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# Bacteria-Based *In Vivo* Peptide Library Screening Using Biopanning Approach

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**Traditionally, library screening has been performed to identify biologically active agents including small molecules or peptides that inhibit target proteins or molecules with therapeutic interests. Due to its chemical nature, library screening is usually performed under *in vitro* environments using purified proteins and molecules. However, active agents identified from *in vitro* screenings often fail to exhibit biological activities in cells. To overcome this inherent limitation, we have developed an *in vivo* peptide library screening system that allows for the identification of dissociative inhibitors of protein interactions of interest. The screening is based on the reconstitution of the cI repressor from bacteriophage lambda with high-density expression peptide library and is entirely performed in bacteria cells. Furthermore, to enhance the efficacy and sensitivity of the screening, a multiple-round biopanning approach was employed for amplification and enrichment of positive peptides. Overall, this *in vivo* screening should provide a fast and efficient tool for identification of biologically active peptide molecules against target protein assembly.**

***Keywords*:** peptide library, *E. coli*, screening, biopanning, lambda repressor

Many biological processes, such as signal transduction, cell cycle regulation, gene regulation, viral assembly and replication, rely on protein-protein interactions. Flow of inter- and intracellular information is largely governed by the combination of protein-protein interactions, and the identification of the network of those interactions among proteins is the key to understand the cellular mechanism of interest (Rual *et al.*, 2005). During the last couple of decades, because of the availability of methodologies like the yeast two-hybrid system (Fields and Song, 1989; Chien *et al.*, 1991) and the phage display system (Smith, 1985), many important protein-protein and protein-peptide interactions have been identified from many different organisms. Revealing the biological function of a gene and its encoded protein can often be achieved by identification of its interacting partner and its place in the cascade of protein-protein interactions. The identification of a critical protein-protein interaction is, however, only the first step. Modulation of the interaction is necessary to produce true insight into its biological purpose (Souroujon and Mochly-Rosen, 1998).

In a previous study (Park and Raines, 2000), we have developed an *in vivo* genetic selection for dissociative inhibitors of protein interactions from a peptide library. The selection was based on the artificial reconstruction of a hybrid repressor (NcI-X) from bacteriophage lambda (Pabo *et al.*, 1979; Hu *et al.*, 1990). When the hybrid repressor was expressed in bacteria cells along with an encoded peptide library, only cells with an inhibitory peptide could survive due to the growth selection using tetracycline resistance reporter. Key components of the selection to be expressed in a bacteria cell included a hybrid repressor, a reporter module ($\lambda P_R$-*lacZ-tet*), and a random peptide library. In the previous study, however, the hybrid repressor and the reporter module were expressed from the same plasmid whereas the peptide library was expressed from a separate plasmid. One drawback of this two-plasmid system is the difficulty in controlling the relative expression level of the hybrid repressor and the reporter.

In this study, we attempted to improve the screening with several modifications. First, the hybrid repressor and the reporter module were expressed from separate plasmids to enable fine tuning of expression levels of these components and to facilitate cloning of various target hybrid repressors. Second, multiple-round biopanning approach (Mandecki *et al.*, 1995; Giordano *et al.*, 2001) was incorporated to enable amplification and enrichment of positive candidates from each round of panning. In successive rounds of panning, the condition for the growth selection became gradually stringent to ensure the survival and propagation of positive candidates. We have confirmed that these modifications enhanced the efficiency, speed, and sensitivity of the selection by monitoring reporter gene activities and biochemical properties of repressors and by analyzing the sequences of candidate peptides from each round of biopanning. Together, this *in vivo* screening should allow for an efficient and high-throughput identification of biologically active peptide molecules against target protein assembly.

## Materials and Methods

### Bacterial strains and plasmids

*E. coli* TG1 strain was used for molecular cloning and library screening. Hybrid repressors to be used for the gel shift assays were expressed in BL21(DE3) strain. pACYC184 plasmid was used as a parental plasmid for the repressor plasmid. *pLacUV5* promoter and the DNA binding domain of cI repressor (NcI) were generated by PCR from pJH391 (Park and Raines, 2002). Leucine zipper domain (Zip) and APIP were generated from pSH29 (Park and Raines, 2000) and

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pCDNA3-APIP (Cho *et al.*, 2004) (a kind gift from Y. K. Jung lab) by PCR, respectively. Each PCR fragment was inserted into pACYC184 at *Cla*1 and *BspH*1 sites. The resulting plasmids were labeled pSH2128 for NcI-Zip and pSH2135 for NcI-APIP. The pSC101 replication origin and the reporter module were prepared from pKD46 (Baba *et al.*, 2006) (a kind gift from J. H. Roe lab) and pSH20 (Park and Raines, 2000), respectively and were used to construct the reporter plasmid pSH2209. Library plasmid was constructed as described in the previous studies (Cwirla *et al.*, 1990; Labean and Kauffman, 1993; Park and Raines, 2000).

