# Application of the Synthetic *Escherichia coli*'s Twin-Arginine Translocation Pathway for the Detection of Intracellular Antibodies against Hepatitis C Viral Protease

Attapol Kamthong[1], Phenbunya Boonyalekha[1], Jeerapond Leelawattanachai[2], Dujduan Waraho-Zhmayev[1*]

[1] Biological Engineering Program, Faculty of Engineering, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand
[2] ThailandNational Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 12120, Thailand.

* Corresponding author. Tel: +66908806979; email: dujduan.war@mail.kmutt.ac.th
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**Abstract:** Hepatitis C is a liver disease caused by hepatitis C virus (HCV) infection. Protease inhibitor (PI) is included in the current oral direct-acting antiviral (DAA) combination therapy. However, these HCV PIs are small molecule drugs; therefore, they could have serious off-target adverse effects. New types of treatment such as antibody drugs that have very high specificity and selectivity would be a better alternative. Our study reports the application of the PROTECT (Protease inhibitor Recognition based On Tat Export after Cleavage Tampering) assay, an *in vivo* detection method for protease inhibiting intracellular antibodies (intrabodies) based on the bacterial twin-arginine translocation (Tat) pathway to the HCV NS3 protease system. This assay was designed such that when protease is co-expressed inside the bacterial cytoplasm, Tat transportation of the uncleaved protease substrate due to protection of the cleavage site by a specific intrabody will result in β-lactam antibiotic resistance. Using the anti-NS3 intrabodies isolated previously, we demonstrated that PROTECT assay could distinguish between the inhibitory and non-inhibitory anti-NS3 intrabodies resulting in selective growth in the presence of β-lactam antibiotics. This method has potential for the screening of agents that inhibit proteolytic cleavage in a bacterial cell-based assay, which may find use in identifying, reconstituting, and characterizing protease inhibitors, identifying mutations on the substrate that can inhibit proteolytic cleavage, and drug screening.

**Key words:** *Escherichia coli*, intracellular antibodies, protease inhibitors, twin-arginine translocation pathway.

## 1. Introduction

Protease inhibitors (PIs) are used to prevent proteolytic cleavage due to proteases, a large enzyme family that catalyzes the proteolysis of a peptide bond. They have been used to treat many disorders and viral infections for many decades. It was estimated that proteases account for 5-10% of all pharmaceutical targets being pursued for drug development [1]. PIs found in nature are commonly proteins or peptides; however, current PI drugs are often synthetic peptide-like or small molecules resembling known protease substrates. For this reason, they can cause off-target effects due to the cross-reactivity of the PI with the

homologues of the target protease containing the same catalytic mechanism and analogous substrate specificity, which may be essential to the human body. Moreover, emerging evidence has suggested that the small-molecule PIs have many side effects. It has been evidenced that HIV PIs are associated with metabolic abnormalities such as dyslipidemia, insulin resistance, and lipodystrophy/lipoatrophy, as well as cardiovascular and cerebrovascular diseases [2]. Some PIs may have significant interactions with other drugs. Consequently, macromolecule drugs such as antibody drugs may represent a better alternative to the current small molecule drugs because of their very high specificity and selectivity, which are the main advantages over the current small molecule drugs which are more promiscuous. Therefore, in terms of safety, biologics have low toxicity and thus are known to cause less serious adverse events [3]. Biotechnological advances allow for large-scale production of biologics in a cost-effective manner. Biologics, especially monoclonal antibodies and antibody-drug conjugates, currently represent mainstream therapeutics. With new approvals and targets, their market share continues to grow every year [4].

