

# A flexible platform for automated high-performance protein purification using micro scale separation technology

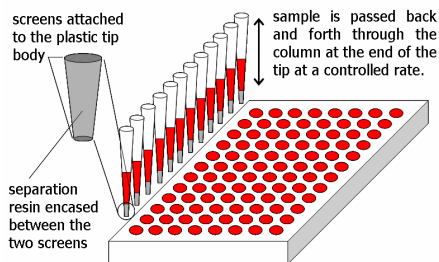
Jeremy Lambert, Murray Anderson, Doug Gjerde, Liem Nguyen, PhyNexus, Inc., San Jose, CA

## Introduction

As the scale and scope of protein analysis continues to expand in number and complexity, the amount of material available for study necessarily decreases and traditional approaches to protein purification fail to maintain the flexibility and performance required for controlled study of protein structure and function. The requirement of decreasing sample scale demands a new approach to protein separation designed for processing small samples while maintaining a high level of control over the purification process. Recent advances in the area of miniaturized high-throughput tools for purification, enrichment and desalting of proteins enable researchers to obtain superior purification results from smaller samples in a fully automated platform providing a high degree of flexibility required for comprehensive protein analysis.

## PhyTip® Columns

PhyTip column technology is tailored specifically to achieve the highest enrichment and purity possible from starting samples of as little as 100  $\mu\text{L}$  to as much as 25 mL. The affinity columns utilize standard protein separation resins that allow for maintenance of protein function, such as agarose-based resins (including Protein A, Protein G, IMAC, glutathione, streptavidin). The high capacity microcolumns are confined within standard pipette tips by encasing the resin (5-160  $\mu\text{L}$ ) between two inert hydrophilic screens situated at the ends of the tips (Fig. 1). These screens contribute virtually no dead volume to the microcolumn, allowing for precise control over the interaction between the sample and the column.



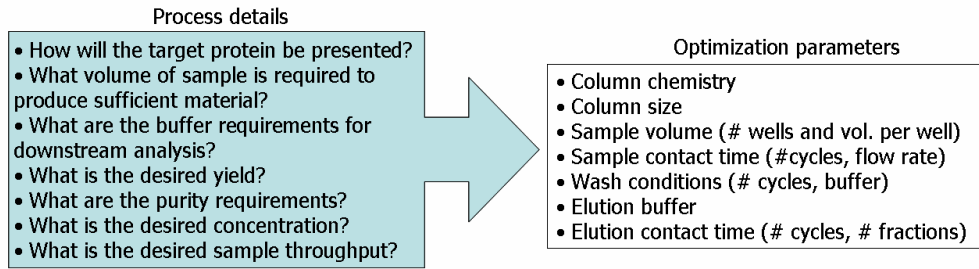
**Figure 1.** PhyTip column format.



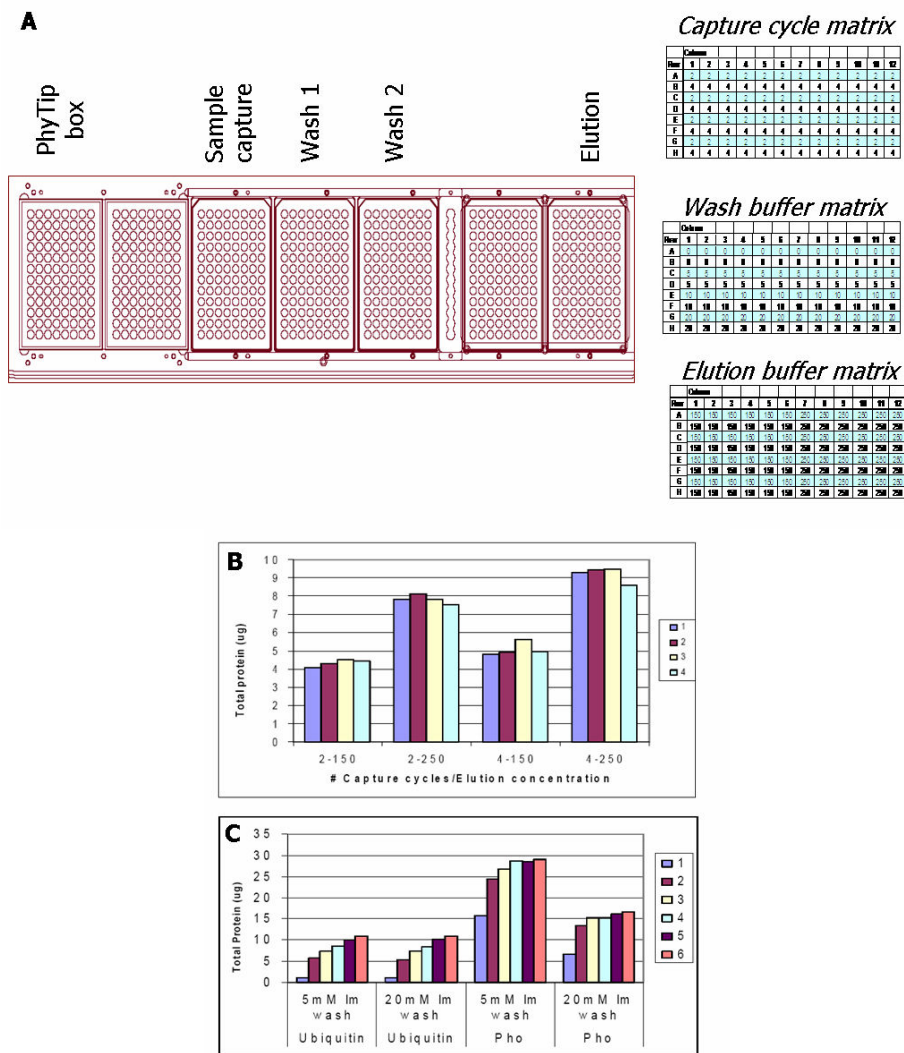
**Figure 2.** MEA Personal Purification System enables fully automated purification of 1-96 samples at a time while maintaining full control of column operation throughout the purification method.

