Applying the E. coli’s twin-arginine translocation pathway to isolation of biomarker-specific nanobodies from a synthetic cameledized human nanobody library

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Abstract. Monoclonal antibodies (mAbs) have been used extensively both for treatment and diagnostics. Phage display has been successfully used for isolation of many mAbs currently sold in the market. However, the main drawback is that it could result in a large number of false positives. In this study, we explored the feasibility of combination of two powerful antibody isolation techniques, phage display and Functional Ligand-binding Identification by Tat-based Recognition of Associating Proteins (FLI-TRAP), to identify nanobodies (Nbs) that are specific to HBsAg, an antigen commonly used for hepatitis B infection diagnostics. A synthetic cameledized human nanobody library was subjected to 2 rounds of biopanning against HBsAg adr subtype, commonly found in southeast Asia. As expected, sequencing analysis of all 12 randomly selected clones from biopanning showed truncated Nbs, representing false positive. Full-length Nb genes were amplified from the phage eluted during the 2nd round of biopanning was subcloned into FLI-TRAP system for isolation. For evaluation, 16 clones were also randomly picked and submitted for sequencing analysis. Interestingly, 15 out of 16 clones had the same sequence and were full-length Nb, so C1 was used to represent these clones. C10, however, was truncated at framework 3. ELISA result of crude extract showed that C1 showed binding activity ≈ 4.5 fold higher than reference Nb and ≈ 1.46 fold lower than commercial purified monoclonal antibodies while its WB result showed that C1 had a higher protein yield than the reference Nb. C10 did not show ELISA signal nor was detected in WB, thus truncation was confirmed since the detection was performed using anti-FLAG antibody specific to FLAG epitope tag fused to the C-terminus of Nb. Nonetheless, our study demonstrated the feasibility to use FLI-TRAP after initial phage display screening to easily identify full-length Nbs. This combined platform would be powerful tool for easy isolation of Nb against new target as well as for affinity maturation.

1. Introduction
In 1975, the hybridoma technique, a technique used for production of monoclonal antibodies (mAbs), was developed which allows production of identical copies of one type of antibody from hybridoma cells. Since an mAb binds to a specific epitope with high affinity they are extremely useful in clinical therapeutics and diagnostics [1]. However, mAbs have limitations, for example, most mAbs are produced in eukaryotic systems resulting in high cost and the size of mAbs is quite large, which may disturb efficient tissue accessibility and penetration. Recently, a novel type of antibody fragment derived from a heavy-chain antibody (HeAb) present in sera of Camelidae, termed single-domain antibody fragment (VHH) or nanobodies (Nbs) has been extensively applied in medical researches [2]. Many valuable biochemical properties of Nbs make them more appealing than the traditional mAbs. First, high
level of Nb expression can be achieved in microorganisms. From previous studies, Nbs can be expressed in *E. coli* with yields that are higher than the expression of conventional antibody fragments because conventional antibody fragments, such as, antigen binding fragments (Fab) or single-chain variable fragments (scFv), have high complexity and multi-domains. Second, Nbs have high thermal and conformational stability. Some Nbs were shown to possess a reversible unfolding behavior at 90 °C and melting point ranging between 60 °C to 78 °C. Also, they can resist to proteases and extreme conditions such as high urea concentration and extreme pH. Finally, Nbs can access epitopes that are inaccessible because of their small size (<15 kDa), long protruding loop at CDR3 region, and cleft-recognition properties [3]. Therefore, these properties make them suitable for applications in many fields, for example, versatile research tools in biotechnology, nanobiotechnology tool kits, as well as targeting therapeutics [4].

For antibody selection from a library, one of the most popular methods is phage display. This method works by presenting polypeptides on the surface of bacteriophages and the desired polypeptides are isolated by binding to the desired target, coated on a solid surface, based on the interaction between the ligand and the receptor [5-6]. Thus, phage display is a very powerful *in vitro* technique for identifying receptors with affinity for a specific ligand from a large number of samples. However, phage display is known to be notorious for acquiring false positives, because of the binding of peptides present on the surface of the phage to non-target-related materials used during the selection (e.g. plastics, albumin, skim milk) and propagation advantages [7]. Moreover, traditional phage display method utilizes Enzyme-Linked Immunosorbent Assay (ELISA) for the highest affinity receptor identification after phage display. This method has high possibility of false positive or negative results due to insufficient blocking of the surface of the microtiter plate with the immobilized antigen [8].

