Protein Expression and Purification 61 (2008) 92–98

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

A high-throughput microtiter plate-based screening method for the detection of full-length recombinant proteins

Matthias Mack [a,*], Maren Burger [b], Patricia Pietschmann [b], Björn Hock [b]

[a] Institute for Technical Microbiology, Mannheim University of Applied Sciences, Paul-Wittsack-Str. 10, 68163 Mannheim, Germany
[b] Merck Serono, Department of Protein and Cell Science, 64271 Darmstadt, Germany

ARTICLE INFO

Article history:
Received 26 March 2008
and in revised form 2 May 2008
Available online 20 May 2008

Keywords:
Protein production
Escherichia coli
Luminex® xMAP™ bead technology

ABSTRACT

The Gram-negative bacterium *Escherichia coli* is an important host for the (heterologous) production of recombinant proteins. The development and optimization of a protocol to overproduce a desired protein in *E. coli* is often tedious. A novel high-throughput screening method based on the Luminex® xMAP™ bead technology was developed allowing a rapid evaluation of a certain expression strategy. A variant of green fluorescent protein (GFPuv) from *Aequorea victoria* was used as a reporter to establish the methodology. The N-terminus and the C-terminus of GFPuv were engineered to contain a His_6- and an HA-tag (YPYDVPDYA), respectively. The double-tagged protein was loaded onto Luminex-microspheres via its His_6-tag, the presence of the HA-tag was verified using an anti-HA antibody. High-throughput detection of full-length proteins (containing both tags) on the beads was performed using an automated Luminex 100IS analyzer. The results were compared to results obtained by classical Western blot analysis. Comparison of the two methods revealed that the Luminex-based method is faster and more economical in detecting full-length (intact) soluble recombinant protein, allowing one to routinely screen a high number of parameters in gene expression experiments. As proof of concept, different protocols to overproduce double-tagged model eucaryotic proteins (human protein S6 kinase 1 and human tankyrase) in *E. coli* were monitored using the new approach. Relevant parameters for optimizing gene expression of the corresponding genes were rapidly identified using the novel high-throughput method.

Comparably rapid growth and protein production rates combine with extensive physiological knowledge and advanced genetic tools to make *Escherichia coli* one of the most powerful and versatile expression systems [1,2]. Notably, the majority of therapeutic proteins have been produced in either mammalian cell-culture systems or *E. coli* [3,4]. Although many alternative organisms and expression systems are now being considered for recombinant protein production [5,6], exciting progress continues to be made with *E. coli*. Nevertheless, the high-level production of functional full-length proteins, especially those from eukaryotic sources, in *E. coli* sometimes is difficult and methods for improving the production of proteins refractory to the typical techniques must be determined empirically [1]. This process is time-consuming and thus expensive. The present study was initiated in order to develop a solid and reliable method for the high-throughput evaluation of different expression strategies. The Luminex® xMAP™ system is a microsphere-based suspension array technology capable of analyzing and reporting up to 100 different reactions in a single reaction vessel [7–9]. This system proved to be an extremely useful platform for the immunological detection of intact (double-tagged) soluble recombinant proteins in cell-free extracts of overproducing *E. coli* strains. As proof of concept, the novel method was used to exemplarily monitor and optimize the heterologous production of two human proteins, protein S6 kinase 1 [10] and tankyrase [11].

Materials and methods

Bacterial strains and growth conditions for the 96-format gene expression experiments

The strains *E. coli* BL21(DE3) (Invitrogen), *E. coli* BL21 Star™ (DE3) (Invitrogen), *E. coli* BL21(DE3)pLysS (Novagen) and *E. coli* BL21-CodonPlus™ (DE3)-RILP (Stratagene) were used for all overexpression experiments. *E. coli* BL21(DE3) strains were designed for high-level protein production using T7 RNA polymerase-based expression systems. The BL21 Star™ (DE3) strains contain a mutation in the gene encoding RNaseE, which is one of the major sources of mRNA degradation in *E. coli*. *E. coli* BL21(DE3)pLysS cells provide tighter control for expression of toxic proteins. *E. coli* BL21-CodonPlus™ (DE3)-RILP cells carry extra genes to resolve the issue of codon bias for organisms with high AT content. The *E. coli* strains were aerobically grown at 37 °C in a nutrient broth (2×TYG) (pH 7.2) containing 1.6% (w/v) bactotryptone (Difco), 1% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl and 1% (w/v) glucose. Plasmid containing strains were cultured in 2×TYG containing 30 μg/ml