HCV infection is one of the most problematic infectious diseases, causing a high burden to public health with an estimated 80 million chronic HVC-infected individuals worldwide [5]. About 48-75% of patients with symptomatic acute hepatitis C and 85-90% of asymptomatic patients develop chronic infections, which are strongly associated with cirrhosis, end-stage liver disease, and hepatocellular carcinoma. Within the past decade, antiviral drugs with direct action against the virus, especially NS3/4A protease inhibitors, have been developed to treat HVC infection. In fact, in 2016, it was reported that five out of the ten most expensive prescription drugs in the United States of America are for hepatitis C infection treatment and two of them (Olysio and Viekira Pak) comprise HCV nonstructural protein 3 (NS3) serine protease inhibitor [6]. Nonetheless, resistance to NS3 protease inhibitor has been observed due to the high HCV replication rate causing a heterogeneous virus population in infected patients. Moreover, most of the NS3 protease inhibitors available in the market currently were designed for HCV genotype 1 and, therefore, show lower potency against HCV genotype 3 [5]. Since HCV genotype 1 is the most prevalent worldwide (83.4 million cases, 46.2% of all HCV cases) while genotype 3 is the next most prevalent globally (54.3 million, 30.1%), it is clear that more drug development for HCV treatment is still needed [7].

The use of monoclonal antibodies as therapeutic agents has led to the concept of intracellular antibodies or intrabodies. They, basically, are the smaller antibody fragments which are engineered to function inside the cells for the targeting of the intracellular proteins that are the cause of disease. In general, most antibody fragments failed to express or lost their ability to bind to the target antigen when expressed intracellularly as they are naturally produced to function outside cells that have an oxidizing environment. Therefore, the development of screening or an isolation method of functional intrabodies is important for drug discovery. Gal-Tanamy et al. have developed a genetic screen used for the isolation of single-chain antibodies acting as intrabodies that can neutralize HCV NS3 serine protease. The method is based on the co-expression of the enzyme β-galactosidase (reporter protein), recombinant NS3 protease, and fusion-stabilized single-chain antibody fragment (scFv) in E. coli [8]. Interestingly, they also showed that the isolated anti-NS3 scFvs could inhibit the autonomous amplification of HCV replicons resistant to the small molecule inhibitors of the NS3/4A protease as well as replicons derived from different HCV genotypes [9]. This type of antibody fragment drug may be very useful for the treatment of HCV because it may also be applied as gene therapy in the future, which can reduce the drug resistance rate that occurs when patients often miss drug doses.

The Tat pathway is a secretion pathway commonly found in bacteria and plant thylakoids. It has useful

unique properties, i.e. the quality control and the hitchhiker mechanisms, which have been applied for the development of many protein engineering platforms [10]-[13]. Our recent work reported the potential of applying the Tat pathway as a tool for the isolation of protease-inhibiting intrabodies, which we later designated PROTECT (Protease inhibitor Recognition based On Tat Export after Cleavage Tampering) [13]. Using a synthetic biology approach, the in vivo selection system was designed by co-expressing three components, i.e., protease activity detector (tripartite fusion of substrate flanking by a twin-arginine translocation (Tat) signal sequence at its N-terminus and a reporter which is TEM-1 β-lactamase (Bla), an antibiotic resistance protein, at its C-terminus), protease generator (C-terminal His tagged HCV NS3 protease) and protease blocking device (C-terminal FLAG tagged protease-inhibiting intrabody), in the cytoplasm of *E. coli*. Based on this design, in the presence of the protease and inhibitors, the Tat-specific export of an uncleaved substrate from the cytoplasm into the periplasm resulting in bacterial cell resistance to the β-lactam antibiotic, such as Ampicillin (Amp) and Carbenicillin (Carb), is used to identify candidate inhibitors. This method was applied to the HIV-1 system and showed that the HIV-1 p17 intrabody specific to the C-terminus of p17, an HIV protease substrate, could protect the p17-p24 cleavage site from proteolysis resulting in β-lactam antibiotic resistance.