Another attractive method that can be used for specific receptor identification is the Functional Ligand-binding Identification by Tat-based Recognition of Associating Proteins or FLI-TRAP method. This method is based on the hitchhiker’s mechanism of the bacterial twin-arginine translocation (Tat) pathway, which is the unique ability to transport two folded proteins from the cytoplasm to the periplasm. The FLI-TRAP technique utilizes this mechanism by designing two proteins, the N-terminal Tat signal peptide (ssTorA) fused to the receptor protein, and the C-terminal β-lactamase (Bla) fused to the ligand. Therefore, if the ssTorA-receptor protein assembles with the ligand-Bla in the cytoplasm then the complex protein will be translocated to the periplasm. In addition, there is an important property of Bla which makes it suitable as a reporter protein: its ability to digest the β-lactam ring antibiotic when present in the periplasm, rendering cells resistant to it. Therefore, we can identify receptor protein that is specific to the target ligand simply by growing bacteria in the medium containing β-lactam ring antibiotic such as Ampicillin (Amp) or Carbenicillin (Carb) [9]. This technique was successfully used for isolation of enhanced scFv mutants specific for the dimerization domain of the yeast (GCN4p) transcription factor and the non-amyloid component region of α-synuclein, which has been associated with Parkinson’s disease [10]. In those cases, FLI-TRAP selections were performed based on scFvs that already bound to their cognate antigens. However, selection of novel receptors from a synthetic library requires a very large size of the library, which will allow for higher chance to find a high affinity receptor. FLI-TRAP is proven to be a powerful tool for drug discovery because it tends to isolate high affinity binders with less false positives under appropriate selection conditions. Unfortunately, its main disadvantage is that it cannot be used for screening from a large library size due to the background growth. Screening a large library size means plating a large number of cells on solid LB-agar plate containing β-lactam ring antibiotic and leakage of active Bla from dead cells can still eave the β-lactam ring antibiotic used as selection pressure resulting in false positives [9-10].

This study aims to develop a new strategy for isolation of Nbs specific for a biomarker by combining the advantage of phage display and FLI-TRAP together. First, biopanning of a synthetic human Nb library against the desired target was performed using the phage display technique in order to pre-screen the library. After that, the ‘focused’ library specific for our target was subcloned into FLI-TRAP format for *in vivo* screening, which can greatly reduce the labor and cost associated with *in vitro* analysis following biopanning in a typical phage display screening.

Biomarker is any substance, structure, or process that can be measured in the body for predicting the incidence or the outcome of disease [11]. As a proof-of-concept study, we were interested in isolation
of Nbs specific for hepatitis B surface antigen (HBsAg), which is used as a biomarker for hepatitis B infection. Hepatitis B infection is a pandemic disease that occurs around the world. In World Health Organization (WHO)’s 2017 global hepatitis report revealed that, in 2015, an estimated 257 million people were living with chronic HBV and there were as many as 720,000 deaths due to chronic liver disease and primary liver cancer occurred as a result of the HBV infection. Chronic HBV infection causes a large burden thus requires greater access to testing and treatment. Unfortunately, access to affordable hepatitis testing is limited. Only 9% of HBV-infected persons, 22 million, have been diagnosed and only 8% of those diagnosed with HBV infection or 1.7 million persons were on treatment [12]. In addition, HBsAg have four major serological subtypes, namely adw, adr, ayw and ayr. Basically, HBsAg has one common determinant for all subtype, namely a, and two pairs of mutually exclusive determinants, dy and wr. The determinant d is specified by amino acid 122 of lysine and y by that of arginine. Similarly, the determinant w is specified by amino acid 160 of lysine, and r by that of arginine. The occurrence of HBV subtypes follows a geographical pattern. Strains from northern Europe and North America are predominated by adw, strains from the Pacific, northern China, and Korea by adr, strains from the Middle East and southeastern Europe by ayw, and strains from the southeastern Asia by adw or adr. There have been reports that the efficiency of anti-HBsAg used for construction of diagnostic test kits is decreased when used with incorrect subtypes [13-14], therefore, the development of a new method to isolate new antibodies which can be used for fabrication of a diagnostic test kit specific to the subtypes found prevalently in Thailand will allow us to detect the disease more accurately and economically and can aid in infectious disease prevention and treatment.