* Corresponding author. Fax: +49 621 292 6420.
E-mail address: m.mack@hs-mannheim.de (M. Mack).

1046-5928/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2008.05.005

kanamycin, 15 μg/ml gentamycin or 30 μg/ml chloramphenicol. Small scale (1 ml) cultures were grown for 16 h in 96 deep-well plates (Millipore) agitated at 1000 rpm in an orbital shaker. Fully grown cultures were diluted 1:25 using fresh 2×TYG. For chaperone co-overproducing *E. coli* strains [12] containing the plasmids pTf16 (producing trigger factor), pGro7 (producing GroES-GroEL), pKJE7 (producing DnaK-DnaJ-GrpE) and pG-KJE8 (producing DnaK-DnaJ-GrpE and GroES-GroEL) (Takara Bio Inc.) the corresponding inducers (arabinose, 25 mM or tetracycline, 10 μg/ml) were added. Subsequently, the strains were aerobically cultivated until the cultures had reached an optical density of 0.8 (OD_{600}). Gene expression was induced by adding isopropylthiogalactopyranoside (IPTG) (30–300 μg/ml). The cells were cultivated for another 4 h and the cell density of the cultures was determined photometrically at 600 nm. The cells were harvested by centrifugation (15 min, 2000*g*, 4 °C) and stored at −20 °C.

General DNA manipulations

For standard molecular biological manipulations, well-described protocols were used [13]. Restriction enzymes, T4 ligase, and DNA polymerase were purchased from New England Biolabs. The thermostable DNA polymerase used in PCRs was *Pfu* polymerase (Stratagene). Oligonucleotide primers were ordered from Sigma–Aldrich. The standard cycling conditions used for PCRs were as follows: 2 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C; and 1 cycle for 10 min at 72 °C. General DNA sequence analysis was performed with the Lasergene software (DNAstar Inc.).

Preparation of cell-free extracts

The frozen cell pellets were dissolved in BugBuster™ protein extraction reagent (Novagen). The amount of reagent was calculated according to the produced biomass (60 μl reagent per OD_{600} of 1.0). The mixture was gently agitated (700 rpm in a thermomixer "comfort", Eppendorf) for 20 min at room temperature. The lysates were centrifuged (4000*g*, 15 min) and subsequently cleared using filter plates (3/0.2 μm; Pall Corporation) and a vacuum station (MWG Biotech).

Western blot analysis

Western blots were prepared by electroblotting SDS–polyacrylamide gels onto nitrocellulose membranes (Bio-Rad) and probing with the mouse-anti-penta-His antibody (Qiagen) (1:1000 dilution) for 4 h, followed by incubation with goat anti-mouse peroxidase (POD) (Jackson Immunoresearch). The Western blots were blocked, washed, and probed at room temperature in 10 mM sodium phosphate (pH 7.4) containing 150 mM NaCl, 0.1% bovine serum albumin, 0.1% ovalbumin, 0.1% Tween 20, and 0.02% sodium azide. The detection of POD was performed using 3,3′-diaminobenzidine (Sigma–Aldrich) as a substrate (as described by the manufacturer).

