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# Column-free purification and coating of SpyCatcher protein on ELISA wells generates universal solid support for capturing of SpyTag-fusion protein from the non-purified condition

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## 1. Introduction

A variety of display technologies, such as Phage or mRNA display, are used for selection of new binders against wide ranges of antigens [1–3]. In Phage display, a DNA library encoding millions of variants of a specific binding scaffold is cloned in fusion to a phage coat protein such that each phage expresses a slightly different specific binder on its surface. The DNA encoding each binder is then contained within each phage, allowing a direct linkage between the DNA sequence and binding functionality of the scaffold to the target antigen. The selection relies on an affinity enrichment process known as Biopanning [3,4]. The Biopanning process includes three parts: binding of phage to the target antigen, washing out the non-binding phage particles, and then elution of the bound phage. The target antigens used for Biopanning are typically highly purified recombinant proteins that are immobilized on either beads [5] or solid supports such as ELISA multi-well plates [6]. Solid-support immobilization methods require less target protein and facilitate the selection of multiple targets simultaneously. However, in both methods, the quality and yield of the recombinant protein have an essential role in the generation of antibodies [7]. For every new Biopanning selection process ~ 1 mg of purified recombinant protein is required (typically 1–100 µg/ml for each well of a microtiter plate). In many laboratory applications, the purification of new target antigen proteins can present a serious bottleneck to developing new binders, since each new target can require an entirely new expression and purification method to be developed.

To circumvent purification steps, several more direct immobilization methods have been established. Lim et al. have developed a method denoted as Yin-Yang panning [8]. This method was developed for affinity selection of a specific protein in a crude lysate without purification. This procedure was done by saturation of non-binder antibodies in non-expressed bacterial lysate, with blocking agents in ELISA

wells, followed by selection of specific binders from expressed lysates in wells. This method was used for the development of a MERS-CoV nucleoprotein specific antibody. Although this method is very cost effective, it must be optimized for every target protein. Hence, there is need for development of a sandwich ELISA method for specific capture of target proteins from crude feedstocks. Antibody-mediated capture is highly specific, but expensive and a specific antibody must be available for every target protein. Protein ligation methods are a valuable alternative to antibodies, which facilitate the formation of covalent bonds during the coating reaction [9]. The most widely used methods are sortase, split intein coupling [10] and SpyTag/SpyCatcher. By immobilization of one partner it is possible to catch the unpurified corresponding partner. The sortase-mediated coupling reaction takes 24 h at 100 µM of enzyme concentration [11]. For split intein, the coupling reaction is limited to 10 µM concentrations of partners [12]. The optimal target protein concentration in phage display is in the nM range [13]. Hence, there is need for a more sensitive method to achieve coupling. The SpyTag/SpyCatcher coupling reaction is an interesting method that is being rapidly developed to fill the gaps of the aforementioned methods. The coupling reaction requirements have decreased from 10 µM concentrations in early work to the recently engineered versions 002 and 003 with reaction requirements of 100 nM and 10 nM respectively and coupling times of a few hours [14,15]. The SpyTag/SpyCatcher derivatives have been applied in a wide range of studies such as cancer-vaccine development [16], cell-specific capturing [17], and enzyme immobilization [18].

Fierle et al. have also used bacterial superglue for specific capture of antigen from crude lysate [19]. This method is based on the highly specific covalent peptide-protein interactions of SpyTag (SpyT) and SpyCatcher (SpyC) from *Streptococcus pyogenes* [20]. In practice, the SpyTag is chemically synthesized and conjugated onto beads, while the corresponding SpyCatcher protein is expressed as a fusion partner with

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several cancer antigens expressed in a recombinant eukaryotic host system. The SpyCatcher fusion protein was specifically captured onto the bead-based solid phase and then used specific-antibody selection. This interaction is efficient and highly specific. However, it is relatively expensive due to chemical synthesis and conjugation of Spy-Tag. The aforementioned studies have inspired us to develop a very simple and cost-effective method for specific capture of antigen from crude lysate. To achieve this purpose, we have designed an indirect sandwich-like ELISA by non-chromatographic purification of faster variant SpyCatcher002 [14] protein and coating it on an ELISA plate to capture a SpyT002 fusion protein (in this work GFP) from a crude lysate. We show that this coated SpyC002 protein has the potential to be used as universal platform for immobilization of SpyT002 fusion proteins.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated. All cloning enzymes were purchased from Thermo Fisher Scientific. All oligonucleotides were synthesized by Macrogen (Seoul, South Korea) and synthetic DNA constructs were synthesized by Generay Biotech (Hongkong). All DNA extraction kits such as plasmid, Gel extraction and PCR clean up were purchased from Dena Zist Asia (Mashhad, Iran). For cloning and expression of recombinant proteins TOP10 and BL21(DE3) (RIL) were used respectively. Antibodies were purchased from BioLegend.

