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Research paper

A simple high-throughput purification method for hit identification in protein screening

Emma Cummins [a,*], Deborah P. Luxenberg [b], Fionnuala McAleese [b], Angela Widom [b], Brian J. Fennell [a], Alfredo Darmanin-Sheehan [a], Matthew J. Whitters [b], Laird Bloom [b], Davinder Gill [b], Orla Cunningham [a]

[a] Wyeth Research Ireland, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland
[b] Wyeth Research, Cambridge MA 02140, USA

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ABSTRACT

Phage and ribosome display technologies have emerged as important tools in the high-throughput screening of protein pharmaceuticals. However, a challenge created by the implementation of such tools is the need to purify large numbers of proteins for screening. While some assays may be compatible with crude bacterial lysates or periplasmic extracts, many functional assays, particularly cell-based assays, require protein of high purity and concentration. Here we evaluate several methods for small-scale, high-throughput protein purification. From our initial assessment we identified the HIS-Select 96-well filter plate system as the method of choice for further evaluation. This method was optimized and used to produce scFvs that were tested in cell-based functional assays. The behavior of HIS-Select purified scFvs in these assays was found to be similar to scFvs purified using a traditional large-scale 2-step purification method. The HIS-Select method allows high-throughput purification of hundreds of scFvs with yields in the 50–100 μg range, and of sufficient purity to allow evaluation in a cell-based proliferation assay. In addition, the use of a similar 96-well-based method facilitates the purification and subsequent screening of large numbers of IgGs and Fc fusion proteins generated through reformatting of scFv fragments.

1. Introduction

As the pharmaceutical industry continues to expand its portfolio of protein therapeutic agents, new methods are continuously being sought to improve protein drug discovery and production. In the discovery phase, efforts are being made to develop high-throughput screening processes analogous to those used for small molecules. Technologies including phage and ribosome display generate thousands of proteins to be screened for lead identification and optimization of protein therapeutics. Like small molecule HTS, the goal of high-throughput protein screening is to enable testing of large numbers of molecules for activity in an assay that examines their biological interaction with the target. A successful HTS campaign will involve ranking of hits through a series of different screening assays that are both high-throughput and informative. Often, as the number of hits is narrowed down, the complexity of the assay or number of data points increases.

While some HTS assays may be compatible with crude bacterial lysates or periplasmic preparations, many functional, cell-based assays require protein of high concentration and purity for several reasons. Firstly, contaminating bacterial proteins and high endotoxin levels may interfere with cellular assays. Generally, the greater the complexity of the assay the greater the interference. Secondly, often a selected protein that does not express well may be missed

Abbreviations: HTS, high-throughput screening; IMAC, immobilized metal affinity chromatography; SEC, size exclusion chromatography; HTRF, homogenous time resolved fluorescence assays.

* Corresponding author. Tel.: +353 1 716 6976; fax: +353 1 716 6981.
E-mail address: cummine4@wyeth.com (E. Cummins).

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in screening assays conducted using crude extracts. Thirdly, the stability of the protein being tested may be compromised in crude preparations, forcing screens to be carried out under strict time constraints. Therefore, production of high quality material becomes a necessity, but also a bottleneck in the HTS process, limiting assays available for use with large numbers of clones. Medium scale systems have been developed such as the AktaExpress, which can purify up to 50 mg of protein from 16 samples in 11 h using a 2-step protocol (Chapman, 2005). In lead discovery, small quantities of protein are often sufficient to generate data to allow ranking of hits. While technical advancements have been made in the area (Chapman, 2005; Smith, 2005), a rapid, robust and low-cost methodology to purify protein at a high-throughput scale has not been reported. Small-scale methods on the market, or described in the literature, include resin and magnetic bead-based technologies in various formats. However, some of the methods described are cumbersome, time-consuming, and expensive, and are not amenable to scale up to large numbers of proteins. Bannister et al. (2006) describe a method for high-throughput purification of scFvs using customized equipment, including a computer-controlled syringe press and 2 liquid handling robots, that allows the generation of 150 μg of protein from 50 ml cultures. However, the system is limited to batches of 48 samples, with sample preparation carried out in 50 ml falcon tubes. This limits the throughput to 2000 samples per month, corresponding to 67 samples per day. Likewise, Su et al. (2007) report a high-throughput method which can process up to 300 scFvs per week per scientist, but requires the use of a dedicated liquid handling robot optimized for protein purification.

