but no tissue, BSA (0.5 ug/ul, highest concentration) as a non-specific protein, and dilution buffer (20mM Tris, 0.02% bromophenol blue). Purified PSA protein (0.01 ug/ul highest concentration) was included as a positive control for the PSA specific assay and a negative control for the Androgen Receptor and PSAP assays. The three prostate tissue samples and controls were diluted 1:2 in dilution buffer and aliquoted into a 384 well plate. The samples were serially diluted 1:2 in dilution buffer for a total of four dilutions (1:16 largest dilution). The samples were then spotted in duplicate using a glass slide microarrayer (V&P Scientific, Inc.) onto 0.1um pore size nitrocellulose membranes (Protran-Schleicher & Schuell) cut into 1 3/4" x 7/8" rectangles. See Liquid Tissue.

Two membranes were spotted for each specific biomarker assayed. The assay for the positive membrane included the specific primary antibody for the protein being measured. The negative membrane omitted the specific primary antibody to control for non-specific reactions with the detection system. Additionally, a separate membrane was spotted and stained with Memcode Reversible Protein Stain (Pierce Biotechnology) for determination the total protein concentration and to insure proper dilutions of the spots had occurred. This protein measurement was used to normalize the relative intensities of the specific spots to the total protein level in the sample.

Signal Development

Rinse membrane w/ TBS pH 7.5
~ 5 minutes
↓
Block
~ 30 minutes
↓
1° Ab
30 minutes
↓
Wash
15 minutes
↓
2° Ab (biotinylated)
30 minutes
↓
Wash
15 minutes
↓
S A Conjugated AP
30 minutes
↓
AP Wash
15 minutes
↓
TBS pH 9.5
5 minutes
↓
BCIP/NBT
30 minutes
↓
Rinse w/ water 3-6x
~ 2 minutes
↓
Air Dry
**Total Time ~3.5 - 4 hours
(~45 minutes hands on time)**



Immunoassay Sandwich
The reaction for detection of the specific proteins in the liquid tissue samples.

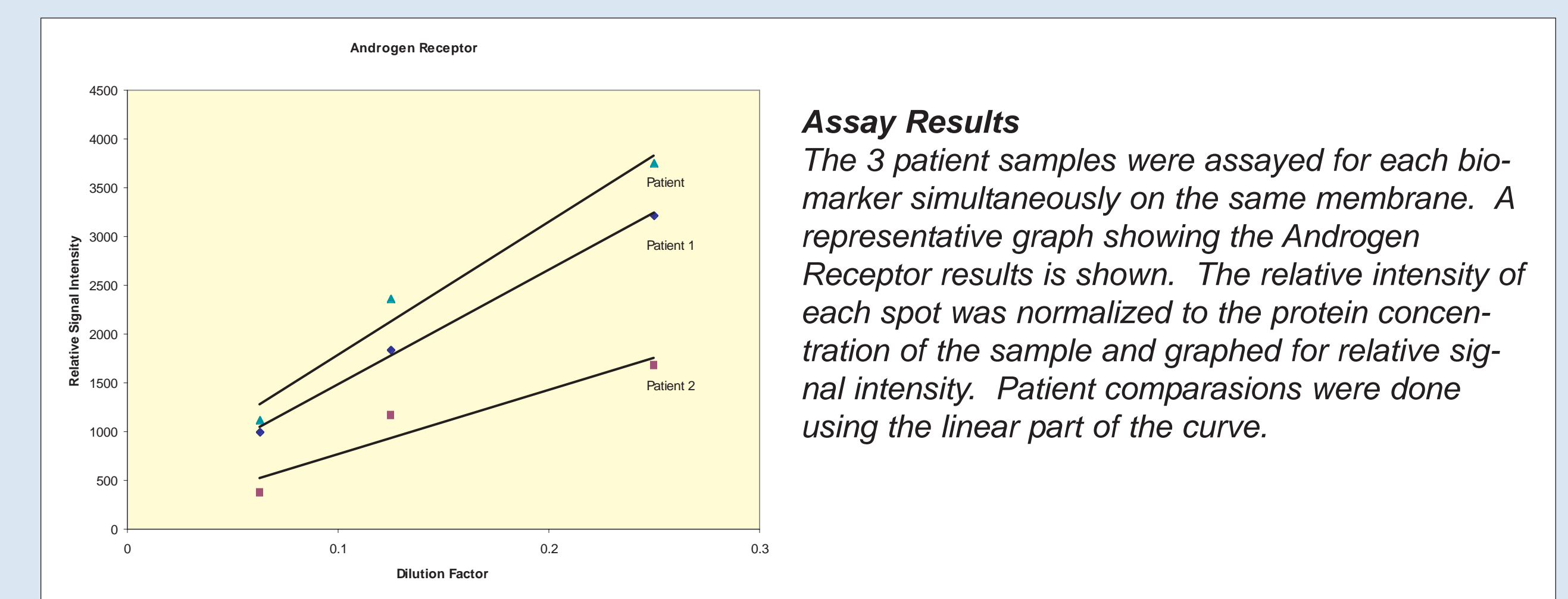
Legend:
● = AP
● = Substrate
● = Streptavidin
● = Protein
● = 1° Ab
● = Biotinylated 2° Ab