2. Materials and methods

2.1. HBsAg-Specific nanobody identification from a synthetic camelpidized human nanobody library by phage display

The nanobody library used in this study was purchased from Creative Biolabs (USA). For the first round of biopanning, 2 μg HBsAg subtype adr (HBs-875, ProSpec-Tany TechnoGene Ltd., Israel) was diluted in 50 μl coating buffer (0.1 M NaHCO3, pH 8.6) and coated onto an ELISA plate overnight at 4 °C. Next morning, coating solution was removed and the plate was blocked with 300 μl blocking buffer (PBS containing 3% skim milk) overnight at 4 °C. Blocking buffer was removed and 100 μL PBS containing 10^{11} phage library particles (phage particles eluted from the 1st biopanning was used in the 2nd biopanning) was added to the plate and incubated for 2 hours at 37 °C. The plate was washed 5 times with washing buffer (PBS with 0.1% Tween 20) and 200 μL elution buffer (100 mM TEA, pH 10.0) was added and incubated for 10 minutes at 37 °C. The elution buffer was pipetted vigorously for 10 times before being transferred into 100 μL Neutralizing Buffer (1 M Tris, pH 7.4). For 2nd round of biopanning, E. coli TG1 cells were used for phage containing Nb library infection. Basically, 6 ml of E. coli TG1 cells were grown in 2×YT medium until OD_{600} ≈ 0.5 and total eluted solution was added to infect the culture by incubating the cells at 37 °C for 30 minutes. Then, 9 ml of 2×YT containing 100 μg/ml ampicillin and 1% (w/v) glucose were added to the culture and incubated overnight at 37 °C. Overnight culture was centrifuged at 4,470 ×g for 15 minutes at 4 °C, and resuspended with 2×YT containing 15% glycerol to prepare a master stock for storage at -80 °C. To amplify the phage, a sample from the master stock was inoculated into 25 mL 2×YT-AG medium, grown to exponential phase (OD_{600} ≈ 0.5) and 2×10^{11} pfu M13KO7 Helper Phage were added to the culture. Infection was allowed to occur by incubating the culture in a 37 °C water bath for 30 minutes. Then, the culture was centrifuged at 5,000 rpm for 15 minutes. The pellet was resuspended in 25 mL 2×YT containing 100 μg/mL ampicillin and 50 μg/mL kanamycin. The culture was transferred into a 250 ml flask and grown overnight at 30 °C with 225 rpm shaking speed. To purify the phage for next round of panning, culture was centrifuged at 4,000 x g for 20 minutes. Then, 1/5 of volume of 5×PEG/NaCl (200 g/l polyethylene glycol-800, 2.5M NaCl) was added to the supernatant and left on ice for 1 hour. Phage was collected as pellet by centrifuging at 12,000 x g for 15 minutes at 4 °C. Finally, the supernatant was discarded, and the pellet phage was resuspended in 1 ml sterile PBS, which was used for 2nd round of biopanning using 0.5 μg HBsAg.
2.2. Library construction

*E. coli* strain DH10β was used for all plasmid constructions. The plasmid used for library cloning was based on pDD18 [9]. Since the Nb genes in the library contain the *Xba*I cut site and the original pDD18-ssTorAscFv-GCN4(GLF)-FLAG contains *Xba*I-*Sal*I cut sites for receptor cloning, a reference Nb gene sequence obtained from Creative Biolab’s manufacturer manual (2017) was synthesized by GenScript (USA) with *Xba*I-*Spe*I cut sites at the 5’ end and *Sal*I cut site at the 3’ end and replacing the DNA sequence representing internal *Xba*I cut site from tct aga to tct tga, but still resulting in the same amino acids. This reference Nb gene was subcloned into pDD18-ssTorAscFv-GCN4(GLF)-FLAG plasmid via *Xba*I-*Sal*I cut sites to create pDD18-ssTorA-ref Nb-FLAG, which was used as template for Nb library cloning as well as negative control because it was expected to be a non-specific receptor. Nanobody genes from 2nd round of biopanning were pooled and cloned into pDD18-ssTorA-ref Nb-FLAG via *Spel*-*Sal*I cut sites. Basically, full-length nanobody genes were amplified from phagemid by PCR technique using a forward primer that is specific for the beginning of the Nb framework region 1 (ggc atg act agt atg gcc cag gtg cag c) and a reverse primer that is specific for the end of Nb framework region 4 (ggc atg gtc gac gct cga gac gtc gac cag).