**Reporter assay**

TG1 cells carrying reporter and repressor plasmids were grown in LB media containing ampicillin (50 μg/ml), kanamycin (25 μg/ml) and chloramphenicol (34 μg/ml). At mid-log phase ($OD_{600}$=0.5-0.8), cells were treated with isopropyl-β-D-thiogalactoside (IPTG; 20 μM) for 3 h. The induced cells were harvested and β-galactosidase activity was measured after permeabilization using SDS/chloroform (Kippert, 1995).

**Library screening and biopanning**

Repressor plasmid (pSH2135) and reporter plasmid (pSH2209) were transformed into *E. coli* TG1 strain. The transformants were again transformed with the library plasmid (200 ng) using electroporation to yield more than $1\times10^8$ transformants. Transformed cells were spread on selection plates with LB agar containing carbenicillin (50 μg/ml), kanamycin (25 μg/ml), chloramphenicol (34 μg/ml), IPTG (20 μM), and tetracycline (15 μg/ml) and were incubated for 16 h at 37°C until $Tet^R$ colonies appeared. Twenty colonies were randomly picked and were subject to plasmid mini-prep for sequence analysis. The $Tet^R$ colonies were eluted from plates with 2 ml of fresh LB media. Washed colonies were harvested by centrifugation at 13,000 rpm for 10 min and library plasmids were purified using a mini-prep kit. For the second round panning, purified plasmid (400 μg) was re-transformed by electroporation and the transformants were spread on selection plates containing tetracycline (20 μg/ml) and were incubated for 16 h at 37°C. The procedure was repeated for the third round panning with selection plates containing tetracycline (25 μg/ml).

**Gel shift assay**

NcI-Zip and NcI-APIP proteins were produced from the BL21(DE3) strain and were partially purified. A fluorescein-labeled, double-stranded DNA probe corresponding to the operator sequence of λPr promoter was synthesized. The gel shift assay was performed as described (Park and Raines, 2000).

**Sequencing and sequence analysis**

At each panning step, twenty colonies were randomly picked and library plasmids were purified by DNA LaboPass purification kit (Cosomo Genetech Inc., Korea). Purified plasmids were sequenced using a sequencing primer 5′-GCTAGTTATTGCTCAGCGG-3′ at the Genome Research Facility in Seoul National University. DNA sequences of screened peptides were translated into protein sequence and were aligned using the VectorNTI software (Insilicogen, Inc., USA) with the Blosum85 algorithm (Corpet, 1988; Mount, 2008). The color scheme in the sequence alignment is as follows: no color for residues with no similarity, pale gray for residues with weak similarity to the consensus residues, dark gray for consensus residues with occurrence of greater than 50% and black color for consensus residues derived from a block of similar residues.

## Results and Discussion

**Modifications of library screening**

To overcome the drawbacks of the previous screening strategy (Park and Raines, 2000), we decided to produce the hybrid repressor and reporter modules in separate plasmids in bacteria cells. For this purpose, the hybrid repressor was expressed from a repressor plasmid harboring a p15A replication origin (Corpet, 1988) and the reporter module was expressed from a reporter plasmid carrying a pSC101 (Manen *et al.*, 1989) replication origin (Fig. 1). In the previous screening system it was difficult to replace and/or change the target hybrid repressor because a large reporter module was co-expressed from the same plasmid. The independent expression of reporter and repressor modules enabled fine tuning of the expression levels of each modules and facile cloning of target hybrid repressors.

