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Choice of Efficient Molecular Biology Techniques for Bacterial Cell Surface Display

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Abstract— For *in vitro* assessment of proteins, cell surface display is a novel technique. Using flow cytometry and / or iterative selection procedures like biopanning, libraries of heterologous polypeptides can de displayed and monitored on the surface of bacterial and yeast cells. However, this is not possible without the choice of efficient strains, reagents etc. and some high-throughput molecular biology techniques. In this short technical note, we discuss the use and reliability of some highly efficient vectors, reagents and techniques that may enhance the expression of metal binding proteins on the surface of Gram-negative bacterial cells. The apt choices of these will facililate the surface display approach as a tool for fundamental and applied research in frontier areas like Bioremediation, Vaccinology and Biotechnology.

Index Terms— Bacteria, bioremediation, heterologous, molecular biology, protein, surface display, vector.

1 Introduction

Today, the exploitation of the prokaryotic (mainly bacteria) and eukaryotic (plants, yeast etc.) cell surfaces is a quarter of astounding continuing research. This novel trend has been intelligently pursued with significant and extensive applications covering different aspects of biotechnology and biological sciences in general ([1], [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15]). In this context, we have provided a detailed and critical account in an earlier review [16]. For instance, in Gram-negative bacteria, majority of the surface display systems target the surface-approachable loops of naturally displayed proteins by introducing external peptides. In Gram-positive bacteria, the thick peptidoglycan layers make them appropriate model candidates for stringent laboratory procedures and challenging field applications [5]. Till date, diverse surface displaying systems have been successfully developed [17], which can be classified into three major groups: C-terminal fusion, N-terminal fusion, and Sandwich fusion, based on their recombinant natures. It is also important to note that the progressive use of displaying metal-binding peptides on the surface of bacterial cells is another emerging trend, resulting in competent metal-binding ability. These recombinant bacteria may then act as biosensors. These may help in screening and quarantine of heavy metals in specialized bioremediation activities. So, it is now possible to synthesize ideal, conceptualized bacteria with these connecting strategies with increased specificity and affinity towards the target metal. This may also effect in considerable helpfulness of these types of bioadsorbents [11]. Thus, the novel cell surface display mechanism has been utilized in different biotechnological schemes [13], and it will continue to act as a promising tool for applied research in years to come. But in order to display a protein or a metal binding peptide on the surface of bacteria, we need competent molecular biology techniques that would result in quick and efficient expression of these on the surface and can be quantified and even progressed to field use. In this short technical note, we review some existing techniques but with careful choice of starting materials and processes, along with their principles.

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2 Expressing Metal Binding Polypeptides

2.1 Construction of target vector

First we need to prepare competent cells that will be transformed with the desired vector. The motivation is to scale up the production of vector for recombinant technology. The transformed cells will be selected with appropriate antibiotic and will be followed by overnight growth in enrichment medium. Isolation of plasmid DNA (vector) is the next step with checking the identity by Agarose gel electrophoresis. Restriction digestion (single / double) of the vector follows for the insertion of the target construct.

2.2 Synthesis of target construct / oligonucelotides

After extracting genomic DNA from bacteria, the restriction sites are analyzed and special oligos / primers are designed based on the target metal-binding protein on the bacterial surface. Amplification of these oligos is done with Polymerase Chain Reaction (PCR) technique to increase the amount exponentially. After restriction digestion with appropriate restriction endonucleses (special enzymes that cut within a nucleotide sequence or DNA), the target constructs are ready to be liagted with the cut vector (as explained in section 2.1).

2.3 Cloning and Expression in bacteria

The target construct is ligated to the cut plasmid with ligase, to produce the recombinant vector that possesses the target sequence to generate the polypeptides. After this cloning step, we again go through the original procedures of competent cell

preparation, transformation and selection to get the cloned cells. The recombinant vector is then isolated and the clones are confirmed with restriction digestion and agarose gel electrophoresis. After this, we check for expression in specialized bacterial cells after induction of protein synthesis by an inducer and extracting and running a Sodium-Dodecyl Poly Acrylamide Gel Elcetrophoresis (SDS-PAGE), to confirm the size of the protein / polypeptides.

3 Choice of Strains, Vector and Targets

The choice of strains varies according to the host organism, i.e. Gram-positive or Gram-negative bacteria. Here we have considered a well-known and robust Gram-negative bacterium *Escherichia coli* (*E. coli*) as a case study and studied the appropriate procedures as discussed in section 2.

