High-throughput and high-yield purification of recombinant proteins expressed in *Escherichia coli*.

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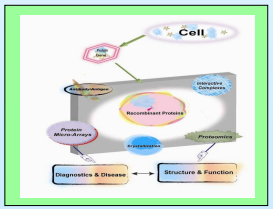
Abstract

Many biotechnologically important microbial processes, drug and biomarker discovery require understanding of protein function in cellular networks. Deciphering protein function creates high demand for production of large number of purified proteins of yield sufficient for protein crystallography, mapping of protein – protein interactions or developing affinity reagents (antibodies, aptamers) towards proteins of interest. Therefore, robust high throughput protein expression and purification processes need to be developed to meet this demand. We have developed a high throughput expression of recombinant proteins containing hexahistidine affinity tag in Escherichia coli followed by one step affinity purification of proteins on Ni $^{2+}$ agarose beads. Genes of interest were cloned using automated high throughput cloning. Proteins were expressed using producing strains of *E. coli* in the scale of 800 ml/clone using BactoliftTM (Lofstrand) or 25 ml/clone in 50 ml Falcon tubes. Proteins were then purified by IMAC on Ni^{2+} agarose from crude lysates either using filtration based low throughput – higher yield automated process on BioRobot 3000 (Qiagen) or using magnetic separation based high throughput - lower yield automated process on Biomek FX^{TM} robot (Beckman Coulter). Thus process parameters of protein purification of sets of 12 or 96 proteins could be compared, respectively. Although both processes provided proteins with comparable purity the Biomek-based process was technically much easier, much higher -throughput and less error-prone. Using those processes for automated high throughput production of milligram quantities of purified recombinant proteins is discussed.

Introduction

Since the number of proteins within an organism are several orders of magnitude greater than the number of genes, the proteins in the biological world is comprehensive and very often, are multi-functional.
To understand the structural and functional relationship of proteins, purified proteins are need for various studies, such as X-ray crystallography, nuclear magnetic resonance (NMR). To get insight into the intracellular chain of events, and to identify the biologically interactive network, proteins are being used in the sophisticated proteomics screening and analysis. Most importantly, a great number of proteins have been selected and applied in medical research, disease fighting and even bio-defense sectors; this includes the developing of antigens for vaccines, thermo-stable protein chips for protein arrays etc.
Taken together, it stands to reason that a large number of proteins in significant amounts are needed for various purposes. Consequently, development and application of a high-throughput (HTP) protein production method attract increased interests today. Furthermore, to achieve high efficiency purification, a variety of affinity tags are constructed into the coded recombinant proteins and over-expressed in *E.coli* or yeast expressing systems.
In our laboratory we have developed a high throughput protein production pipe-line which includes the expression of His-tag fused proteins followed by the use of an automation platform for purification.
In this presentation, we demonstrate our production pipe line at different stages, and compare the purification between a low throughput, large-scale mode with a high throughput, mini-scale mode. Our results suggest that the high throughput protein production method is indeed feasible and provide an assurance for the supply of the increased demand of high quality proteins.

Proteins in Play



Methods & Results

High-throughput (HTP) cloning

The hypothetical proteins from *S. oneidensis* were amplified from genomic DNA. The amplified fragments were cloned into a pMCSG7 vector . Genes encoding proteins containing signal sequences transmembrane regions were removed from the heterologous expression targets set. PCR primers were designed to amplify the coding region including a N-terminal 6xHis-tag and a TEV protease recognition site. After construction, the plasmid were then used for transformation into E. coli BL21 (DE3).

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Protein Expression

In the process of developing a HTP-purification, two different procedures were used for over-expression of proteins in *E.coli* cells.
The first one is the procedure for a large scale purification using the Bactolift expression system (see picture).
The second process is designed for the HTP –purification in an automation mode. Cells were cultured in 50 ml flacon tubes.