### 2.2. Plasmid construction

Primer sequences used for plasmid construction in this study are available in Table 1. First, the sequence encoding the RTX tag (BRT<sub>17</sub>) [21] was synthesized by Generay and then cloned with *NcoI* and *EcoRI* into pET32a (which contains Trx solubility enhancer tag) to generate pET32-RTX. SpyC002 (Addgene ID:102827) and GFP were amplified and cloned by *EcoRI* and *HindIII* into pET32-RTX to make pET32-RTX-SpyC002 (Also referred as SpyC) and pET32-RTX-GFP respectively. The SpyT002 encoding plasmid was constructed according following steps: First, SpyT002-MBP was amplified from the pMAL-c5X vector and cloned into pET28(a) to make pET28-SpyT002-MBP. In parallel, RTX tag was cloned into a pMAL-c5X by *NcoI* and *EcoRI* (pMALp5X-RTX) and then RTX from pMALc5X-RTX subcloned into pET28-SpyT002MBP by *SacI* and *EcoRI* (for retrieve pMAL linker) to make pET28-SpyT002-MBP-RTX. Finally, the GFP DNA sequence was amplified and cloned by *EcoRI* and *HindIII* to generate pET28-SpyT002MBP-RTX-GFP (also referred as SpyC). For a negative control, GFP was cloned into pMAL-c5X-RTX to generate pMALc5x-RTX-GFP.

### 2.3. Protein expression

All protein expression experiments were performed in the *E. coli* strain BL21(RIL). Cells harboring pET32-based plasmids (pET32-RTX-GFP and pET32-RTX-SpyC002) were cultured in Luria Broth (LB) media supplemented with 100 µg/mL ampicillin and 30 µg/mL

chloramphenicol, while pET28based plasmids (pET28-SpyT002-MBP-RTX-GFP) were cultured in LB media supplemented with 50 µg/mL kanamycin and 30 µg/mL chloramphenicol. The expression cells were cultured in 5 mL LB overnight at 37 °C. The cultures were diluted 1:100 (v/v) into 200 ml LB media supplemented with the appropriate antibiotics. The cells were then grown at 37 °C until OD600 reached to 0.6–0.8, at which point 0.5 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for protein expression induction at 18 °C for 20 h.

### 2.4. Lysis and recovery

Cells were harvested by centrifugation at 5000 × g for 10 min at 4 °C. The cell pellets were resuspended in Low Salt Buffer (40 mM Tris-HCl, 200 mM NaCl at pH 8.5). Low Salt Buffer was used for further steps, which include washing and dissolving steps. In each case, the cell pellet was resuspended in 1/20 of original culture volume. The resuspended cultures were then sonicated for 10 cycles of 30 s sonication at a setting of 4–5 W, with 30 s on ice. The resulting lysate was then clarified by centrifugation at 18,000 rpm for 30 min at 4 °C. The supernatant was recovered for purification of the target proteins.

### 2.5. RTX-mediated protein purification and protein analysis

This step was done according to the original Fan et al. procedures [21,22]. Briefly, calcium chloride was added to the cleared lysate of pET32-RTX-GFP, pET32-RTX-SpyC002 and pET28-SpyT002MBP-RTX-GFP to a final concentration of 25 mM (diluted from a 2 M stock solution). The sample was then mixed by inverting, and then incubated at room temperature for 15 min. The sample was then centrifuged at 16,000 g for 5 min and the supernatant was discarded. The remaining pellet was resuspended in Low Salt Buffer (40 mM Tris-HCl, 200 mM NaCl at pH 8.5) by a short sonication. The sample was then centrifuged again at 16,000 g for 5 min and supernatant was discarded. The pellet was washed according previous step for 4 times and final pellet then dissolved in Low Salt Buffer containing 25 mM EDTA. Diluted samples were centrifuged again to separate remaining aggregates and soluble protein. The final supernatant was transferred to new tube and labelled as purified protein. Products of the TRX-RTX-SpyC002 purification steps, as well as purified Trx-RTX-GFP and SpyT002-MBP-RTX-GFP proteins were analyzed by SDS-PAGE.

Protein concentration was measured using a Bradford Assay [23] with Bovine Serum Albumin (BSA) used as a standard. The standard curve was generated according nanodrop 2000 protocol by using 125, 250, 500, 1000 and 2000 µg BSA/mL water. Clarified lysate samples were diluted 1:100 and purified product samples were diluted 1:50 in water.