## 2. Materials and Methods

### 2.1. Plasmid Constructions and Bacterial Strains

*E. coli* strain DH5α was used for all plasmid constructions. The plasmid used for tricistronic expressions was based on pDD18 [11]. The HCV NS3 system was cloned into the tricistronic expression vector, pDD18 scFvp17::FLAG ssTorA::p17Δp24::Bla HIV-PR::His [13], which required 3 steps. First, to create the protease blocking device comprising anti-NS3 scFv (scFv 35 or scFv 53Y) fused with a C-terminus FLAG tag, the scFvp17 was excised using *Nde*I-*Sal*I and replaced with PCR-amplified anti-NS3 scFvs from pMALc-TNN-35 or 53Y (kindly provided by Itai Benhar). Second, to create the NS3 protease activity detector device (ssTorA::NS5A/B::Bla), primers encoding a short peptide representing the NS5A/B cleavage site (SEDVVCCSMSY) were synthesized with *Xba*I and *Not*I cut sites flanking the short peptide, phosphorylated, and ligated into the digested vector resulting from step 1 thus replacing p17Δp24. Finally, to create the protease generator device, PCR-amplified truncated HCV NS3 protease (ΔNS3) [14] from pMGT14 MBP-NS3 (also kindly provided by Itai Benhar) was generated with a C-terminus His tag flanking by *Hind*III cut sites and cloned to replace HIV-PR::His. All the constructs were sequenced to confirm.

### 2.2. Selective Growth Assays

*E. coli* strain MC4100 was used for the *in vivo* spot plating assay. Briefly, MC4100 cells were co-transformed with pDD18 and pDD322Kan TatABC plasmid as overexpression of Tat ABC proteins can improve transport efficiency. Cells were then grown overnight at 37°C in Luria Bertani (LB) medium supplemented with 25 μg/ml chloramphenicol (Cm) and 50 μg/ml Kanamycin (Kan). The next day, the β-lactam antibiotic resistance of bacteria was evaluated by spot plating 5 μl of serially 10-fold diluted overnight cells (normalized in fresh LB to OD600 = 2.5) on to LB agar plates supplemented with 0.001-0.01% arabinose and varying amounts of Carbenicillin (Carb; 0–1000 μg/ml). Spot-plated bacteria were incubated at 30°C or room temperature for 24 h.

### 2.3. Protein Analysis

*E. coli* strain MC4100 containing both pDD18 and pDD322Kan plasmids were subcultured in fresh LB media at 37°C until the cells reached an OD600 ~0.4–0.5 then induced by adding arabinose to a final concentration of 0.01% wt/vol and protein expression was continued at 30°C and harvested 3 h after induction. Samples were normalized by $OD_{600}$ = 75 before being subjected to subcellular fractionation, which was performed using the ice-cold osmotic shock procedure to release periplasmic proteins, followed by sonication to release cytoplasmic proteins [12]. Each fraction was separated by SDS/PAGE and Western blotting was performed according to standard protocols. Membranes were probed with rat anti-FLAG (1:3,000; Sigma-Aldrich) to detect anti-NS3 intrabodies and mouse anti-Bla (1:500; Abcam) to detect the ssTorA::NS5A/B::Bla fusion. Secondary antibodies used were goat pAb anti-rat conjugate HRP (1:5,000; Abcam) for rat anti-FLAG, and anti-mouse conjugate HRP (1:2,500; PROMEGA) for mouse anti-Bla.