## Tailoring the Enrichment Process

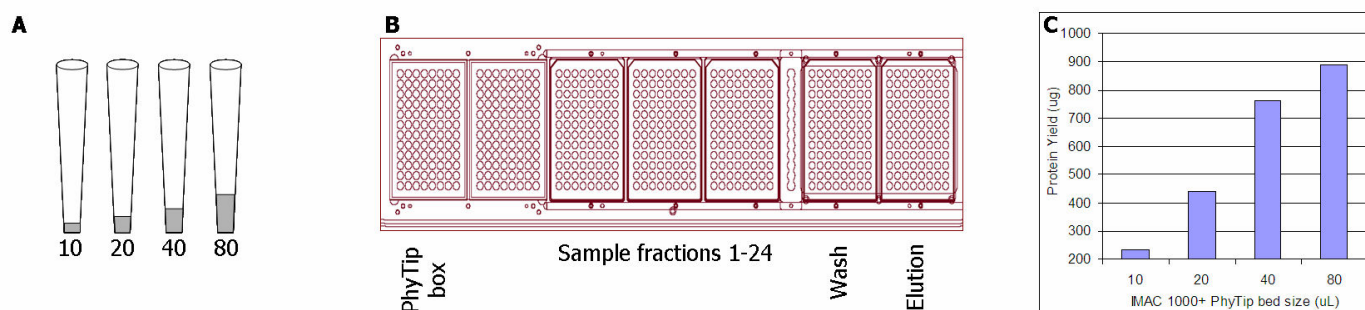
In order to facilitate the optimization of the capture, wash and elute process, the MEA Personal Purification System (Fig. 2) has been designed as a dedicated platform to purify and enrich proteins using a 12 channel system to sequentially prepare a full 96-well plate. In order to accommodate a wide range of sample volume and column capacity, the system can be used for either 200  $\mu\text{L}$  tips (containing 5-20  $\mu\text{L}$  of resin) or 1 mL tips containing 10-160  $\mu\text{L}$  of resin) by simply changing the pipetting station. The system has been specifically engineered to accurately and reproducibly perform the processes involved in capturing, purifying and enriching protein samples. This is an ideal platform to rapidly and automatically perform the necessary steps involved in optimizing the protocols for a given resin and a specific protein of interest (Fig. 3). Examples of the effect of capture, wash, and elution conditions for IMAC purification of 6XHis-tagged recombinant proteins from *E. coli* lysate are shown below.



**Figure 3.** The versatility of the PhyTip column format facilitates examination of a wide range of variables when selecting appropriate purification conditions depending on the sample format and subsequent analysis requirements.