To test the SpyT-SpyC interaction, 200 µg of purified SpyT002-MBP-RTX-GFP and 1 mg Trx-RTX-SpyC002 were mixed and incubated for 2 h at 30 °C. The samples then analyzed by SDS-PAGE. To test the SpyT-SpyC interaction, 200 µg of purified SpyT002-MBP-RTX-GFP and 1 mg of purified MBP-RTX-GFP (negative control) were mixed with 1 mg Trx-RTX-SpyC002 and incubated for 2 h at 30 °C. The samples then analyzed by SDS-PAGE.

**Table 1**  
Sequence of primers in this study.

	Primer name	Sequence (5'-3')
1	SpyC002-F-EcoRI	TTACTGCATATGGAATTCGGTAGTGGTAGTGCCATGGTAACACCTTATCA
2	SpyC002-R-HindIII	TTATCAAAAGCTTAGTATGAGCGTCACCTTTAGT
3	GFP-F-EcoRI	AACATGGAATTCGTGAGCAAGGGCGAGGAGCT
4	GFP-R-HindIII	ATCGAAAGCTTGGCGCCGCTTACTCGAGTAACCTCGTCCATGCC
5	SpyT002-MBP-F-NcoI	ATGGCCATGGATGTGCCTACTATCGTATGGTGGATGCCTACAAGCGTTACAAGGGTAGTAAAATCGAAGAGGTAACCTG
6	MBP-R-EcoRI	TCCTTTGAATTCATGTGAAATCCTTCCCTCGATCC

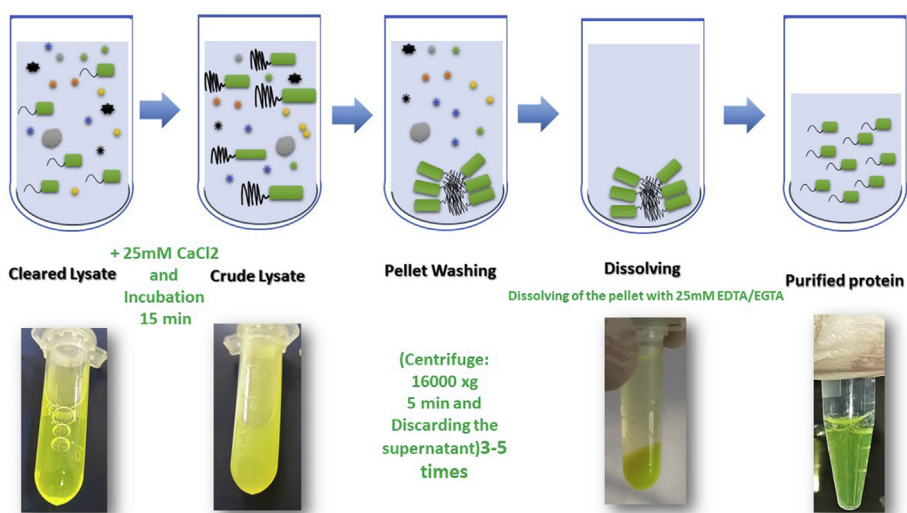


Fig. 1. RTX-Mediated purification procedure.