RESULTS

Three biomarkers for prostate cancer were measured in FFPE tissue from patients with non-metastatic adenocarcinoma of the prostate. See chart below for pathology information. Two of the markers, PSA (prostate specific antigen) and AR (androgen receptor) are known to positively correlate with cancerous prostate tissue. The third marker, PSAP (prostatic acid phosphatase) has been shown to negatively correlate with prostate cancer. The biomarker protein corresponds to the Gleason Grades from pathology studies. The results have been normalized for the total protein concentrations of the Liquid Tissue preparations spotted on the microarray to allow comparison of the biomarker protein levels in each of the patient samples.

These assays show two patients with similar profiles including higher PSA and AR levels with lower PSAP. The third patient has comparatively lower levels of PSA and AR, along with comparatively higher PSAP levels. These results show that it is now possible to measure a range of biomarkers in multiple patient samples. The advantage of using formalin fixed paraffin embedded tissue blocks as a source of research material is that frequently there is very good documentation of patient history and outcomes. **This enables the researcher to use FFPE to develop and test the validity of biomarker panels that may be correlative and predictive in patient diagnosis and treatment schemes.**

Pathology of Prostate Tissue Samples					
Sample	Gender	Age	Diagnosis	Gleason Grade	Lymph Nodes
1	Male	62	Adenocarcinoma	4 + 5	Negative
2	Male	54	Adenocarcinoma	3 + 3	Negative
3	Male	62	Adenocarcinoma	3 + 4	Negative



Assay Results
The 3 patient samples were assayed for each biomarker simultaneously on the same membrane. A representative graph showing the Androgen Receptor results is shown. The relative intensity of each spot was normalized to the protein concentration of the sample and graphed for relative signal intensity. Patient comparisons were done using the linear part of the curve.

ProteoSol™ Tissue Extraction

HEMATOXYLIN & EOSIN STAINING
Melt paraffin in 58 - 60° C oven
30 - 60 minutes
↓
Deparaffinization & Rehydration
~ 25 minutes
↓
Hematoxylin Stain
~ 7 - 17 minutes
↓
Eosin counterstain
~ 5 minutes
↓
Tissue section evaluation
**Total time ~ 1.5 - 2 hours
(~60 minutes hands on time)**

CELL PROCUREMENT

Melt paraffin in 58 - 60° C oven
30 - 60 minutes
↓
Deparaffinization & Rehydration
~ 25 minutes
↓
Sample collection (needle dissection)
~10 minutes
↓
Buffer A incubation @ 95° C
1.5 hours
↓
Cool on ice
1 - 2 minutes
↓
Buffer B incubation @ 37° C
60 minutes
↓
Incubation @ 95° C
5 minutes
**Total Time ~4 - 4.5 hours
(~40 minutes hands on time)**

Liquid TISSUE Preparation Microarray

KEY:	1	2	3	4	5	6	7
● Patient Sample 1	●	●	●	●	●	●	●
● Patient Sample 2	●	●	●	●	●	●	●
● Patient Sample 3	●	●	●	●	●	●	●
● Liquid Tissue preparation/no tissue - Negative Control	●	●	●	●	●	●	●
● BSA (0.5 ug/ul in well A) - Negative Control	●	●	●	●	●	●	●
● PSA (0.01 ug/ul in well A)	●	●	●	●	●	●	●
● Dilution Buffer - Negative Control	●	●	●	●	●	●	●

Three patient samples and controls were diluted 1:2 in dilution buffer and aliquoted into row A of a microarray plate. A 1:2 serial dilution was made in dilution buffer down to a 1:16 dilution.