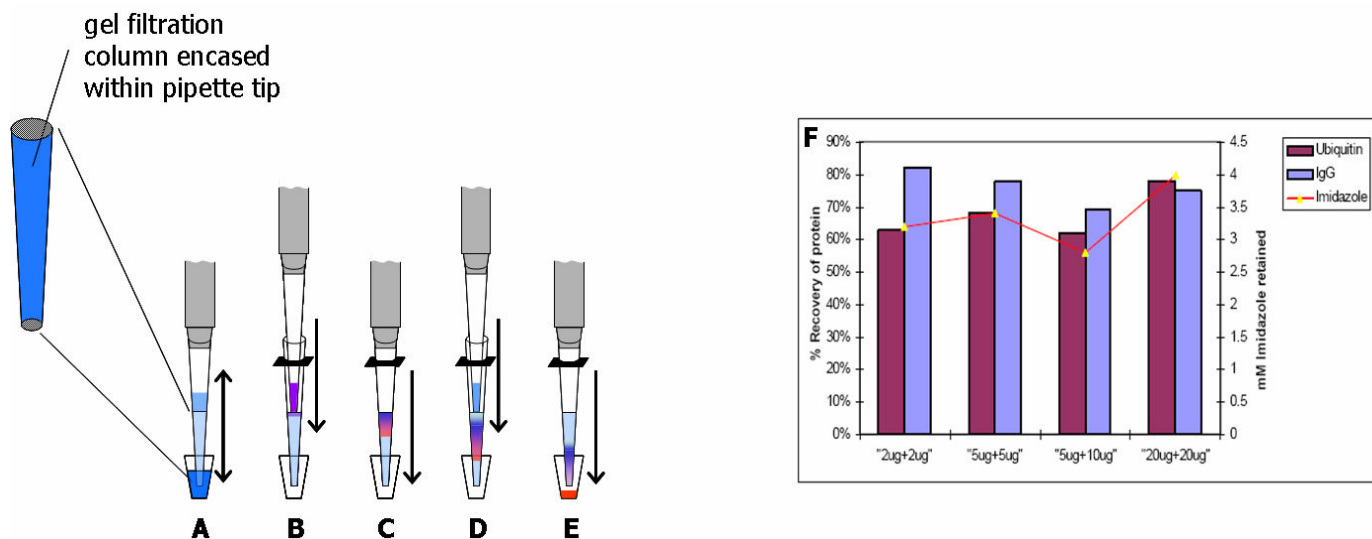
**Figure 4.** Experimental design and MEA deck layout of IMAC optimization matrix (A). The experiment is designed to optimize for three independent variables of the enrichment process; capture cycles, concentration of imidazole in wash buffer and concentration of imidazole in elution buffer. Results of automated IMAC optimization study (B). The amount of ubiquitin purified from each condition was quantified by HPLC. For the variable imidazole wash concentrations 1 = 0 mM imidazole, 2 = 5 mM imidazole, 3 = 10 mM imidazole and 4 = 20 mM imidazole. The x-axis shows 2 cycle capture with either 150 or 250 mM imidazole elution or 4 cycle capture with 150 or 250 mM imidazole elution. Further optimization of purification conditions for 6XHis-ubiquitin and 6XHis-Pho polymerase (C). Comparison of imidazole concentration in the wash buffer and elution buffer for two proteins. The legend indicates the concentration of imidazole in the elution buffer. 1 = 100 mM, 2 = 200 mM, 3 = 250 mM, 4 = 300 mM, 5 = 400 mM and 6 = 500 mM.



**Figure 5.** The availability of a range of PhyTip column sizes provides flexibility in scale of the purification process – in both sample volume and binding capacity. A total of 24 mL of sample was processed with columns containing 10, 20, 40, or 80 µL of IMAC resin (A) by performing four capture cycles from each of 24 1mL fractions in deep-well plates (B). Twelve unique 24 mL samples can be processed simultaneously using this configuration. The graph in panel C shows 6XHis-ubiquitin yield as a function of IMAC column size. A total of 24 mL of sample was processed with each column (4 capture cycles per fraction, 1000 µL/min flow rate).

## Buffer Exchange

PhyTip 5K columns contain gel filtration media selected to exclude all proteins larger than 5 kDa, allowing proteins to retain functionality and pass through the column while small molecules such as salts will be retained by the gel filtration media. Samples and other liquids are transferred to the top of the PhyTip 5K desalting column using the MEA Personal Purification System with liquids pumped through the column in a single direction at a specific flow rate.