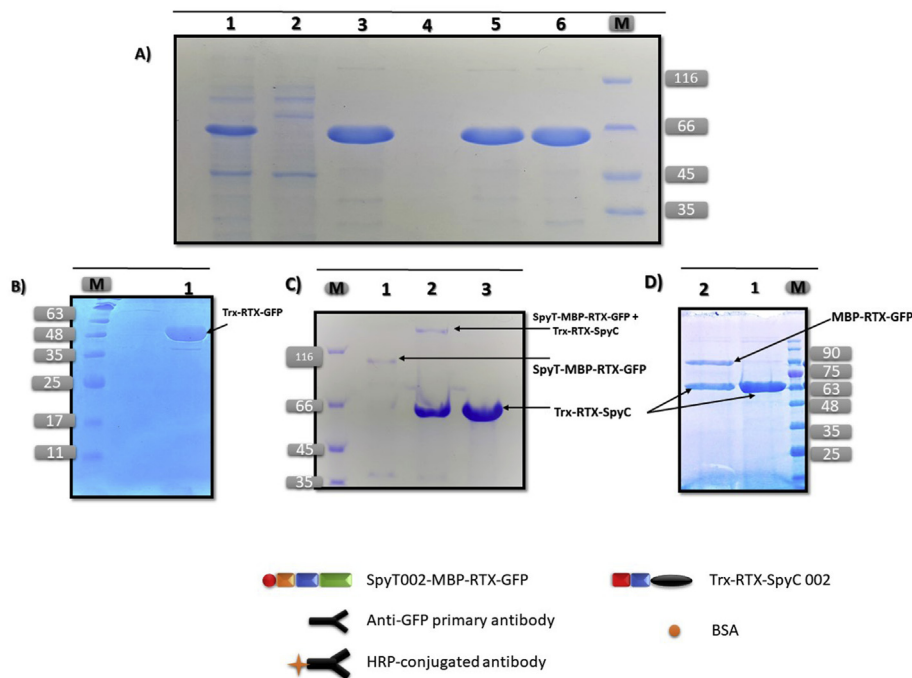


Fig. 2. SDS-PAGE result of purification of recombinant proteins in this study. (A) Purification of Trx-RTX-SpyCatcher002: Lane 1, cleared lysate; Lane 2, supernatant; Lane 3, Tag purity after 3x washing; Lane 4, supernatant after 3x wash; Lane 5, Tag purity after 4x wash; Lane 6, Tag purity after 5x wash; Lane M, protein maker. (B) Purified Trx-RTX-GFP: Lane M: Protein marker; Lane 1, Pure protein. (C) SpyT-SpyC reaction: Lane M, Protein marker; Lane 1 Purified SpyT002-MBP-RTX-GFP; Lane 2, Mixture of SpyT002-MBP-RTX-GFP and Trx-RTX-SpyCatcher002; Lane 3, Purified Trx-RTX-SpyCatcher002. (D) negative control of SpyT/SpyC reaction: Lane M: Protein marker, Lane 1 purified Trx-RTX-SpyC, Lane 2 Trx-RTX-SpyC and MBP-RTX-GFP which shows no reaction.

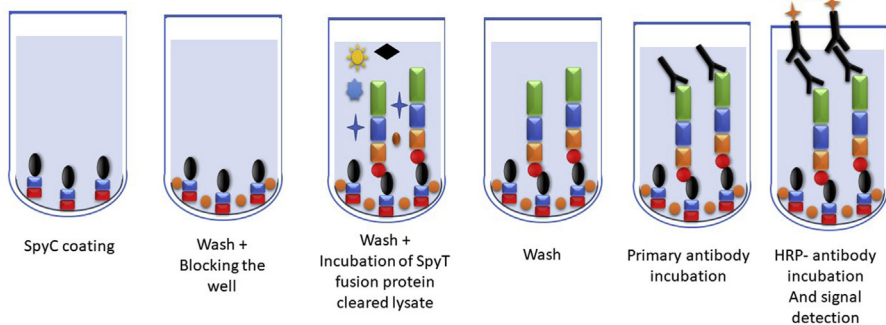
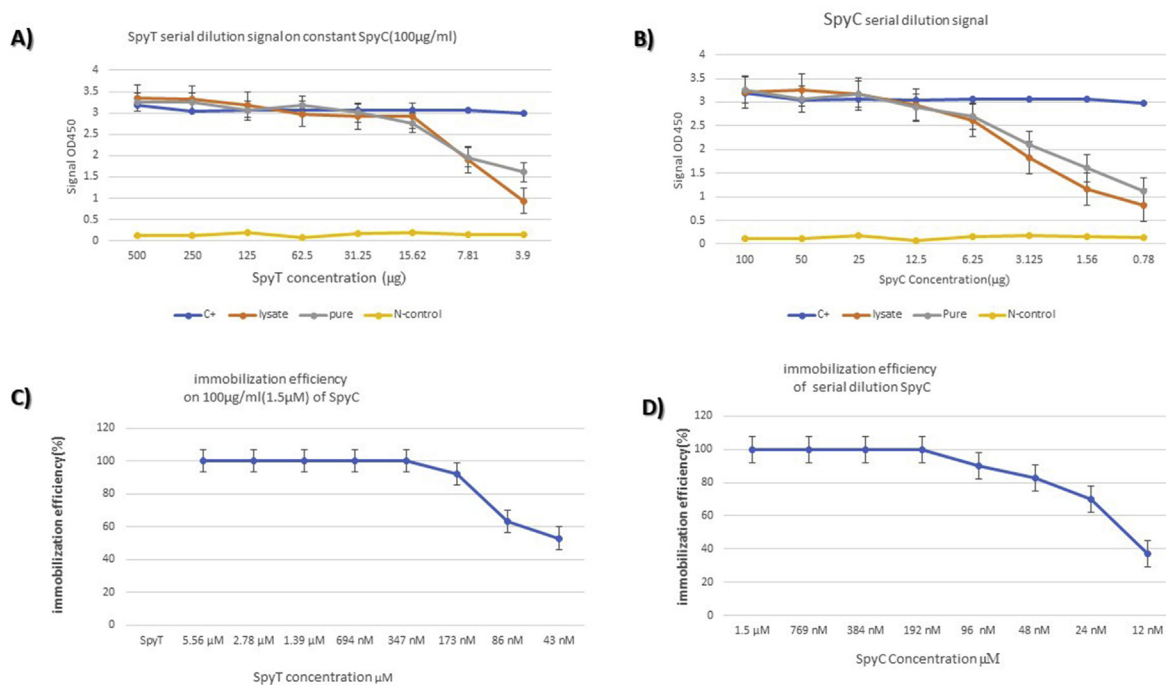


Fig. 3. Coating SpyC on ELISA well and capturing and detection of recombinant SpyT fusion protein.