Expression in Bactolift

Bactolift is an air sparge fermentor (Lofstrand LTD, Gaithersburg, MD). The culture can be performed in 12 to 24 culture bottles with a medium capacity of 800 ml- 1 liter for multiple clones simultaneously. The agitation was achieved by pressing the air through the medium during culture using a flow-controlled air pump. The culture continued at 37oC for 3 hours. The cells were recovered by direct centrifugation of the bottles.

HTP-Expression in 50 ml tubes

When a large number of clones are being expressed at the same time (HTP-expression), the culture was performed in 50 ml falcon tube containing 25 ml LB medium in the presence of carbenicillin. The tubes were incubated at 30°C after induction with IPTG (Isopropyl-β-D-thiogalactopyranoside). Routinely, protein expressions of twenty-four or forty-eight clones were performed in a batch.

General characteristics of cell culture with Bactolift

Cells transformed with different plasmid were grown in 800 ml LB medium in Bactolift. Optical density of cells was measured at 600 nm before incubation and during the harvest

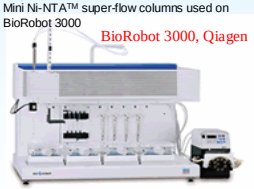
	OD_{600} at Induction	OD_{600} at Harvest	Cell Mass (g)	Average Protein (mg/ml)	Purified Protein (mg)
Mean± SD	0.9?3± 0.39	2.0?± 0.35	4.9?± 0.89	6.3± 2.7	11.8± 12.1
Number culture	34	34	34	34	34

Comments

Depending on the expression level of proteins the amounts of purified proteins were varied, ranging from 0.5 mg to 50 mg from a culture bottle.

Large-scale Purification for Bactolift Expression

Mini Ni-NTA™ super-flow columns used on BioRobot 3000



Expressed 6xHis-tag proteins are bound to a pre-packed Ni-NTA mini- column (1.5 ml bed volume)

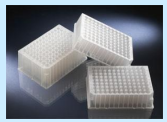
Cell lysis and purification

Different lysis procedures were used for the Bactolift expression and HTP-expression.

For Bactolift expression - Cell lysate was supplemented with 5 mM 2-Mercaptoethanol. Lysis of cells was completed by using sonication of the samples. The cell lysate was supplemented with 10 mM imidazole and 5% glycerol and applied to a pre-packed mini Ni-NTA™ super-flow column (1.5 ml, Qiagen). Either the robotic purification using Qiagen 3000 or manual gravitation filtration was used.

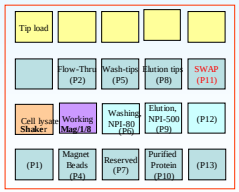
For HTP-expression - Aliquots of cell suspension corresponding to 30 OD at 600 nm were spun down. The pellets were either directly undergone lysis with the MagneHis™ Cell lysis reagent (Promega) or lyzed as suspension with FastBreakTM lysis reagent.

Different formats of 96-wells are used for HTP-purification



Working station for robotic HTP- purification

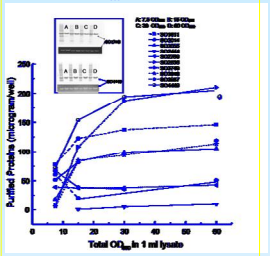
Labware and Alps



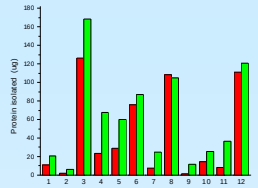
Robotic purification

Depending on forms of cells during harvest, two different cell lysis reagents, provided by Promega were used for two different experimental design.
As cell suspension - Cell suspension (0.9 ml) was transferred to a well of a 96-deep well plate (2.2 ml). 0.1 ml of 10x concentrated FastBreak™ Cell Lysis Reagent was then added to the suspension.
As cell pellets – MagneHis™ Cell Lysis Reagent (1ml) was directly add to the cell pellets that were previously presented in the wells of a 96-deep well plate.
The plate obtained either from a or b was incubated at room temperature for 30 minutes. The robotic purification was initiated by addition of 1 ml MagneHis™ Ni-particles (Promega) to the wells. Other purification parameter were entered into the programs. The steps for binding, washing and elutions were executed in sequence by the Biomex. During washing and elution, the MagneHis Ni-particles were captured with a Magnabot. The clear lysate or eluted proteins (purified product), respectively, were then robotic transferred to another clean 96-well plate.
The eluted protein were stored at -80°C in the presence 40% glycerol.