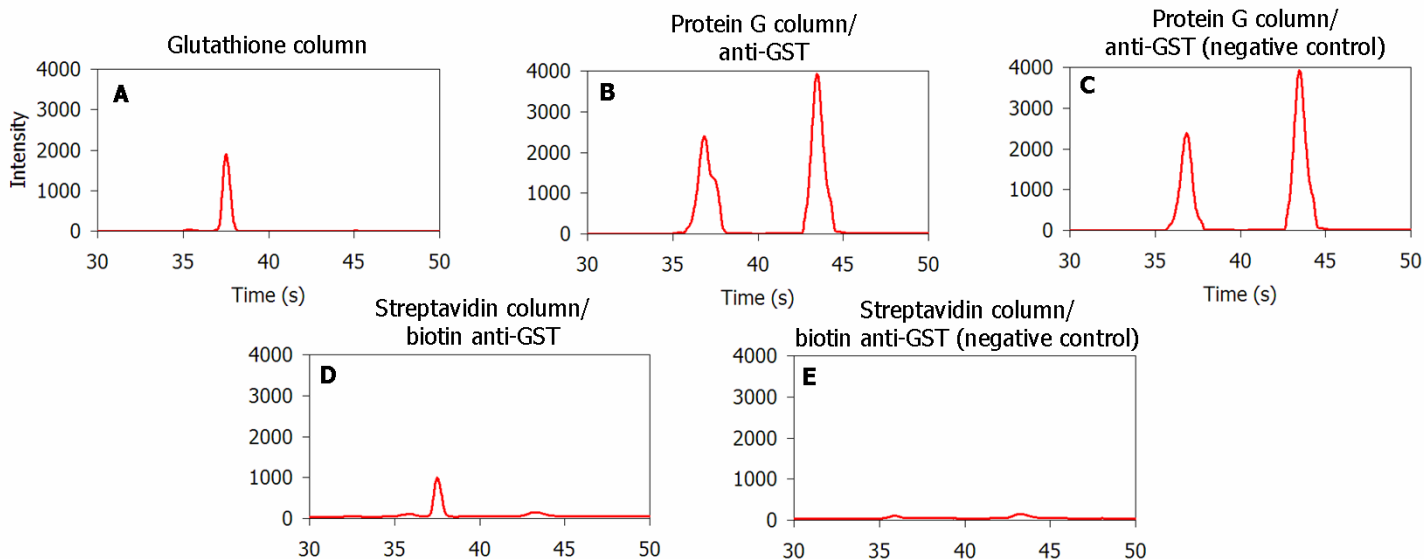


**Figure 6.** Desalting and buffer exchange with PhyTip 5k columns. The columns contain 80 µL of gel filtration media housed within a 200 µL pipette tip. Columns are conditioned with water or buffer (A) depending on whether the process involves desalting or buffer exchange. The conditioned column is placed into a holder positioned above a 96-well collection plate and protein samples (20 µL) to be desalted are loaded onto the top of the column using a transfer pipette tip (B) and pushed into the bed using the pipettor (C). A volume of chaser liquid (20 µL) is loaded onto the top of the column (D). Water is used as the chaser for desalting, or in the case of buffer exchange the appropriate buffer is used as the chaser liquid. The protein is pushed through the bed and recovered in the collection plate (E).

Protein recovery from buffer exchange as a function of mass applied to the column is shown in panel F. The columns were conditioned with PBS for buffer exchange from an initial sample containing 500 mM imidazole. The mass of ubiquitin or IgG was varied from 2 µg to 20 µg. Recovery of protein is plotted on the y-axis on the left. Imidazole concentration is plotted on the right. Greater than 95% of the imidazole is removed in the process while recovering an average of 70% of the protein. Analysis was performed by quantitative HPLC.

## Immunoaffinity Purification

In addition to enrichment of recombinant proteins, PhyTip columns also provide a format for high-performance isolation of native proteins via immunoaffinity columns. Target-specific antibody or antigen complex can be captured either directly on Protein A or Protein G columns or by capturing a biotinylated antibody using Streptavidin columns. Results from purification of GST-ubiquitin using a monoclonal anti-GST antibody are shown below.