## 3. Results and Discussion

### 3.1. Construction of the Detection System

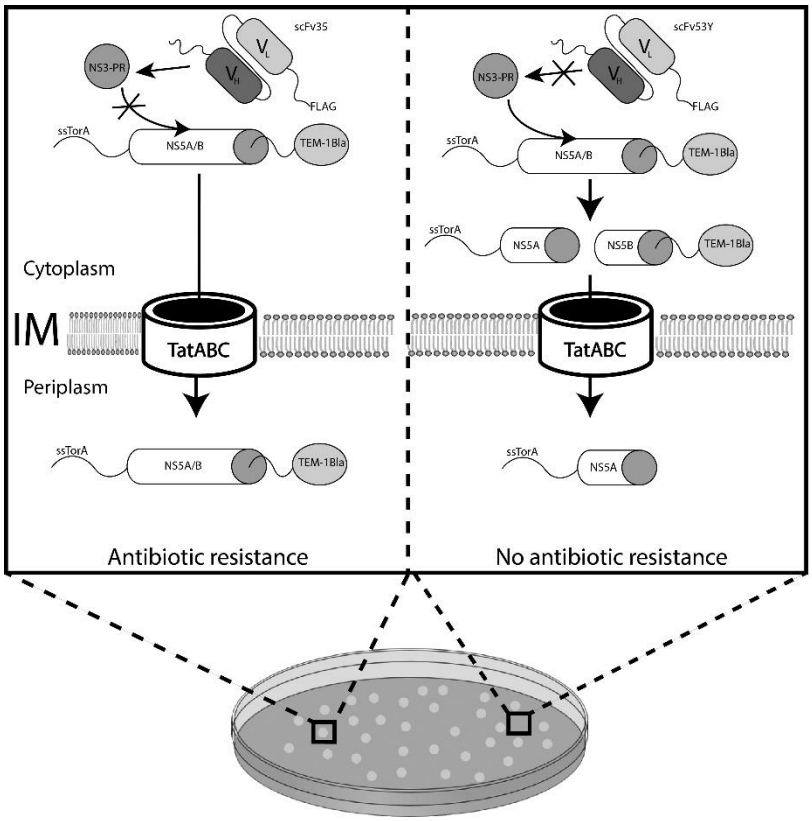


Fig. 1. Schematic illustration of the PROTECT assay used for the isolation of protease-inhibiting intrabodies.

The detailed concept of the PROTECT system has been illustrated previously [13]. Fig. 1. illustrates the schematic representation for this specific system. The anti-HCV NS3 intrabodies were obtained from previous work by Gal-Tanamy *et al.* for use as our proof-of-concept system for HCV protease inhibition. These intrabodies are in single-chain variable fragment (scFv) format and were shown to bind specifically to single-chain NS3 protease (scNS3). scFv 35 was selected as the positive control as it showed high %

inhibition and could inhibit NS3-mediated cell proliferation whereas the non-inhibitory scFv 53Y was used as the negative control [8]. These antibodies were cloned with a C-terminal FLAG tag to aid their detection using the Western blot technique. A short peptide (SEDVVCCSMSY), representing the NS5A/B cleavage site of NS3 protease, was used as the substrate in our experiment. This peptide is derived from the amino acid sequences of the C-terminus of HCV NS5A and of the N-terminus of NS5B proteins where NS3 cleaves more efficiently than at other cleavage sites in the HCV polyprotein and has been used in previous studies [8], [15]. This NS5A/B cleavage site was constructed as a tripartite fusion in between the N-terminal Tat signal sequence derived from Trimethylamine-N-oxide reductase (ssTorA) and the C-terminal reporter protein, Bla, an enzyme that degrades β-lactam antibiotics. Finally, the HCV ΔNS3 protease was cloned with a C-terminus His tag. The truncated protease was used because the full-length NS3 protease has low solubility in the cytoplasm of *E. coli* whereas the expression of the protease domain of NS3 protein encompassing amino acids 1027-1218 (ΔNS3) showed greater activity than other constructs [14]. All of these components were constructed in the same plasmid and under the same arabionose-inducible, araBAD promoter, as a tricistronic construct.

### 3.2. Protease Inhibiting Intrabody Can Block HCV NS3 Catalysis *in Vivo*

When we first developed the assay, we successfully applied it to the HIV-1 PR system. Here, we aim to demonstrate that PROTECT can be applied to various types of targets. In our previous work, we used the non-interacting intrabody as a negative control, whereas in this current work, we compared between two intrabodies. Both can bind to the protease but one scFv has a catalysis inhibitory effect (scFv 35, used as a positive control) while the other does not (scFv 53Y, used as a negative control). Fig. 2. illustrates that cells containing scFv 35 clearly showed higher β-lactam antibiotic resistance than the cells containing scFv 53Y. The positive control showed high cell resistance as a single level colony ($10^{-6}$ dilution) could grow even at 1000 μg/mL Carb, which means a large amount of ssTorA::NS5A/B::Bla was transported to the periplasm, probably due to the low solubility of the protease when produced in the cytoplasm of *E. coli* as reported previously, even though the HCV ΔNS3 construct was used [8], [14]. For the negative control, a higher induction level and temperature seemed to result in higher background growth, probably because high translation rates can cause the protease to form inclusion bodies resulting in the even lower solubility of ΔNS3. Nonetheless, at the best conditions tested, which were induced with 0.001% arabinose, plated on 1000 μg/mL Carb, and incubated for 24 h, the differences between the positive (grew at $10^{-6}$ dilution) and the negative controls (grew at $10^{-3}$ dilution) are about 1000-fold.