Effect of Cell Lysis : Purified Proteins as a function of total OD_{600} cells used for lysis

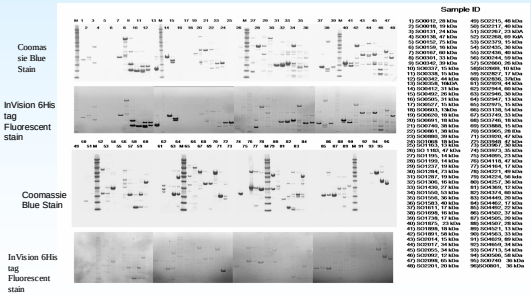


Comparison of Lysis between MagneHis™ cell lysis reagent and FastBreak ™ Lysis reagent



Red bar: lyzed in Fast Break reagent (Promega)
Green bar: lyzed in MagneHisTM cell lysis reagent (Promega)
1,SO3949; 2,SO3973; 3,SO4095; 4,SO4118; 5, SO4221; 6,SO4369; 7,SO4502; 8, SO4504; 9, SO4563; 10,SO4659; 11,SO4713; 12, SOA0160

Protein Pattern of HTP-purification on Automation Mode



Comparison of expression and purification between large scale (Bactolift) and mini-scale (HTP-mode)

	Large Scale	HTP-purification
culture volume (ml)/total cell mass for purification	800 (1600 OD)	30 OD/well
Purification methods	1.5 ml Ni-NTA mini-column	100µl MagneHis Ni-particles
Total number of samples	47	96 (in one 96-well plate)
Time required (including all prep steps and QC)	Two weeks	One week
Total average protein (mg)	13 (n = 46) (~ 800 ml)	0.25 (n = 96) (one well = 200 µl)
Yield µg/ OD at protein purity >90%	Mean= 8.7 (µg/OD) SD = 7.8 (µg/OD) N= 31	Mean = 8.02 (µg/OD) SD = 3.59 (µg/OD) N = 30
Yield µg/ OD at protein purity >80%	Mean= 8.46 (µg/OD) SD = 7.7 (µg/OD) N= 34	Mean = 7.33 (µg/OD) SD = 3.61 (µg/OD) N = 39

Analysis and Data Processing

Concentration of proteins in samples were assayed with Bradford Protein assay solution (BioRad, Hercules, CA). Purity of proteins was examined by running the samples in pre-NuPAG Pre-Caste SDS 4-12% polyacrylamide gels (Invitrogen, Carlsbad, CA) and stained with Gelcode Blue Plus (Pierce). His-tag proteins in gels were visualized using InVisionTM His Tag (Invitrogen) reagent.
Data about the protein purification and sample processing were maintained by spreadsheets and in house developed laboratory information management system (LIMS).

Summary

1. We demonstrate the production of purified proteins in two different modes: The large-scale, low throughput (Bactolift), and the mini-scale, high throughput automation. Statistically, there are no significant differences in yield, i.e. the amounts of purified proteins per cell mass, in term of the optical density of cell suspension at 600 nm.
2. While the Bactolift system with a production capacity to 50 mg, can be used for purification of proteins aiming at crystallography and NMR-studies the automation mode in a 96-well plate with a capacity of 0.25 mg/well can be used in expression screening for a large number of unknown proteins, for use in proteomics, antigen/antibody interactions and finally for identification of interacting protein within a protein network in cells.
3. With our current state, we are able to purify proteins from 24 to 96 clones weekly and able to deliver pure proteins from varied expression level, ranging from 0.1 to 50 mg/clone/well.
4. Further enhancement of the high throughput and speed in purification turn over is in progress by including direct cell culture in 96-deep well plate.
5. The presence of Data processing and management (Freeze-tracking and pLIMS) make it possible for storage and handle large amounts of samples.