2.3. Identification of library selection conditions and selective growth assay

*E. coli* strain BL21(DE3) was used for the *in vivo* spot plating assay, which was used for identification for library selection condition. Initially, BL21 (DE3) cells were co-transformed with pDD18-ssTorA-ref Nb-FLAG and pDD322Kan-TatABC::HBsAg-Bla plasmids. TatABC proteins were overexpressed in FLI-TRAP system as overexpression of Tat translocation proteins can improve transport efficiency. Cells were grown overnight at 37 °C in Luria Bertani (LB) medium containing 25 µg/ml chloramphenicol and 50 µg/ml kanamycin. The next day, β-lactam antibiotic resistance of bacteria was measured by spot plating 5 µl of 10-fold serially diluted overnight cells culture (normalized in fresh LB to OD<sub>600</sub> = 2.5) onto LB agar plates containing varying amounts of arabinose (0.01-1%) and Carbenicillin (0-200 µg/ml). Spot-plated bacteria were incubated at 30 °C and 37 °C for 24 hours. For the selective growth assay, BL21(DE3) cells were co-transformed with pDD18ssTorA-anti-HBsAg Nb (library)-FLAG and pDD322Kan TatABC::HBsAg-Bla and grown overnight at 37 °C. Cells were centrifuged and normalized in fresh LB to OD<sub>600</sub> = 2.5 followed by direct plating of 100 µl of diluted cells onto LB containing arabinose and carbenicillin as previously determined by reference Nb spot plating. Hits were randomly picked after 24 h incubation and were spot plated to confirm carbenicillin resistance prior to sequencing analysis.

2.4. Protein analysis by ELISA and Western blotting

For protein production, pDD18 ssTorA-Nb-FLAG containing hits were cured from the co-transformed cell by CaCl<sub>2</sub> transformation of miniprep of mixed plasmids into *E. coli* strain BL21(DE3) and plated on LB Agar plate containing only Cm for pDD18 selection. Next day, single colonies were patched onto LB Agar plates containing Cm or Kan. Colonies that grew on the plate containing Cm but not Kan were supposed to only contain the pDD18 plasmid, not the pDD322Kan plasmid. After that, cells containing only pDD18 ssTorA-Nb-FLAG were subcultured in fresh LB media at 37 °C until OD<sub>600</sub> ≈ 0.5 then induced by adding arabinose to a final concentration of 0.1 or 1% wt/vol and protein expression was continued at 37 °C before harvesting cells 4 hours after induction. Cells were harvested by centrifugation and were resuspended in 1xPBS and normalized to OD<sub>600</sub> = 25. Soluble protein was extracted by sonication and collected as whole cell lysate. ELISA and Western blotting were performed according to standard protocols using rat anti-FLAG (1:3000; Sigma-Aldrich) and goat pAb anti-rat HRP conjugated HRP (1:5000; Abcam) for detection.
3. Results and discussion

3.1. Analysis of Nbs specific for HBsAg using traditional phage display

The nanobody library used in this study was a well-established human single domain antibody (sdAb) library, named HuSdL-1, purchased from Creative Biolabs (USA). SdAb is also known as a nanobody. It represents the smallest antibody fragment that still possess high specificity and affinity. The size of HuSdL-1 is 1.5 x 10^9 clones. It was generated based on camelized human VH3 in FR2. Its diversity was created by random mutagenesis in different CDR regions as well as elongated human HCDR3. The library was created with phage display format which would allow rapid discovery of large numbers of high-potency nanobodies. Even though phage display is a very common and powerful method that has been used to identify six human mAbs approved for therapeutic use and other candidates are in advanced phases of clinical studies [15], many studies showed that combination of phage display with other screening methods such as bacterial display method or next-generation sequencing can enhance the effectiveness of antibody isolation [16-17]. Here, we performed 2 rounds of biopanning using HBsAg adr subtype as antigen then randomly picked 12 colonies for further analysis. As expected, since we only analysed a small number of clones, we found several false positives. In fact, sequencing analysis revealed that all of them have a truncated DNA sequence in the framework 1 (Figure 1) causing complication in amino acid sequence analysis. This could be due to the incomplete Nb synthesis during the construction of this synthetic library. Since it is difficult to identify where the Nb sequence begins as a start codon was not recognised in the sequencing results, these samples may have not fold properly thus exposing hydrophobic patch which basically bound non-specifically to the plate.