2.6. Indirect sandwich-like ELISA

The purified Trx-RTX-SpyC002 was diluted in PBS buffer to a final concentration of 100 µg/ml. It was coated on ELISA wells either at constant (100 µg/ml) or ½ serial dilution concentrations (100, 50, 25,

12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml) in 100 µl samples at 4 °C overnight. The wells were then washed with TBST buffer (Tris-buffered saline, pH 7.4, with 0.1% (v/v) Tween 20) 5x times. The coated wells were blocked with 1.0% BSA in PBST buffer (PBS with 0.1% (v/v) Tween 20) for 1 h at 37 °C. The wells were then washed with TBST



**Fig. 4.** ELISA and Immobilization results. The experiment was repeated in two replicates. Error bars represent Standard Error (SE) of mean; Blue; positive control (Trx-RTX-GFP) (SE 0.02, +SD 0.06), Grey: Pure protein (Trx-RTX-SpyC002) (SE 0.24, +SD 0.63), Red: Cleared lysate (SE 0.33, +SD 0.84), Yellow: Negative and Non-specific controls (SE 0.01, +SD 0.01). (A) ELISA signal (OD 450) of  $\frac{1}{2}$  serially diluted SpyT (500–4 µg/ml) analysis on 100 µg/ml coated SpyC wells, (B) ELISA signal analysis in  $\frac{1}{2}$  Serial dilution of SpyT (500–4 µg/ml) of  $\frac{1}{2}$  serially diluted SpyC (100–1 µg/ml), (C) Immobilization efficiency analysis in 100 µg/ml coated SpyC, (D) Immobilization efficiency analysis in Serial dilution of SpyC.

buffer 3x times. At this point coated SpyC is ready for capture of the SpyT fusion protein. To assess Maximum signal of anti-GFP in different concentration, purified Trx-RTX-GFP was used as positive control and serially diluted from 100 to 0.75 µg/ml and coated according to the previous steps described above. As SpyT reacts with SpyC in a 1:1 Molar ratio, the signal of reacted Spy-T-GFP to SpyC at different concentrations with a respective positive control indicates the reaction rate for each respective concentration. For negative and non-specific control 3 type of coated SpyC, empty blocked well with 1% BSA and empty blocked wells SpyT cleared lysate treated (to assess blocking efficiency) were used.

SpyT002-MBP-RTX-GFP in either pure and cleared lysate form (from step 2.4.) was diluted in PBS-T buffer to final concentration of 500 µg/ml. Then, serially diluted samples at 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 µg/ml in 100 µl buffer volume were incubated with SpyC coated wells for 2 h at 30 °C. The wells were then washed with TBST buffer 5x times, and 100 µl of a 1:5000 dilution of Primary antibody (anti-GFP) was added and incubated for 1 h at 37 °C. The wells were then washed with TBST buffer 5 times and a 1:5000 dilution HRP-conjugated antibody (anti-Rat) was added and incubated for 1 h at 37 °C. The wells were then washed with TBST buffer 5 times. To develop a signal, 100 µl TMB substrate was added to each well and incubated for 15 min in darkness. The reaction was then stopped by addition of an equivalent volume of 0.3 M H<sub>2</sub>SO<sub>4</sub>. The wells were then read at 450 nm.

### 3. Results

#### 3.1. Protein purification

The RTX (BRT<sub>17</sub>) tag sequence (GGAGNDTLY)<sub>9</sub> is derived from the consensus block V RTX domain from the adenylate cyclase toxin (Cya A) of *B. pertussis*. It reversibly aggregates and becomes insoluble in the presence of low concentrations of calcium (25 mM) and remains insoluble until the calcium is removed by EDTA or other strong chelating

agent. At this calcium concentration most bacterial proteins remain soluble, making it possible to isolate RTX-fusion proteins from whole lysates by simple centrifugation. By washing the RTX fusion pellet several times, it is possible to achieve pure a protein. Addition of metal chelators such as EDTA/EGTA in equivalent concentration reverts the RTX tag to soluble form. The RTX-based purification is illustrated in Fig. 1. Addition of a solubility enhancer (in this work Trx) enhanced the production yield. For pET32-RTX-SpyC002, ~6.5 mg of pure protein was purified from 200 ml of shake-flask culture, where the pellet was washed 5 times. It was noted that after the third round of washing the purity of the protein didn't change, indicating that 3 wash cycles are suitable for protein purification (Fig. 2A). This procedure was repeated for Trx-RTX-GFP (Fig. 2B) and SpyT002-MBP-RTX-GFP (Fig. 2C), where 3.3 mg and 1.5 mg of purified protein was achieved respectively. For SpyT002-MBP-RTX-GFP, after every round of washing the pellet size decreased significantly, and for this reason the yield of this protein is lower than the others (see Fig. 3).