The screening method was also improved by incorporating the multiple-round biopanning strategy (Mandecki *et al.*, 1995; Giordano *et al.*, 2001; Ellis *et al.*, 2011). Traditionally, biopanning strategy was often adopted in *in vitro* screening methods such as phage display system to amplify and enrich positive candidates (Sternberg and Hoess, 1995; Chen *et al.*, 1996; Ehrlich *et al.*, 2000). It was believed that biopanning was not technically compatible with *in vivo* screening methods due to

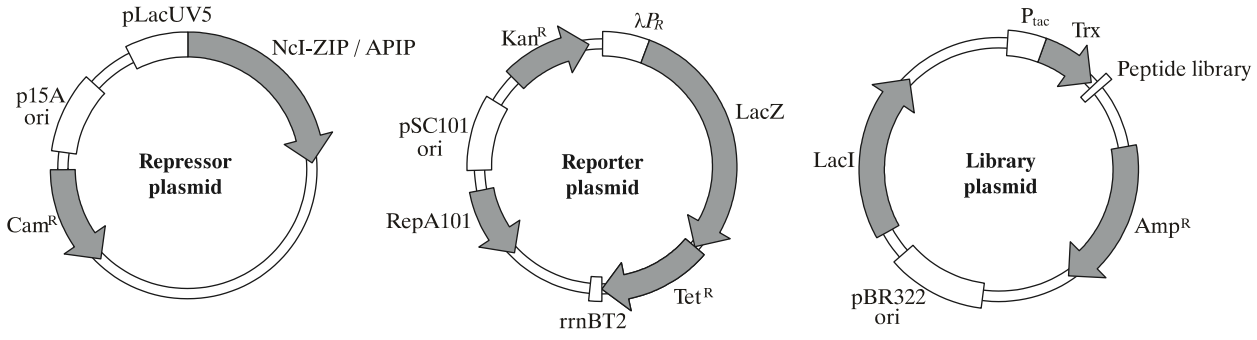


**Fig. 1.** Map of the repressor plasmid, the reporter plasmid and the library plasmid used in the screening. All three plasmids carry different replication origins and therefore are compatible in bacteria cells.

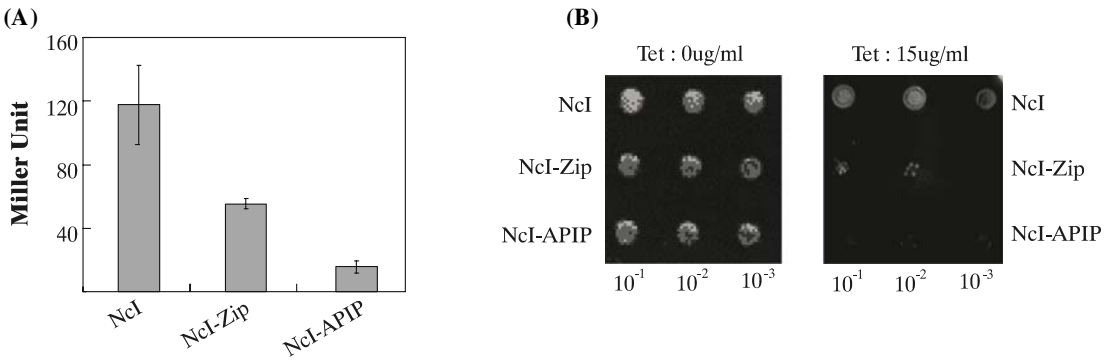


**Fig. 2.** Validation of activity of the reporter genes used in the screening. Cells carrying the λPr-*LacZ-tet* reporter module and hybrid repressors were tested for LacZ activity and tetracycline sensitivity. APIP and ZIP proteins were chosen as model dimerizing proteins and were fused to the NcI domain to create hybrid repressors, NcI-APIP and NcI-Zip, respectively. (A) LacZ activity was monitored for NcI alone, NcI-ZIP and NcI-APIP repressors. NcI-ZIP and NcI-APIP showed a noticeable decrease in the enzyme activity as compared to the NcI anone control. (B) Cells carrying hybrid repressors were tested for the sensitivity to tetracycline. Cells were plated at various dilutions onto LB-agar containing tetracycline at the concentration of 0 (left) and 15 μg/ml (right). In the right panel, cells carrying NcI-ZIP and NcI-APIP repressors fail to survive indicating that hybrid repressors were functional and turned off the $Tet^R$ reporter to convey $Tet^S$ phenotype.

chemical and biological heterogeneity of live cells. In our system, the tight growth selection in which only cells with positive candidates can survive allowed for the biopanning with multiple rounds of screenings. In each round of panning, only cells with positive candidates exhibited the tetracycline resistance and were carefully recovered for the next round of panning. In successive rounds of panning, the stringency of selection was gradually increased for amplification and enrichment of positive clones. The application of biopanning strategy greatly improved the efficiency and sensitivity of the screening.