3.2. Applying FLI-TRAP method to enhance selection of Nbs

Phage display is an in vitro technology, which permits the creation of libraries containing up to 10^{11} different variants [17]. The library used in our study has the size of 1.5x10^9 clones. As it is a synthetic library, it could provide Nbs that can virtually bind to any target antigen. However, the possibility to find one such Nb could be increased when other method is coupled with phage display as previously reported [16-17]. However, those methods require advanced and expensive equipment, for example, bacterial display may require a flow cytometer while NGS requires a next-generation sequencing machine. The method we used for this study is very simple and cheap as this in vitro method was designed such that the transport of binding proteins is linked to antibiotic resistance thus the selection is based on the β-lactam ring antibiotic concentration. Basically, FLI-TRAP only requires LB medium, Agar, and a β-lactam ring antibiotic. Specifically, FLI-TRAP has proven its potential as a protein drug discovery tool as i) it gives less false positives (as compared to the classical yeast two hybrid system) when selected at appropriate conditions, ii) allows for a single-selection in vivo (selection is performed in its natural conditions and bypasses complications associated with protein purification or

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**Figure 1.** Sequence analysis of 4 out of 12 Nb sequences isolated from traditional phage display method showing truncated DNA sequence in framework 1.
immobilization), and iii) selects for stable (weak association maybe dissociated during transport) and soluble (authentic quality control of the Tat pathway to discard the unfolded insoluble proteins) complexes [9-10]. Nonetheless, the main pitfall of FLI-TRAP is that the selection is limited to a much smaller library size when compared with an in vitro method like phage display due to the background growth arising from the leakage of the reporter protein, the β-lactamase enzyme, to the medium. This research was the first study to combine the advantage of the phage display technique, which is its ability to screen a very large library size, with the FLI-TRAP ability to select strong binding proteins with low false positives occurrence. Figure 2(A) illustrates the overall steps performed in this study. Basically, phage display allowed for screening from a very large library (1.5x10⁹ clones) which then was subcloned into FLI-TRAP (2.5x10⁶) for further screening, thus resulting in a small manageable number of binders which could be further analysed. Typically, 3 - 4 rounds of biopanning are required for high affinity antibody isolation. However, using more rounds also means the elution will contain a limited number of clones as the degree of the enrichment cannot be controlled during the biopanning process, which has been regarded as major drawback of phage display. Therefore, we performed 2 rounds of biopanning before subcloning the ‘focused anti-HBsAg nanobody library’ into our FLI-TRAP system (Figure 2(B)), in order to enrich the library but not to limit its diversity. Also, as evidenced by the sequencing results from eluted phage from 2nd round of panning, many isolated Nbs were truncated which represents false positives. This problem can easily be solved by designing the primers used for subcloning the focused anti-HBsAg Nb that would bind specifically to the start of framework 1 and the end of framework 4 to ensure that only full-length Nb genes from the eluted phage would be amplified.

For isolation of Nbs specific for HBsAg using FLI-TRAP, we first had to identify appropriate conditions for selection. Since the background growth depends on how much soluble antigen-Bla is present in the

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**Figure 2.** Application of FLI-TRAP for isolation of anti-HBsAg Nb library. (A) Schematic representation of the overall method for combination of phage display and FLI-TRAP for isolation of specific Nbs from synthetic camelized human nanobody library (B) Schematic representation of the FLI-TRAP assay used in this study (C) Spot plating of E. coli BL21(DE3) co-transformed with pDD18-ssTorA-ref Nb-FLAG and pDD322Kan TatABC::HBsAg-Bla.
cytoplasm of *E. coli*, it is important to identify appropriate conditions as well as number of cells to be placed on selective plates to minimize false positives. In order to do so, spot plating was performed by serially diluting overnight culture 10-fold and spotting samples on the surface of solid LB-agar plate containing various inducer levels (Arabinose) and selection pressure (Carbenicillin), and incubating at various temperatures. The extrapolation calculation was described previously [18]. Figure 2(C) shows the spot plating used for library selection condition identification. It shows that the background growth when ssTorA-ref Nb-FLAG are co-expressed with HBsAg-Bla was low in all conditions. From previous study, high arabinose induction could be more efficient since Nb is fused with ssTorA therefore it could be exported to the periplasm before having a chance to bind with antigen-Bla unless the amount of ssTorA-Nb-FLAG fusion protein accumulation in the cytoplasm is high enough to allow for antibody-antigen complex to form. Therefore, 1% arabinose was selected. For antibody maturation in which initial antibody exhibits affinity towards the target antigen, FLI-TRAP could be performed at high Carb concentration, for example, at 500 µg/mL Carb for scFv-GCN4 while the wild-type could grow up to 125 µg/mL Carb [9]. In our case, we were searching for Nbs that could bind to HBsAg from a synthetic library not an immunized library, which means only small amount of Nbs in this library may bind to our target antigen, therefore, we chose to start the isolation under low selection pressure, i.e., at 25 µg/mL Carb. At 1% arabinose, 25 µg/mL Carb and at 37 °C incubation temperature, background growth from ssTorA-ref Nb-FLAG with HBsAg-Bla grew at 10^{-1} dilution therefore we could plate cells at 10^{-2} dilution, representing 2.5x10^{7} cells per plate. We plated 20 plates and obtained total of 73 hits.