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

The dilutions were spotted in duplicate using a microarrayer spotter on to 0.1 um nitrocellulose membranes (Protran-Schleicher & Schuell).

Membrane with microarrayed samples:

●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●
●	●	●	●	●	●	●	●

Developed Microarray Membrane

	1	2	3	4	5	6	7	8
A	●	●	●	●	●	●	●	●
B	●	●	●	●	●	●	●	●
C	●	●	●	●	●	●	●	●
D	●	●	●	●	●	●	●	●

MATERIALS AND METHODS

Tissue Evaluation

Three FFPE prostate tissue samples from patients with non-metastatic prostate adenocarcinoma were sectioned and stained with hematoxylin and eosin (H&E). The area of the slide containing the tumor was circled with a marker to identify the cells to be collected in a serial section from the tissue block.

Cell Procurement

A serial section was cut from the FFPE block and placed on a untreated standard glass microscope slide. The thickness of the section and area to be collected were used to calculate the approximate number of cells to be prepared according to the product insert. The system has been optimized for use with 60,000 cells in 20 ul reaction volume. More cells can be processed by scaling the size of the reaction. The samples were deparaffinized and rehydrated according to product insert. Using the H&E stained slide as a reference, the appropriate areas of the tissues were collected by needle dissection.

Note: It is possible to use laser microdissection for tissue procurement when a subset of cells too small for the needle dissection procedure will be selected for processing in the Proteosol Tissue Extraction System.

Detection

KPL reagents were used for the development and detection of the microarrayed samples and controls. The membrane was rinsed with Tris Buffered Saline, pH 7.5 (TBS) for 5 minutes then blocked in 0.5% casein Blocker Solution for 30 minutes. The primary antibodies were diluted in the blocking solution to the manufacturers' recommended concentrations for Western blots. Negative membranes were incubated in blocking solution only. The membranes were washed three times for five minutes each in 1X Wash Solution. The biotinylated secondary antibody was diluted 1:250 in Blocker Solution, incubated with the membranes for 30 minutes, and then washed as above. The alkaline phosphatase/streptavidin conjugate was diluted 1:1000 in Blocker Solution, incubated for 30 minutes, and washed as above. The pH of the membranes were brought up to the pH optimum for alkaline phosphatase by soaking for 5 minutes in TBS, pH 9.5. The buffer was drained from the membrane without drying. The BCIP/NBT substrate was incubated with the membranes for 30 minutes then the membranes were rinsed with distilled water for between 2 to 5 times to stop the reaction and clear the background. Membranes were air dried and protected from light.

Data Analysis

A grayscale image of the positive, negative, and protein stained membranes was captured with a CCD camera as a TIFF file. Grid Grinder image analysis software for microarrays (gridgrinder.sourceforge.net) was used to measure spot intensity and background. The data was imported into an Excel spreadsheet for graphing and statistical analysis. The negative membrane was used for correction of non-specific signal and the samples were normalized for the amount of total protein for each spot.

CONCLUSION

Currently, it has been estimated that there are over 5 billion blocks of formalin fixed paraffin embedded tissue (FFPE) in existence. A large percentage of these samples have complete histories and known clinical outcomes. Researchers doing basic research, clinical diagnostics, and drug discovery would benefit from a method that enables a high throughput membrane based immunoassay method to compare levels of protein expression. The Proteosol products provide the researcher the ability to solubilize the cross-linked proteins from specific areas of FFPE tissue allowing them to control the selected in each individual tissue. When the results are normalized for protein concentration, relative quantitation of protein expression can be compared between samples. Previously, a single immunohistochemistry slide would show the detection of a single biomarker. This membrane based method enables multiple biomarkers to be measured from a single sample preparation that can be made from the same tissue section. Additionally, this method makes it possible to measure multiple patient samples for the same target protein in one experiment.

The Proteosol products provide a very enabling new technology for proteomic biomarker research from FFPE tissue by providing a method for solubilizing proteins from these samples in a way that was previously not feasible.

Proteosol is a trademark of KPL, Inc.
Liquid Tissue is a trademark of Expression Pathology.