## Validation of screening reporters

The lambda repressor protein (cI) used in the screening binds to its operator DNA as a homodimer. The cI repressor has two distinct domains: the N-terminal DNA binding domain (NcI) and the C-terminal dimerization domain (Pabo *et al.*, 1979; Hu *et al.*, 1990). The reporter genes used in the screening were the β-galactosidase gene (*LacZ*) and the tetracycline resistance gene ($Tet^R$), When a hybrid repressor, in which dimerizing or interacting proteins were fused to the NcI domain, is functional inside a cell the transcription of reporters should be repressed and thus bacteria cells should exhibit low LacZ activity and sensitivity to tetracycline. To validate the reporter functions, we tested leucine zipper domain (Zip) and APIP, which are known as dimeric proteins, in hybrid repressor. When fused to NcI domain in hybrid repressor, both Zip and APIP showed a dramatic decrease in the LacZ activity and sensitivity to tetracycline (15 μg/ml) compared to the control of a mutant repressor with NcI domain alone (Fig. 2). The hybrid repressors of NcI-APIP and NcI-ZIP efficiently repressed the expression of reporters.

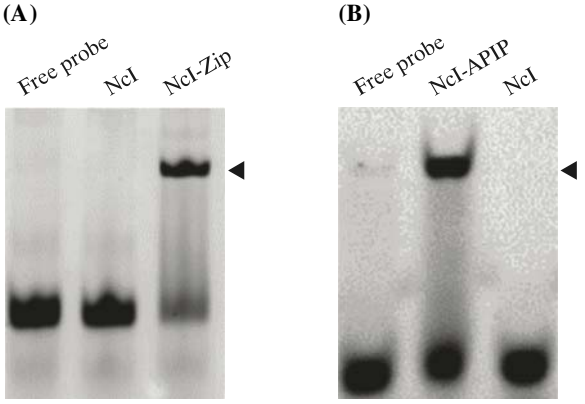


**Fig. 3.** Gel shift assay using a fluorescently labeled DNA probe corresponding to the $\lambda O_{R1}$ operator. The binding of NcI-Zip (A) and NcI-APIP (B) hybrid repressors to the probe DNA was monitored. The hybrid repressors caused a electrophoretic retardation compared to controls such as free probe and NcI alone.

## Gel shift assay

To biochemically validate the function of hybrid repressors, gel shift assays were performed in which a fluorescently labeled DNA was used as a probe along with partially purified hybrid repressor proteins. Functional repressor proteins should bind to the probe DNA based on the operator of $\lambda P_R$ promoter and should cause the retardation in the migration of DNA probe. NcI-APIP and NcI-Zip hybrid repressors clearly showed the retardation in electrophoretic mobility of probe DNA as compared to the control of free probe and NcI do-

**Table 1.** Results of the three rounds of panning. Colony counts and tetracycline concentration are indicated. At each panning, concentration of tetracycline was increased by 5 μg/ml. Twenty colonies were randomly picked and were subject to further analyses including sequencing and sequence alignment at each panning step.

| | Concentration of tetracycline (μg/ml) | Number of $Tet^R$ colonies | Number of total transformants |
|---|---|---|---|
| 1st panning | 15 | 1270 | $2.37\times10^8$ |
| 2nd panning | 20 | 322 | $1.29\times10^8$ |
| 3rd panning | 25 | 184 | $3.28\times10^8$ |