3.3. Analysis of anti-HBsAg Nb selected from FLI-TRAP.

Out of 73 hits, 16 colonies (C1-C16) were randomly picked for spot plating to ensure that the hits could grow at 10^{6} dilution (~12 cells per 5 µL), which represent single cell level meaning they can grow due to Tat export of antibody-antigen complex not from background growth. Figure 3(A) shows that all isolated Nbs could grow at 10^{6} dilution at 25 µg/mL Carb, which was at the selection condition. At Carb concentrations higher than the selection conditions, the hits could grow when large number of cells were placed but not at a 10^{6} dilution. All of the hits showed much more resistance to Carb when compared with the reference Nb as we expected that these hits contained ssTorA-anti-HBsAg Nb that could bind specifically to HBsAg-Bla allowing for the complex proteins to be transported from the cytoplasm to the periplasm. For evaluation, since all these clones grew at single colony level, all clones were submitted for sequencing analysis. Interestingly, 15 out of 16 clones from C1 to C16 except C10 had the same sequence, which means only 2 distinct clones were obtained in our first selection. The only clone with different sequence, namely C10, had a one base-pair deletion at the beginning of framework 3 causing frameshift and a stop codon in framework 3 (Figure 3(B)). However, we continued to analyse both C1 (representing the rest of the clones which have the same sequence) and C10 using ELISA and WB. Figure 4(A) shows ELISA results from a crude extract.
from BL21(DE3) cells expressing C1, C10, and reference Nb, and compared to purified anti-HBsAg mAb purchased from ProSpec-Tany TechnoGene Ltd., Israel (1:5000 dilution). In this experiment, 0.5 µg of HBsAg (adr subtype), which was the same concentration used for biopanning, was coated on the plate. C1 showed binding activity 4.5-fold higher than reference Nb and 1.46-fold lower than purified monoclonal antibodies while its WB result (Figure 4(B)) showed that C1 has a higher soluble protein yield than the reference Nb. C10 did not show ELISA signal nor was detected in WB, thus truncation was confirmed since the detection was performed using anti-FLAG antibody specific to FLAG epitope tag fused to the C-terminus of the Nb.

![Figure 4](image)

**Figure 4.** Nbs property testing (A) Binding activity testing of isolated anti-HBsAg Nb compared to reference Nb and a commercial monoclonal antibody by ELISA. (B) Western blot analysis of isolated anti-HBsAg Nb and reference Nb. Blot probed with anti-FLAG.

4. Conclusions

Our study demonstrated the feasibility to use FLI-TRAP after initial phage display screening to easily identify full-length Nbs. FLI-TRAP is an *in vivo* selection method which utilizes an existing natural transport mechanism to our advantage to screen for Nbs of which its solubility and function, i.e., ability to bind specifically to the target, can be selected at the same time. Since the selection is based on coupling Tat export of binding proteins with cell resistance to β-lactam ring antibiotic, the selection is very simple and cheap to perform. No requirement for an expensive equipment apart from equipment usually available in a typical molecular biology laboratory. We expect that this combined platform would be very useful for easy isolation of Nbs against other targets as well as for affinity maturation of the isolated Nb from the initial library.

**Acknowledgment**

This research was supported by Graduate Development Scholarship 2019, National Research Council of Thailand (to A.K.), and the research subsidy fund of fiscal year 2019 from King Mongkut’s University of Technology Thonburi (to D.W-Z.).

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