Luminex-based detection of full-length proteins

The LiquiChip Ni-NTA-Beads (Qiagen) could directly be used in the Luminex assay for binding of His_6[1]-tagged proteins. In parallel, a second method to bind His_6-tagged proteins to microspheres (beads) using mouse-anti-penta-His antibodies (Qiagen) was evaluated and carried out as follows. PBS (10 mM $Na_2HPO_4{\cdot}2H_2O$, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl) was used to wash and resuspend the beads. Luminex carboxylated microspheres (LiquiChip Carboxy Beads, L100-C075-10; Luminex Corp.) (2.5×10^6 microspheres in 200 μl PBS) were separated from each other by vigorous shaking and treatment (30 s) in an ultrasound bath. The carboxy functions of the microspheres were activated by 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) to form an unstable amine-reactive *O*-acylisourea intermediate. Upon addition of *N*-hydroxysulfosuccinimide (Sulfo-NHS, 50 mg/ml in dimethylsulfoxide) the latter intermediate was stabilized by converting it to an amine-reactive Sulfo-NHS ester. After mixing at room temperature for 20 min (700 rpm) the microspheres were washed in PBS, resuspended in 200 μl antibody solution (50 μg/ml in PBS) and separated from each other as described above. After incubation for 2 h (700 rpm) the antibody conjugated microspheres were washed twice in PBS to remove unbound antibodies and resuspended in 100 μl storage buffer. The microspheres were counted (Luminex 100IS) as suggested by the manufacturer. The microspheres were stored at 8 °C and stable for at least 6 months.

Lysates (100 μl) derived from the recombinant *E. coli* cultures were mixed with 1500 microspheres in 30 μl CBS (PBS containing bovine serum albumin, 10 g/l). Binding of the His_6-tagged proteins to the beads occurred by incubation for 16 h (4 °C, 700 rpm). We found, however, that an incubation time of 1 h worked equally well. All subsequent washes were performed using 100 μl CBS. The loaded beads were washed three times and resuspended in 60 μl CBS. The primary antibody (rat anti-HA high affinity) was added (30 μl in CBS) and the mixture was incubated for 1 h (room temperature, 700 rpm) in the dark. After washing three times the beads were resuspended in 90 μl CBS. The secondary antibody was added (30 μl anti-rat R-PE; diluted 1:200). After incubation for 45 min (room temperature, 700 rpm) the supernatant was removed, the beads were washed three times, resuspended in 120 μl CBS, mixed (5 min, room temperature, 700 rpm) and 50 μl were subsequently analyzed with the Luminex 100IS system according to the instructions of the manufacturer (100 events/50 s per cavity). The PE fluorescence (MFI) measured from each bead was proportional to the number of double-tagged protein fusions.

Fluorescence spectroscopy

Fluorescence of GFPuv in diluted lysates (1:20 in PBS) of recombinant *E. coli* strains was determined using the Multilabel-counter Victor3™ 1420 (Perkin Elmer) (390 nm excitation and 510 emission). The measurements (100 μl) were performed in triplicate in black 96-well black bottom microtiter plates. The data were analyzed (i.e. mean values and standard deviations) using Microsoft Excel 2000.

Plasmid construction

For cloning using Gateway technology, the desired target DNA-fragments were amplified using PCR and *Pfu* polymerase. The PCR-products were ligated to the entry vector pDONR207 (Invitrogen) by BP-reaction using BP clonase (Invitrogen) following the manufacturer's instructions. All plasmid constructs were subjected to DNA sequencing and no secondary mutations were detected. The genes for GFPuv (GenBank Accession No. CVU62637), S6K1 (GenBank Accession No. 55953116) and TNKS (GenBank Accession No. AB051528) were introduced into pDONR207 using oligonucleotides, which also contained the information for TEV and HA. The

[1] *Abbreviations used:* His_6, hexahistidine HHHHHH; c-myc or myc, affinity tag derived from human myc protein, EQKLISEEDL; GFPuv, recombinant green fluorescent protein from *Aequorea victoria*; HA, affinity tag derived from influenza hemagglutinin-protein, YPYDVPDYA; TEV, tobacco etch virus protease cleavage site; ENLYFQG; GST, glutathione-S-transferase; MBP, maltose binding protein from *Escherichia coli*; S6K1, protein S6 kinase 1, TNKS (tankyrase).