It should be noted that the fundamental difference of this system compared to the HIV system previously reported [13] is that these scFvs target the protease itself while the positive-control scFv in the HIV system targets the substrate of the protease around the cleavage site. Therefore, from these two studies we have established that our PROTECT assay has the potential to identify protease-inhibiting scFvs that can protect against proteolytic cleavage either by binding to the protease or to the substrate thus tampering the cleavage. There are many advantages associated with this assay compared to the typical *in vitro* protease inhibitor screening technique currently employed. Firstly, this assay is performed *in vivo*. Therefore, it can bypass the cumbersome protein purification step usually required in an *in vitro* assay. Secondly, the assay uses *E. coli* as a host, thus allowing for large library construction and screening as well as opting for cheap large-scale production. The isolation strategy is based on linking its function to the antibiotic resistance; i.e.,

protection against protease catalysis will result in cell survival in the presence of selective media containing the β-lactam antibiotic so the assay can be conducted easily and inexpensively by plating cells on LB agar containing β-lactam antibiotics such as Amp or Carb. This method has potential for screening agents that inhibit proteolytic cleavage in a bacterial cell-based assay, which may find use in identifying, reconstituting, and characterizing protease inhibitors, identifying mutations on the substrate that can inhibit proteolytic cleavage, and drug screening.

