



High Throughput Purification and Characterization of a Protein Variant Library

Introduction

New methods to expedite processes that lead to the development of innovative medicines are investigated and put to use in pharmaceutical research every day. Centocor's Programmed Protein Libraries Platform™ enables scientists to quickly optimize protein biotherapeutic molecules for improved pharmacological and biophysical properties. The robust screening assays used to efficiently and effectively rank and select lead candidates often require purified protein samples. In order to harness the full potential of our PPL™ platform, we needed to identify and implement high throughput methods for purifying and characterizing protein samples to enhance our selection process.

For the past few years, we have used PhyNexus, Inc.'s high throughput protein purification technology for the small-scale purification of large numbers of protein variants from both mammalian and bacterial sources using Protein A and IMAC PhyTips™. However, analyzing the results of large numbers of small-scale purifications using a standard SDS-PAGE protocol was time-consuming and a bottleneck in our process. Caliper Life Sciences, Inc. offers a technology where, through the employment of high throughput microfluidic chips, hundreds of samples can be analyzed in the time it would take to run a single SDS-PAGE gel.

We present here a marriage between PhyNexus' PhyTip technology and Caliper's LabChip GXII that has afforded us the possibility of not only high throughput purification, but also high throughput purification analysis, greatly increasing the numbers of protein samples that we can process in a single day.

Materials and Methods

PhyTip columns containing ProPlus Affinity Resin (Catalog# PTZ 92-20-07) were purchased from PhyNexus, Inc. (San Jose, CA).

A LabChip GXII instrument and HT Protein Express Kits including, chips and reagents, were generously provided by Caliper Life Sciences, Inc. for this analysis.

Two libraries of protein variants based on two different parent molecules were created by making single amino acid substitutions at various positions in each protein. A pool of 9 amino acids was used resulting in one library of approximately 270 total variants and another of 248 variants. For this analysis, 179 variants from the combined libraries plus 4 controls were transiently transfected in HEK293F cells. Transfections were done in duplicate in a 48-well plate format. A total of 366 protein samples were purified in 4 purification runs using PhyNexus PhyTips with 20 μ L beds of ProPlus affinity resin. The purification protocol was run on a Caliper Sciclone ALH 3000 automated liquid handling system using a 96 tip High Volume Head (volumes up to 200 μ L).

Briefly, protein variants were captured on PhyTip columns equilibrated with 10mM Na-Phosphate, 0.14M NaCl, pH7.4. Non-specific and unbound proteins were removed following the capture step by washing with the same buffer for 10CV's. Tips were conditioned prior to elution with 10CV's of 0.14M NaCl, pH7.4 buffer. Purified proteins were eluted with 60 μ L of 10mMNa-Phosphate, 140mM NaCl, pH 2. Samples were neutralized by adding 12 μ L of 2M Tris, pH7.

Following purification, samples were analyzed by high throughput electrophoresis using a Caliper LabChip GXII. Sample plates were prepared according to the manufacturer's directions. Protein variants were run under non-reducing conditions and were not heated prior to electrophoresis. The protein chip was also prepared according to manufacturer's directions. Samples and chip were placed in the instrument and analyzed using the HT Protein Express 100 assay.



Caliper's LabChip GXII System

Results and Discussion

For this analysis, we were able to recover purified protein for each variant. Amounts ranged from approximately 6 μg to 30 μg total protein. Gel images generated by the LabChip GXII showed that the variants were greater than 90% pure (Figure 1). A comparison to the image of a standard SDS-PAGE gel (Figure 2) shows the resolution that the LabChip GXII is capable of achieving. Differences in the apparent molecular weights of the proteins are most likely due to the different matrices used for separation and the methods of detection used for each process – conventional Coomassie blue staining versus laser induced fluorescence.

Figure 1. A comparison of LabChip GXII gel images of duplicate samples on the same plate and on different plates. The banding is due most likely to differences in glycosylation that we commonly see in recombinant proteins from mammalian expression systems.

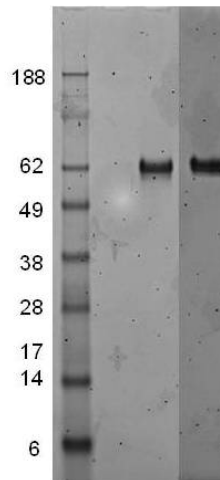
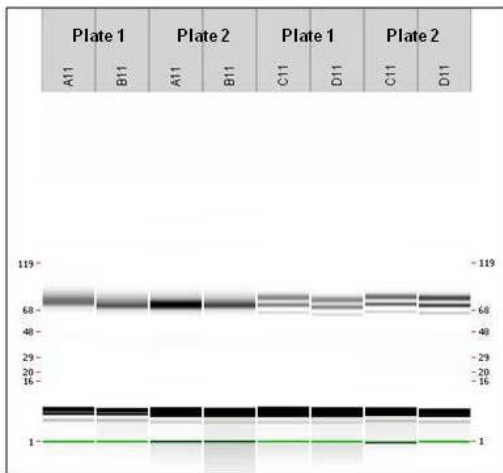


Figure 2. Non-reduced protein variants analyzed using standard SDS-PAGE

PhyTip columns provide a reliable and reproducible way of purifying large numbers of protein variants allowing us to focus on more complex protein libraries without worrying about time constraints. Our current purification protocol enables us to purify 96 samples in approximately 45 minutes. Purification methods are adaptable to a number of automated liquid handlers and columns can be made with varying bed sizes and resins to purify a variety of proteins expressed in bacterial or mammalian systems. The methods developed using PhyTip columns can also be scaled up to purify proteins from larger volumes of starting material. The amount of protein recovered from a PhyTip is generally sufficient for downstream screening assays; however, it is largely dependent on the expression levels of the various proteins being tested.

Analyzing large numbers of purified proteins generated by high throughput purification methods is a time consuming process using standard methods such as traditional SDS-PAGE. High throughput instrumentation such as the LabChip GXII enables researchers to analyze large numbers of samples in a relatively short period of time – 41 seconds per sample or just over a hour to process a 96-well plate. Set up and operation of the instrument are easy and straightforward and samples can be run in either a 96 or 384 well format. Results can be viewed in real time while the instrument is running or at the completion of the run using the analysis software. The software calculates the percent purity of each band on the gel and provides an estimation of concentration that can be useful for downstream assays. A more accurate calculation, however, still requires reading the samples' absorbance at 280nm in a plate reader.

The analysis software allows data from several plates to be compared simultaneously. Data within a plate and across plates can easily be compared too. Reproducibility and reliability are good both between duplicates on a plate and duplicates between plates. Best of all, only a small amount of sample, 2 μL , is required for analysis and this amount is sufficient for several injections of the same sample.

Conclusion

The LabChip GXII provides a fast and efficient way of analyzing large numbers of protein variants. Its sensitivity, resolution and precision enabled the analysis even the lowest expressing variants in the library without using a significant amount of sample. Combined with PhyTip columns for small-scale purification of protein libraries, the two technologies provide a way to automate and miniaturize this part of the screening process improving the speed of data acquisition and ultimately the selection of candidates with desired therapeutic properties.

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