In our experience, 10 μg of scFv will often suffice for dose-dependent titration experiments to begin ranking clones. This shifts the emphasis from the quantity of protein purified per clone, to the number of clones that can be processed in a purified format at an early stage of screening. Here, we compare several high-throughput protein purification methods, amenable to standard automation, with yields of 50–100 μg of scFv from just 10–20 ml of culture, with a throughput of hundreds of scFv per day. A similar high-throughput method was also successfully optimized for the purification of IgGs and Fc fusion proteins. The ability to purify hundreds of scFvs and bivalent antibody formats in a time- and cost-effective manner enables earlier screening of proteins in more biologically relevant formats, providing more information for ranking hits.

2. Materials and methods

2.1. Expression of scFvs

ScFvs were expressed under the control of the LacZ promoter. They contain both hexahistidine and c-myc tags at the C-terminal and are secreted into the periplasm under the control of an *OmpA* leader sequence. *E. coli* TG1 colonies were selected on 2YT agar plates supplemented with 100 μg/ml carbenicillin, and incubated overnight at 37 °C. A single colony from each plate was inoculated in 10 ml of 2YT broth, supplemented with 100 μg/ml carbenicillin and 0.1% glucose, in a 50 ml Falcon tube. Each culture was grown at 37 °C shaking at 250 rpm until it reached an OD600 of 0.6. Expression was induced with 0.2 mM IPTG and cultures were incubated overnight at 30 °C and 250 rpm. Cultures were centrifuged at 1258 *g* for 10 min and supernatants discarded. The pellets were resuspended with 1.5 ml of ice-cold periprep buffer (50 mM Hepes, 0.5 mM EDTA, 20% sucrose, pH 7.5), followed by addition of 1.5 ml of a 1/5 dilution of the periprep buffer in water. Following incubation on ice for 30 min, the samples were centrifuged at 3220 *g* for 10 min. The supernatants were collected and $MgCl_2$ added to each to a final concentration of 10 mM. The periplasmic preparations were stored on ice. For large-scale purifications, periplasmic extracts were prepared in the same way using 500 ml culture and 20 ml of each periprep buffer to resuspend the resulting pellet.

For automation of periplasmic preparation, for samples purified in experiments represented in Figs. 3 and 4, the Qpix II robot (Genetix, New Milton, UK) was used to inoculate single colonies into 1 ml cultures in 20 replicated deepwell plates. The plates were then grown in a Multitron multi-plate incubator (Infors AG, Bottmingen, Switzerland). The extracts were prepared and pooled using the MiniTrak (Perkin Elmer Inc, Waltham, MA) and Multidrop (Titertek, Huntsville, AL) liquid handling robots.

2.2. Purification of scFvs

Purifications were carried out using pooled periplasmic preps and are described below. Each method was tested on a set of 10 different scFvs in duplicate.

2.2.1. IMAC Phytip™

The Phytip column technology (Phynexus Inc, San Jose, Ca) uses pipette tips pre-packed with immobilized metal affinity chromatography (IMAC) resin held between 2 inert screens. Protein-containing sample is drawn back and forth through the resin allowing equilibration, capture, washing and elution of the protein. Product application notes suggest that the tip capacity is 40 μg of protein for 200 μl tips and 100 μg with 1000 μl tips. The smaller tips used in this study are compatible for use with the MiniTrak microplate processing system. A protocol was optimized for purification of sample based on the manufacturer's instructions. In brief, 96-well deepwell plates were set up containing one well with 300 μl equilibration buffer (50 mM Tris, 300 mM NaCl, pH 8.0), 2 wells with 170 μl wash buffer (1/20 dilution of Phytip wash buffer; 50 mM phosphate buffer, 0.1 M imidazole, 1.5 M NaCl, pH 7.4), and 2 wells with 150 μl elution buffer (200 mM imidazole, 50 mM Tris, 300 mM NaCl, pH 8.0) for each sample. In this case, as there was 3 ml of periplasmic extract for each sample, 2 'capture' wells were set up with 1.5 ml extract per well. A program was designed on the MiniTrak as follows: 6 pre-rinse cycles, 25 capture cycles per capture well, 1 wash cycle per wash well, and 6 elution cycles per elution well, where each cycle represented an aspiration step followed by a dispense step at low pressure. Twenty-five cycles at a slow flow rate appeared to capture most of the recoverable scFvs, while 2 washes were sufficient to remove background proteins. Roughly two-thirds of the protein eluted in the first eluate. Samples were stored on ice until they were buffer exchanged.