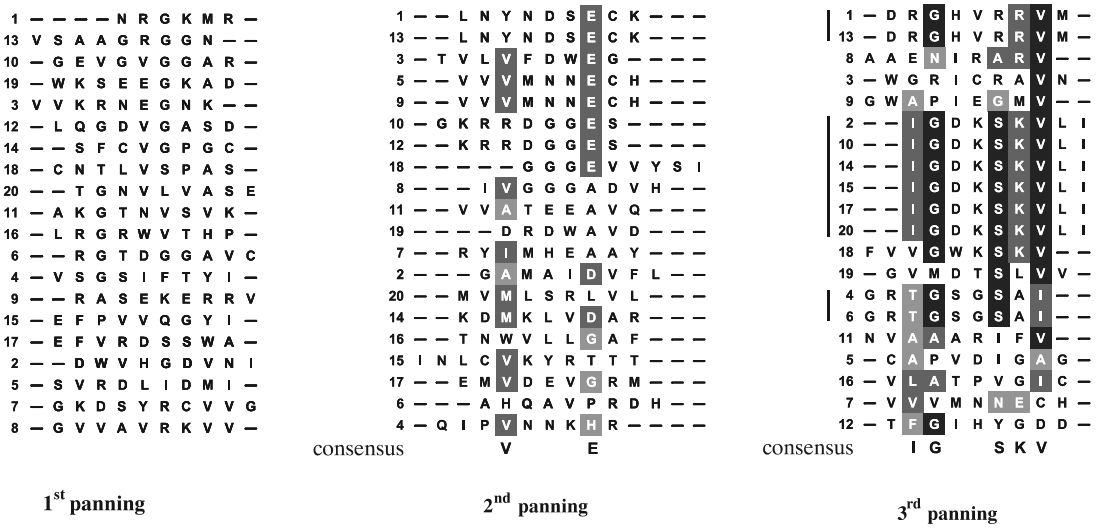


**Fig. 4.** Sequence alignment of random twenty candidates from three panning steps. Peptide sequences were aligned using the Blosum85 algorithm. The result clearly showed that sequences of candidate peptides converged into the consensus sequence with high survival probability during successive rounds of biopanning indicating amplification and enrichment of peptides took place. Color scheme of aligned residues was described in the 'Materials and Methods'.

main alone (Fig. 3). The result indicates that the hybrid repressors tested in the assay can recognize and bind to the operator of the $\lambda P_R$ promoter and turn off the reporter genes. Thus, leucine zipper domain and APIP seem to be suitable for the library screening as a target protein.

### Library screening and biopanning

A peptide library in which nine random amino acids residues were fused to the C-terminus of thioredoxin (Trx) from *E. coli* (Houghten, 1993) was used in the screening. The library contained about $5\times10^8$ independent clones. APIP was tested as a model target of the screening. Bacteria cells harboring both reporter plasmid and NcI-APIP repressor plasmid showed $LacZ^-$ and tetracycline sensitive ($Tet^S$) phenotypes as shown in the Fig. 2. Then, $LacZ^-$, $Tet^S$ cells were transformed with the library plasmid and were monitored for $Tet^R$ phenotype by incubation in the media containing tetracycline at 15 μg/ml. Of $2.37\times10^8$ transformants, 1270 colonies showed $Tet^R$ phenotype at the first round panning. The $Tet^R$ cells were recovered and pooled for the second round panning in which the concentration of tetracycline was increased to 20 μg/ml. The panning was performed in three rounds and the result of each panning was summarized in the Table 1. In successive rounds of panning, the number of $Tet^R$ colonies gradually decreased indicating that the candidate pool was becoming homogeneous and the enrichment of library peptides was in progress.

### Sequence analyses of library peptides

To validate the enrichment of library peptides during biopanning, twenty $Tet^R$ candidates at each panning were subject to sequence analyses. The sequences of 20 candidate peptides from $Tet^R$ colonies were aligned each other using the Blosum85 algorithm (Fig. 4). Peptides from the first round panning did not exhibit a noticeable sequence similarity. It is not surprising that the alignment resulted in low sequence similarity considering that only one in $10^5$ cells survived the selection in the first round panning. Peptides from the second round panning showed a moderate similarity whereas peptides from the third round panning showed a strikingly high similarity in sequences. Of twenty aligned, ten peptides were identical in sequence as indicated with vertical blocks in the Fig. 4 (right panel). The presence of identical peptides with a significant abundance suggests that multiple rounds of biopanning enforced convergence of amplified candidates into certain sequences with high probability of survival. The consensus sequence derived from the alignment was I G x x S K V. The non-identical peptides also showed a clear sequence similarity with the identical ones. This result strongly indicated that library peptides were amplified and enriched during the multiple rounds of biopanning. We believe that application of biopanning approach to the *in vivo* screening should provide an efficient and sensitive tool for identification of biologically active peptides that specifically disrupts target protein complexes.

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