3.1 Choice of cloning and expression strains

Among many strains available, BL-21 could be selected as the *E. coli* strain which should be used for the expression of metal-binding protein. This is based on certain advantages [18]. It has demonstrated higher levels of protein expression. There is increased stability of the recombinant protein which is being expressed. BL-21 strains represented as DE3 are used for expression from vectors utilizing the T7 promoter. Also, there is tighter control of 'leaky' expression with pLysS and pLysE strains. For the cloning purposes, DH-5α is the best choice [19]. This is backed by documented results. High Transformation efficiency has been observed: From $1x10^6$ to $>1x10^9$ transformants/micrograms. Due to endA1 mutation, there is an increased plasmid yield and quality. Also, the stability of the inserts is guaranteed due to recA1 mutation.

3.2 Choice of vector / plasmid DNA

pET-28c (5.367 Kb) from Novagen could be selected as the vector for cloning of target construct and subsequent transformation into BL21 (DE3) strain of *E.coli*. This is due to its efficient properties [18]. It has been the most powerful system yet developed for the cloning and expression of recombinant proteins in *E. coli* ([20], [21]). Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals. It is also possible to attenuate the expression level simply by lowering the concentration of inducer. It has the ability to maintain target genes transcriptionally silent in the uninduced state.

3.3 Choice of target / model insert

Poly-(histidine – cysteine) peptide (containing 6 histidine and 6 cysteine residues) may be used as the model insert for two imprtant reasons [15]. They are very good chelators for divalent metal ions, such as Cd^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, and Pb^{2+}, and therefore may be used as biosorbents for heavy metal removal. The permissive size limit of the polypeptide to be fused to the external loop of OmpC can be easily examined by inserting different numbers of copies of His and Cys linkers.

3.3 Choice of target metal-binding protein

External loop 7 of the Outer membrane protein (Omp) C of E. coli may be good candidate as the point of insertion for foreign peptides (like poly-his-cys-peptides) for cell surface display [15]. In general, the amino acid sequences of the external loops are less conservative. These loops may also be relatively tolerant to insertion and deletion.

4 Principle of Major Techniques / Steps

4.1 Competent Cell and Transformation

Competence can be artificially induced in the cells by treating with $CaCl_2$. The calcium ions help in destabilizing the cell membrane and it also makes the DNA, which is taken in during transformation, resistant to special class of enzymes called DNases (these chew off DNA) by forming a calcium phosphate DNA complex. *E. coli* cells become permeable to DNA molecule when they are harvested during exponential growth phase and treated with calcium salts at 0^oC. Such cells are said to be competent and are capable of being transformed with circular plasmid or phage DNA [22].

During transformation the plasmid DNA is mixed with the competent cells at 0^oC and the mixture is gently heated for about 90 seconds at 42^oC (heat shock). During this time the DNA enters into the cells. After the uptake of DNA the cells are in the growth media to allow the expression of the plasmid–coded genes prior to plating of the selective medium.

4.2 Plasmid DNA Isolation (miniprep)

Competence can be artificially induced in the cells by treating with $CaCl_2$. The calcium ions help in destabilizing the cell membrane and it also makes the DNA, which is taken in during transformation, resistant to special class of enzymes called DNases (these chew off DNA) by forming a calcium phosphate DNA complex. *E. coli* cells become permeable to DNA molecule when they are harvested during exponential growth phase and treated with calcium salts at 0^oC. Such cells are said to be competent and are capable of being transformed with circular plasmid or phage DNA. Alkaline lysis depends on a unique property of plasmid DNA: its ability to rapidly anneal following denaturation [23]. This is what allows the plasmid DNA to be separated from the bacterial chromosome. Typically, *E. coli* cells are grown that contain the plasmid of interest. Then the cells are lysed with alkali and the plasmid DNA is extracted. The cell debris is precipitated using SDS and potassium acetate. This is spun down, and the pellet is removed. Isopropanol is then used to precipitate the DNA from the supernatant, the supernatant is removed, and the DNA is resuspended in buffer (often 1X Tris-EDTA). A mini prep usually yields 5-10 micrograms of plasmid DNA. This can be scaled up to midi prep or maxi prep, which will yield much larger amounts of DNA.