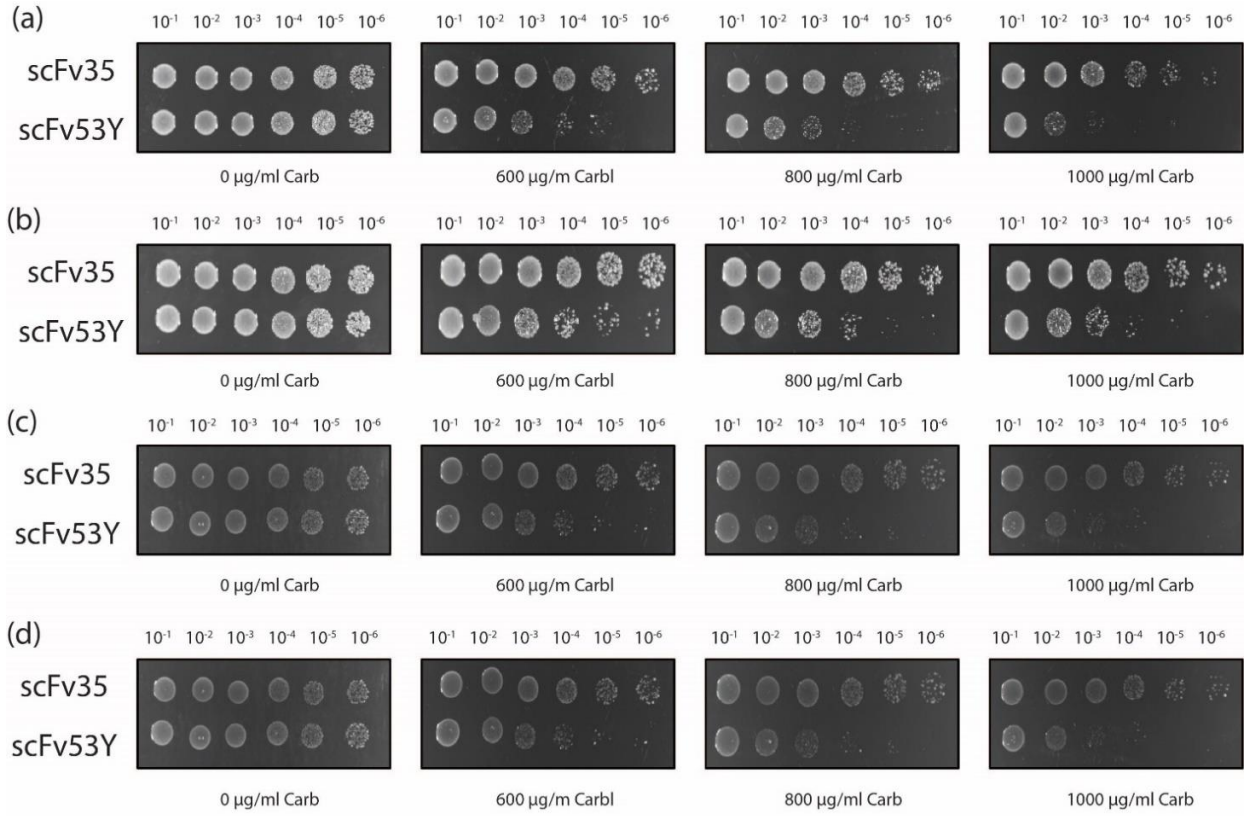


Fig. 2. Selective plating illustrating the differences in β-lactam antibiotic resistance between the inhibitory and non-inhibitory HCV NS3 intrabodies. Each spot corresponds to 5 µL of serially diluted cells containing scFv 35 or scFv 53Y co-expressing with ssTorA::NS5A/B::Bla and truncated NS3 protease plated on Carb as indicated. Cells were induced with 0.001% arabinose and incubated at: (a) room temperature; or (b) 30°C, or with 0.01% arabinose and incubated at: (c) room temperature; or (d) 30°C.

### 3.3. Localization of the Protease Activity Detector

Nonetheless, it is important to confirm that the cell resistance observed in the *in vivo* plating assay is actually based on the Tat transport of the uncleaved tripartite fusion protein. In this case, we investigated the localization of the tripartite fusion protein in each sample using anti-Bla antibody. As expected, the presence of tripartite protein fusion in the periplasm was only observed in the cells expressing inhibitory scFv. This is because scFv 35 binding to the protease can inhibit proteolytic cleavage allowing for the transport of the whole tripartite fusion protein via the Tat signal sequence at the N-terminus into the periplasm, where Bla (fused to the C-terminus) can cleave Carb resulting in cell resistance. Typically, after the Tat transport, the signal sequence will be cleaved off leaving only the 32-kDa NS5A/B::Bla detectable in

the periplasm. As the protection was not complete, some of the 31-kDa cleaved NS5B::Bla was also detected in the cytoplasm of the positive control. In the cells expressing scFv 53Y, the scFv inhibits NS3 catalysis; therefore, the tripartite fusion protein was cleaved leaving the part of NS5B::Bla in the cytoplasm, which was detected using anti-Bla antibody, while only the ssTorA::NS5A part was transported into the periplasm and was undetectable by anti-Bla antibody. Anti-FLAG antibody was used to detect the presence of the anti-NS3 scFvs. It is interesting to observe that the solubility of the scFv 53Y was a lot higher than that of the scFv 35, which was probably why the inhibition was quite successful, as we did not observe any tripartite fusion protein bands in the periplasmic sample when detecting with anti-Bla.