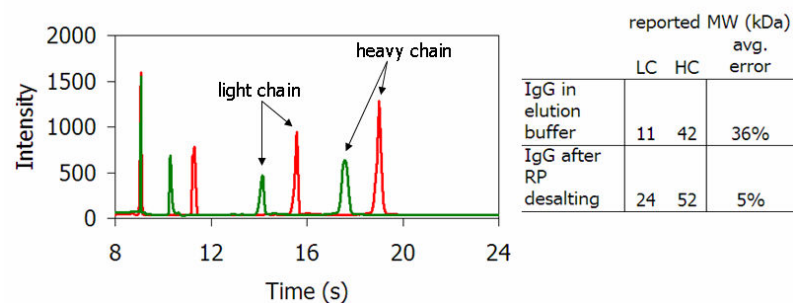


**Figure 7.** GST-ubiquitin fusion protein was purified from *E. coli* lysate directly on a glutathione PhyTip column (A) or by first incubating the lysate with anti-GST followed by purification using Protein G columns. Microfluidic analysis demonstrates that co-elution of the IgG and target protein is observed when using the Protein G column (B) compared with a negative control lysate lacking the fusion protein (C).

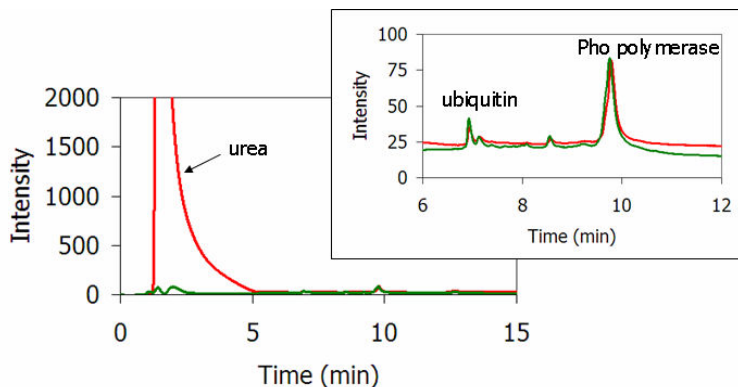
The anti-GST mAb was captured on a Protein G column and conjugated with biotin by passing NHS-LC-biotin reagent through the column. Excess biotin was removed by washing the column and the resulting labeled antibody was eluted using low pH buffer. The biotinylated antibody was then incubated with the lysate samples followed by capture on a streptavidin PhyTip column and elution at pH 2. The resulting product shows little interference from the antibody in the microfluidic analysis due to the retention of the antibody on the streptavidin column after eluting the target protein (D,E).

## Reverse Phase Desalting

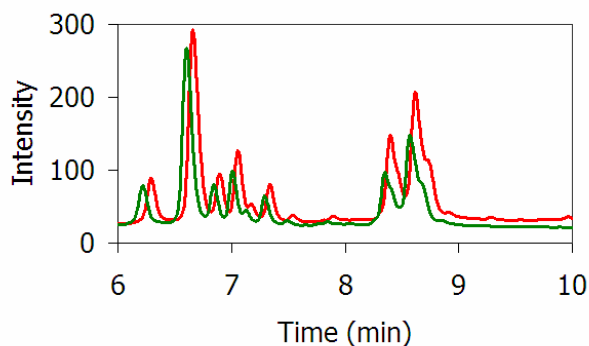
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**Figure 8a.** Microfluidic analysis of IgG sample eluted from Protein A column before (green) and after (red) desalting on a reverse phase column. The high concentration of sodium phosphate in the original sample interferes with accurate determination of the MW of IgG chains (reducing conditions). The estimation of MW is improved by desalting prior to analysis.



**Figure 8b.** 6XHis-ubiquitin and 6XHis-Pho polymerase purified from *E. coli* lysate using IMAC PhyTip column under denaturing conditions (8M urea). HPLC analysis of the sample before (red) and after (green) purification on a reverse phase PhyTip column (10  $\mu$ L bed size). Average protein recovery from the 20  $\mu$ g sample was greater than 90%.



**Figure 8c.** A 20  $\mu$ g sample of GST was digested with trypsin and the resulting peptide fragments were analyzed by HPLC. Comparison of the integrated peak areas demonstrated an average yield of 85% after desalting using reverse phase PhyTip columns.

## Summary

- The use of micro-scale affinity columns processed with the MEA Personal Purification System provides a unique platform for rapid optimization of protein purification and desalting methods. The versatile platform allows automated optimization of variables including capture cycles, column size, wash and elution conditions.
- The availability of multiple column bed sizes and separation chemistry provides a range of capabilities to produce purified protein samples suitable for a wide range of downstream applications including biochemical, functional, and structural studies. The automated process can be set up to purify proteins from sample sizes ranging from 100  $\mu$ L to 25 mL.