#### 3.2. Indirect sandwich-like ELISA

Spy peptide-protein interaction depends on two factors; SpyT:SpyC ratio and their concentrations in the reaction. In general, a 1.5 to 3-fold excess ratio is recommended for one partner, with a 2 h reaction time and 10 µM concentration of the limiting protein at room temperature. This time can be increased to 16 h for crowded surfaces [24]. To achieve the maximum reaction in short time, the ratio and temperature were increased to a 3.5-fold molar excess and 30 °C respectively. Although higher temperature such as 37 °C accelerates the reaction, it may not be suitable for some proteins due to stability concerns. The remaining factor was binding partner concentration. The Coated SpyC proteins were divided into two groups. In one group the concentration of the SpyC was constant at 100 µg/ml (1.5 µM). In another group, The SpyC was  $\frac{1}{2}$  serially diluted from 100 µg/ml (1.5 µM) to 0.78 µg/ml (~10 nM) and then coated onto ELISA wells to determine threshold of reaction at low concentration [25]. For coating, PBS buffer was used

with simple dilution, without need of protein dialysis. Due to the sensitivity of the RTX to calcium, skimmed milk was not used as blocking agent. As the Spy peptide-protein interaction occurs in a pH range from 4 to 8 [24], SpyT fusion proteins were diluted in PBS-T buffer and ½ serially diluted from 500 to 3.9 µg/ml.

To assess the maximum possible signal at different concentrations of reacted SpyT002- GFP fusion protein to the coated SpyC, the purified Trx-RTX-GFP test protein was ½ serially diluted from 100 to 0.78 µg/ml (from A-H) and was used as positive control. The signal of the positive control was approximately 3 (OD 450 nm) in all concentrations. At a constant concentration of SpyC (100 µg/ml), the signal of SpyT at concentrations ranging from 500 to 15 µg/ml was similar to the positive control. The signal decreased significantly at 7.8 µg/ml (86 nM) and 3.9 µg/ml (43 nM) of SpyT concentration. The SpyT lysate signal was somewhat lower due to impurity, but followed pure protein pattern (Fig. 4A red line). In serially diluted SpyC, the signal was maximum at 200 nM concentration of SpyC (Fig. 4B). At lower concentrations the signal decreased to about 40% of positive control at 12 nM. The immobilization efficiency was assessed by calculating the coated SpyT signal relative to the positive control signal. As soluble SpyT is required for SpyC coupling, the immobilization efficiency was calculated by saturation of the coated SpyC. According to the result of the SpyT purified and lysate samples, for 100 µg/ml concentration of coated SpyC, the minimum concentration of SpyT that is required for a complete reaction in 2 h at 30 °C is ~173 nM of SpyT protein (in this work ~15 µg/ml) (Fig. 4C). However, it is possible to use lower concentrations, but with a lower immobilization efficiency (down to 50 nM). Serial dilution of SpyC results showed that the minimum concentration of SpyC required for a complete reaction is between 100 and 200 nM (~6.25–12.5 µg/ml) and in this case at least ~350–700 nM of SpyT (in this work ~31–62.5 µg/ml) is required (Fig. 4D). These results match the original Spy002 article, which reports that the reaction rate in 100 nM SpyC is ~70% [14].

#### 4. Discussion

The Spy tag system has been successfully used for basic research and applied science, such as cell inner/outer localization [26], enzyme thermal stabilization [27], enzyme immobilization [28], vaccine development [29,30] and protein detection [31] and purification [32]. Since the development of this method in 2012, its applications are expanding along with its potential [33]. In this work we have combined a non-chromatographic method with the spy catcher002 protein to generate a potentially cost-effective universal capture method for immobilization of SpyT-fusion recombinant proteins from unpurified feeds. The RTX-mediated protein purification takes 1–2 h, without any specific equipment. As mentioned above, we produced ~6 mg of purified fusion protein in one example, which is theoretically suitable for the generation of hundreds of ELISA wells. Conversely, the sample size is also small, which subsequently decreases the required culture volume. In addition, this system is sensitive to small amounts of recombinant protein, which is ideal for low-expressing recombinant proteins or in mammalian transient expression systems. However, low concentrations of recombinant protein (100 nM for both partners) may show different results due to reaction rate dependence of Spy variant 002 on protein concentration. Recently, variant 003 of this method has been developed [15]. Increased sensitivity of this method to even subnanomolar concentrations may be possible by applying Spy003. As both ELISA and Spy tag-protein are compatible with urea, it is possible to use this method with urea to dissolve inclusion bodies and continue process steps with immobilized protein. This method is also helpful for the study of SpyT fusion vaccines and for studies of immunization or in lab-on-chip methods.