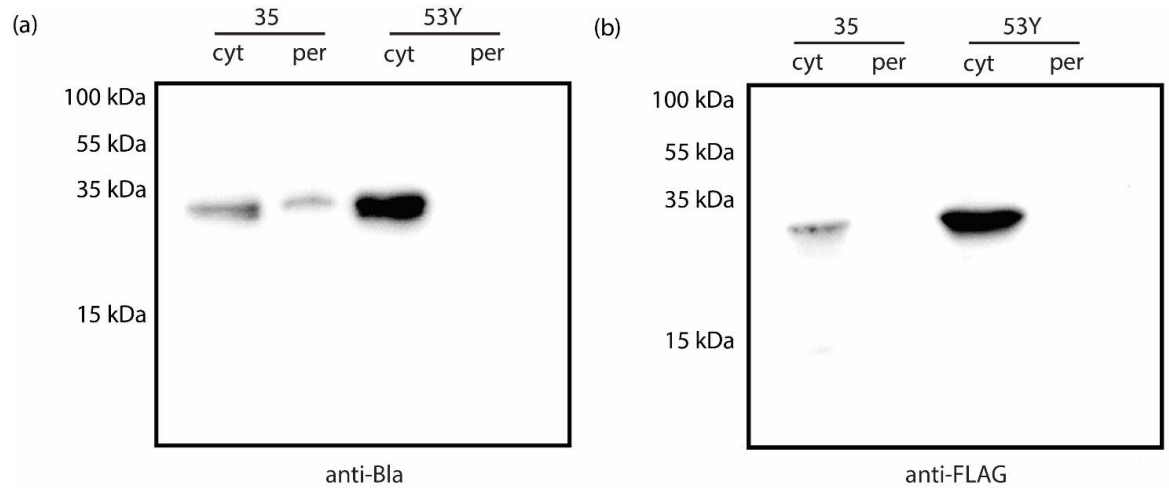


Fig. 3. Transport of uncleaved ssTorA::NS5A/B::Bla fusion protein to the periplasm. Western blot analysis of the cytoplasmic and periplasmic fractions generated from *E. coli* cells carrying pDD322Kan TatABC and pDD18 co-expressing NS3 protease, ssTorA::NS5A/B::Bla, and scFv 35 or scFv 53Y as indicated. Detection of ssTorA::NS5A/B::Bla constructs was with anti-Bla antibody and detection of scFvs was with anti-FLAG antibody. Note that, for ssTorA::NS5A/B::Bla detection, cytoplasmic samples were diluted 20-fold as the amount of the fusion protein in the cytoplasmic fractions was significantly greater than the amount transported into the periplasm.

## 4. Conclusion

Original techniques used for screening of NS3-inhibiting intrabodies required 2 steps, i.e., isolation by virtue of NS3 binding followed by the catalysis inhibition testing step. Gal-Tanamy *et al.* previously described a method that can be used to isolate NS3-inhibitory scFvs by virtue of their catalysis inhibition based on the activity of β-galactosidase [8]. This approach is based on color, i.e., blue-white screening of colonies plated on X-gal plates. Our approach is also a single-step *in vivo* method. However, by linking proteolytic inhibition to the antibiotic resistance, more colonies can be tested and isolation is still very convenient, as it only requires the plating of cells on to β-lactam antibiotic plates. Moreover, another isolation platform based on the Tat pathway has shown that the method favors selection of very stable protein interactions [11], and thus it would be particularly useful for drug screening.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

AK conducted the research, analyzed the data, and wrote the paper; PB designed and conducted the research; JL aided in data interpretation; DW-Z designed and directed the research, analyzed the data, and wrote the paper; all authors had approved the final version.

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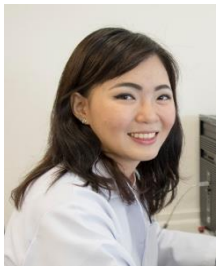
**Attapol Kamthong** was born in Bangkok, Thailand on in 1994. He received a B.S. degree in microbiology from King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, Thailand in 2016. He is currently pursuing a M.S. in biological engineering program at KMUTT.



**Jeerapond Leelawattanachai** was born in Bangkok, Thailand in 1985. She received her B.S. degree in physics from Mahidol University, Bangkok, Thailand in 2007 and her M.S. and Ph.D. degree in biomedical engineering from Cornell University, Ithaca, New York, USA in 2014. She is a researcher in Nanoneedle Research Team (NND), Responsive Material and Nanosensor Research Group (RMNS) at National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani, Thailand. Her main research areas focus on diagnostic platform development, nanobiotechnology, and microneedle technology. Dr. Leelawattanachai is a member of Biomedical Engineering Society.

**Phenbunya Boonyalekha** was born in Bangkok, Thailand in 1992. She received her B.S. degree in Microbiology from King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, Thailand in 2014 and her M.S. in Biological Engineering from KMUTT in 2018. She is a Technology Liaison Officer at Research Innovation and Partnerships Office (RIPO), KMUTT. Her role is to build strategic partnerships with various industries focused on KMUTT main research capabilities including sustainable bioeconomy, digital transformation, innovative material, and smart manufacturing

**Dujduan Waraho-Zhmayev** was born in Bangkok, Thailand in 1980. She received her B.Eng. degree in chemical engineering from Royal Melbourne Institute of Technology (RMIT), Melbourne, Australia, in 2002, and her Ph.D. degree in chemical engineering from Cornell University, Ithaca, New York, USA in 2009. She is an assistant professor in biological engineering program, faculty of engineering at KMUTT, Bangkok, Thailand. Her main research interests include synthetic biology, antibody engineering, drug development and biosensors.