4.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique most commonly used for separation based on molecular size in the presence of electric current. Ethidium bromide, a fluorescent dye that is used to detect DNA in agarose, reduces the electrophoretic mobility of linear DNA by about 15%. Larger molecules of linear double stranded DNA migrate more slowly because of their greater frictional drag and pass through the pores of the gel less efficiently than smaller molecules. Super helical (form – I), nicked circular (form- II) and linear (form-III) DNA of the

same molecular weight migrate through agarose gels at different rates [24]. The relative mobilities of the three forms depend primarily on the agarose concentration in the gel, but the strength of applied current, the ionic strength of the buffer and the density of the super helical twists in the form – I DNA also influence them. TAE is the most commonly used buffer. Agarose gels are usually run in horizontal configuration. If electric field is applied across the gel, DNA, which is negatively charged at neutral pH, migrates towards the anode. The samples get separated based on their molecular size and charge. Hence by using a marker DNA, the size can be found out. Agarose gels have a lower resolving power than the polyacrylamide gels but have a greater range of separation.

4.4 Restriction digestion

Restriction enzymes, also known as restriction endonucleases, are enzymes that cut a DNA molecule at a particular place. They are essential tools for recombinant DNA technology. The enzyme "scans" a DNA molecule, looking for a particular sequence, usually of four to six nucleotides. Once it finds this recognition sequence, it stops and cuts the strands. This is known as enzyme digestion [25]. On double stranded DNA the recognition sequence is on both strands, but runs in opposite directions. This allows the enzyme to cut both strands. Sometimes the cut is blunt; sometimes the cut is uneven with dangling nucleotides on one of the two strands. This uneven cut is also known as sticky ends. For instance, a blunt end may look like this (N: any nucleotide, A: Adenine, T: Thymine):

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5' NNNNNNNATT      AATNNNNNNNNN 3'
3' NNNNNNNTAA      TTANNNNNNNNN 5'
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On the other hand, a sticky end may look like this:

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5' NNNNNNNNNNNG        AATTCNNNNNN 3'
3' NNNNNNNNNNNCTTAA        GNNNNNN 5'
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Restriction enzyme digestions are performed by incubating double-stranded DNA molecules with an appropriate amount of restriction enzyme, in its respective buffer as recommended by different companies, and at the optimal temperature for that specific enzyme [22]. The optimal sodium chloride concentration in the reaction varies for different enzymes, and a set of three standard buffers containing three concentrations of sodium chloride are prepared and used when necessary. Typical digestions included a unit of enzyme per microgram of starting DNA, and one enzyme unit usually is defined as the amount of enzyme needed to completely digest one microgram of double-stranded DNA in one hour at the appropriate temperature. These reactions usually are incubated for 1-3 hours, to insure complete digestion, at the optimal temperature for enzyme activity, typically 37°C. Factors that influence restriction enzyme activity are [26]: buffer composition, incubation temperature, influence of DNA methylation, star activity etc.

4.5 Genomic DNA extraction

Phenol extraction and ethanol precipitation of DNA is the most commonly used method of purifying and concentrating DNA preparations [27]. The DNA solution is first extracted with a phenol/chloroform/isoamyl alcohol mixture to remove protein contaminants, and then precipitated with 100% ethanol. The DNA is pelleted after the precipitation step, washed with 70% ethanol to remove salts and small organic molecules, and resuspended in buffer at a concentration suitable for further experimentation. Phenol/chloroform is used to purify the DNA (removes proteins and inactivates DNAses). The isoamylalcohol in the phenol/chloroforn is used for better seperation between phenol-layer and water layer (containing the DNA). The second chloroform step aims to remove all remaining phenol from the water phase [22].

4.6 Designing Primers / oligonucleotides for PCR

Optimal length of PCR primers should be between 18-22 base pairs (bp). But it may extend up to 40 bp, depending upon the circumstances. Melting Temperature (T_m) by definition is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability [22]. Primers with melting temperatures should be in the range of 52-58°C for achieving the best results. Primers with melting temperatures above 65°C will have a tendency for secondary annealing. The Guanine-Cytosine (GC) content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be between 40-60%. The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer. Presence of the secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. They adversely affect primer template annealing and thus the amplification and greatly reduce the availability of primers to the reaction. Primers should be designed such that they do not form self dimer (formed by intermolecular interactions between the two same sense primers, where the primer is homologous to itself) or cross dimer (formed by intermolecular interaction between sense and antisense primers, where they are homologous) [22].