#### Authorship statement

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below: Conception and design of study, acquisition of data, analysis and/or interpretation of data, Drafting the manuscript, revising the manuscript critically for important intellectual content, Approval of the version of the manuscript to be published (the names of all authors must be listed).

#### Declaration of competing interest

The authors declare no conflicts of interest.

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#### References

- [1] R. Peltomaa, E. Benito-Peña, R. Barderas, M.C. Moreno-Bondi, Phage display in the quest for new selective recognition elements for biosensors, *ACS Omega* 4 (2019) 11569–11580.
- [2] P.E. Saw, E.W. Song, Phage display screening of therapeutic peptide for cancer targeting and therapy, *Protein Cell* 10 (2019) 787–807.
- [3] M. Arslan, D. Karadag, S. Kalyoncu, Protein engineering approaches for antibody fragments: directed evolution and rational design approaches, *Turkish journal of biology = Turk biyoloji dergisi* 43 (2019) 1–12.
- [4] Y. Tan, T. Tian, W. Liu, Z. Zhu, C.J. Yang, Advance in phage display technology for bioanalysis, *Biotechnol. J.* 11 (2016) 732–745.
- [5] A.C.W. Ch'ng, Z. Konthur, T.S. Lim, Magnetic bead-based semi-automated phage display panning strategy for the directed evolution of antibodies, *Methods Enzymol.* 630 (2020) 159–178.
- [6] S. Yun, S. Lee, J.P. Park, J. Choo, E.K. Lee, Modification of phage display technique for improved screening of high-affinity binding peptides, *J. Biotechnol.* 289 (2019) 88–92.
- [7] R. Jara-Acevedo, P. Diez, M. Gonzalez-Gonzalez, R.M. Degano, N. Ibarrola, R. Gongora, A. Orfao, M. Fuentes, Methods for selecting phage display antibody libraries, *Curr. Pharmaceut. Des.* 22 (2016) 6490–6499.
- [8] C.C. Lim, P.C.Y. Woo, T.S. Lim, Development of a phage display panning strategy utilizing crude antigens: isolation of MERS-CoV nucleoprotein human antibodies, *Sci. Rep.* 9 (2019) 6088.
- [9] Z.A. Wang, P.A. Cole, Methods and Applications of Expressed Protein Ligation, in: M. Vila-Perelló (Ed.), *Expressed Protein Ligation: Methods and Protocols*, Springer US, New York, NY, 2020, pp. 1–13.
- [10] A. Romero-Casañas, V. Gordo, J. Castro, M. Ribó, Protein Splicing: From the Foundations to the Development of Biotechnological Applications, in: M. Vila-Perelló (Ed.), *Expressed Protein Ligation: Methods and Protocols*, Springer US, New York, NY, 2020, pp. 15–29.
- [11] S.A. McConnell, B.R. Amer, J. Muroski, J. Fu, C. Chang, R.R. Ogorzalek Loo, J.A. Loo, J. Osipiuk, H. Ton-That, R.T. Clubb, Protein labeling via a specific lysine-isopeptide bond using the pilin polymerizing sortase from *Corynebacterium diphtheriae*, *J. Am. Chem. Soc.* 140 (2018) 8420–8423.
- [12] A.J. Stevens, Z.Z. Brown, N.H. Shah, G. Sekar, D. Cowburn, T.W. Muir, Design of a split intein with exceptional protein splicing activity, *J. Am. Chem. Soc.* 138 (2016) 2162–2165.
- [13] C.R. Geyer, S.S. Sidhu, *Phage Display in Biotechnology and Drug Discovery*, CRC Press, 2015.
- [14] A.H. Keeble, A. Banerjee, M.P. Ferla, S.C. Reddington, I.N.A.K. Anuar, M. Howarth, Evolving accelerated amidation by SpyTag/SpyCatcher to analyze membrane dynamics, *Angew. Chem.* 56 (2017) 16521–16525.
- [15] A.H. Keeble, P. Turkki, S. Stokes, I.N.A. Khairil Anuar, R. Rahikainen, V.P. Hytonen, M. Howarth, Approaching infinite affinity through engineering of peptide-protein interaction, *Proc. Natl. Acad. Sci. U.S.A.* 116 (52) (2019).
- [16] W. Wang, Z. Liu, X. Zhou, Z. Guo, J. Zhang, P. Zhu, S. Yao, M. Zhu, Ferritin nanoparticle-based SpyTag/SpyCatcher-enabled click vaccine for tumor immunotherapy, *Nanomed. Nanotechnol. Biol. Med.* 16 (2019) 69–78.
- [17] J.O. Fierer, G. Veggiani, M. Howarth, SpyLigase peptide-peptide ligation polymerizes antibodies to enhance magnetic cancer cell capture. *Proc. Natl. Acad. Sci.* 111 (2014) E1176.
- [18] Y. Lin, W. Jin, J. Wang, Z. Cai, S. Wu, G. Zhang, A novel method for simultaneous purification and immobilization of a xylanase-lichenase chimera via SpyTag/SpyCatcher spontaneous reaction, *Enzym. Microb. Technol.* 115 (2018) 29–36.

- [19] J.K. Fierle, J. Abram-Saliba, M. Brioschi, M. deTiani, G. Coukos, S.M. Dunn, Integrating SpyCatcher/SpyTag covalent fusion technology into phage display workflows for rapid antibody discovery, *Sci. Rep.* 9 (2019) 12815.
- [20] B. Zakeri, J.O. Fierer, E. Celik, E.C. Chittock, U. Schwarz-Linek, V.T. Moy, M. Howarth, Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin, *Proc. Natl. Acad. Sci. U.S.A.* 109 (2012) E690E697.
- [21] O. Shur, K. Dooley, M. Blenner, M. Baltimore, S. Banta, A designed, phase changing RTX-based peptide for efficient bioseparations, *Biotechniques* 54 (2013) 197–206.
- [22] Y. Fan, J.M. Miozzi, S.D. Stimple, T.-C. Han, D.W. Wood, Column-free purification methods for recombinant proteins using self-cleaving aggregating tags, *Polymers (Basel)* 10 (2018) 468.
- [23] S.R. Gallagher, *Quantitation of Nucleic Acids and Proteins*, (2008), pp. 2.2.1–2.2.29.
- [24] A.H. Keeble, M. Howarth, Insider information on successful covalent protein coupling with help from SpyBank, *Methods Enzymol.* 617 (2019) 443–461.
- [25] A.V. Lin, Indirect ELISA. *Methods in molecular biology* (Clifton, NJ), 1318 (2015) 51–59.
- [26] D. Hatlem, T. Trunk, D. Linke, J.C. Leo, Catching a SPY: using the SpyCatcher-SpyTag and related systems for labeling and localizing bacterial proteins, *Int. J. Mol. Sci.* 20 (2019).
- [27] M. Si, Q. Xu, L. Jiang, H. Huang, SpyTag/SpyCatcher cyclization enhances the thermostability of firefly luciferase, *PLoS One* 11 (2016) e0162318.
- [28] Y. Lin, G. Zhang, Target-specific covalent immobilization of enzymes from cell lysate on SiO<sub>2</sub> nanoparticles for biomass saccharification, *ACS Appl. Nano Mater.* 3 (2020) 44–48.
- [29] A. Marini, Y. Zhou, Y. Li, I.J. Taylor, D.B. Leneghan, J. Jin, M. Zaric, D. Mekhaie, C.A. Long, K. Miura, S. Biswas, A universal plug-and-display vaccine carrier based on HBsAg VLP to maximize effective antibody response, (2019), p. 10.
- [30] D. Li, H. Zhang, L. Yang, J. Chen, Y. Zhang, X. Yu, Q. Zheng, J. Hou, Surface display of classical swine fever virus E2 glycoprotein on gram-positive enhancer matrix (GEM) particles via the SpyTag/SpyCatcher system, *Protein Expr. Purif.* 167 (2020) 105526.
- [31] D. Dovala, W.S. Sawyer, C.M. Rath, L.E. Metzger, Rapid analysis of protein expression and solubility with the SpyTag–SpyCatcher system, *Protein Expr. Purif.* 117 (2016) 44–51.
- [32] I.N.A. Khairil Anuar, A. Banerjee, A.H. Keeble, A. Carella, G.I. Nikov, M. Howarth, Spy&Go purification of SpyTag-proteins using pseudo-SpyCatcher to access an oligomerization toolbox, *Nat. Commun.* 10 (2019) 1734.
- [33] S.C. Reddington, M. Howarth, Secrets of a covalent interaction for biomaterials and biotechnology: SpyTag and SpyCatcher, *Curr. Opin. Chem. Biol.* 29 (2015) 94–99.