4.7 Polymerase Chain Reaction (PCR)

It is the amplification of a small amount of DNA into a larger amount. The procedure is quick, easy, and automated. Four things are needed to perform PCR on a sample [22]: (i) *The target sample*: the biological sample needed for amplifying the DNA. (ii) *Primers*: Short strands of DNA that adhere to the target segment. They identify the portion of DNA to be multiplied and provide a starting place for replication. (iii) T*aq DNA polymerase*: This is the enzyme that is in charge of replicating DNA. This is the polymerase part of the name polymerase chain reaction. (iv) *Nucleotide*: Nucleotides (dNTPs) must be added so that the DNA polymerase has building blocks to work with.

There are three major steps to PCR [28] and they are repeated over and over again. This is where the automation is most appreciated. Thermal cycling profile for standard PCR: (i) *Initial denaturation*: Initial heating of the PCR mixture for 2 minutes at 94–95°C is enough to completely denature complex genomic

DNA so that the primers can anneal to the template as the reaction mix is cooled. If the template DNA is only partially denatured, it will tend to 'snap-back' very quickly, preventing efficient primer annealing and extension, or leading to 'self-priming', which can lead to false-positive results. (ii) *Denaturation step during cycling*: Denaturation at 94–95°C for 20–30 seconds is usually sufficient, but this must be adapted for the thermal cycler and tubes being used. If the denaturation temperature is too low, the incompletely melted DNA 'snaps-back' as described earlier, thus giving no access to the primers. Use a longer denaturation time or higher denaturing temperature for GC-rich template DNA. (iii) *Primer annealing*: For most purposes, annealing temperature has to be optimized empirically. If the temperature is too high, no annealing occurs, but if it is too low, non-specific annealing will increase dramatically. (iv) *Primer extension*: For fragments up to 3 kb, primer extension is normally carried out at 72°C. T*aq* DNA Polymerase can add approximately 60 bases per second at 72°C. A 45-second extension is sufficient for fragments up to 1 kb.

In an optimal reaction, less than 10 template molecules can be amplified in less than 40 cycles to a product that is easily detectable on a gel stained with ethidium bromide. Most PCRs, therefore, include only 25 to 35 cycles.

4.8 PCR purification

The Gen EluteTM Gel Extraction Kit is designed for the rapid purification of 50 bp to 10 Kb linear DNA fragments and plasmids from standard or low-melting agarose gels. This kit can also be used to purify DNA from polyacrylamide gels.

This kit combines silica-binding technology with the convenience of a spin or vacuum column format [37]. DNA fragments of interest are extracted from slices of an agarose gel by solubilizing the gel. The Gel Solubilization Solution can dissolve an agarose slice from gels run in either TBE or TAE buffer. This solution also contains a pH indicator that allows the gel slice to be visualized easily and indicates whether the pH is optimal for DNA binding. The extracted DNA fragments are then selectively adsorbed onto a silica membrane in the presence of the Gel Solubilization Solution. Contaminants are removed by a simple spin or vacuum wash. Finally, the bound DNA is eluted in Tris Buffer. The isolated DNA is suitable for a variety of downstream applications, such as automated DNA sequencing, restriction digestion, cloning etc.

The rest steps like ligation (with T4 DNA Ligase), protein induction (with IPTG) and SDS-PAGE to detect protein size are common and one can follow standard molecular biology protocols [Sambrook and Russell, 2001]. The principles of the above mentioned steps are more or less common for any application. However, the choice of target protein, vactor, bacterial strains will vary depending on the array of factors mentioned in section 1.

5 Conclusions

In this technical note, we have reviewed the most efficient molecular biology techniques like transformation, plasmid isolation, phenol-chloroform extraction, restriction digestion, PCR etc. along with choice of some productive reagents and starting material required to express foreign peptides to the metal binding proteins on the surface of bacterial cells. While the techniques and materials discussed here are not absolute and exhaustive, but nonetheless, for a small scale project, this may be useful information. Often it's a common problem to choose the right molecular biology components to achieve effective surface display and there lies a gap between the right knowledge and high-throughput resources available. This study helps to bridge that gap.

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