공학석사학위논문

Development of a fluorescent sensor "Quenchbody" for rapid analysis of procollagen type III N-terminal peptide

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이준엽의 공학석사 학위논문을 인준함

2021년 8월

위 원 장 _____

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위 원 _____

Abstract

Procollagen type III N-terminal peptide (PIIINP) is a major biomarker of growth hormone which is on the prohibited substances list by the World Anti-Doping Agency (WADA). The recently developed and conducted PIIINP analysis methods are radio immunoassay (RIA) or fluorescence-based sandwich enzyme-linked immunosorbent assay (ELISA), and these have issues with radiation safety, time-consuming, and expensive equipment. Therefore, the development of an analysis method that can overcome these shortcomings is required. To this end, we tried to develop a high-throughput doping analysis method using a fluorescence-based antibody sensor called “Quenchbody. The quenchbody consists of a single chain variable fragment (scFv) and a fluorophore which emits fluorescence depending on the presence or absence of an antigen The sequence of anti-PIIINP scFv was obtained from a hybridoma cell and cloned scFv was expressed in *E. coli*. Inclusion body refolding was performed to obtain more scFv, and fluorescence was conjugated to native and refolded anti-PIIINP scFv that were confirmed to have antigen binding affinity against PIIINP. Finally, dose-dependent fluorescence signal were confirmed with a fluorescence spectrophotometer. The best limit of detection (LOD) and limit of quantitation (LOQ) were calculated as 1.64 nM and 3.89 nM for TAMRA-labeled quenchbody with native anti-PIIINP scFv, according to the five-point logistic curve regression. Furthermore, with 2 nM of quenchbody, the analysis could be performed within 30 minutes from the experimental preparations to validations. Thus, we confirmed the high-throughput and high sensitivity capabilities of quenchbody required for a new doping analysis method.

Key Words: Doping control, Procollagen type III N-terminal peptide, fluorescence-based antibody sensor, quenchbody

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NTA wash of inclusion body, SI: solubilized inclusion body, FT: Ni-NTA flow through, W: Ni-NTA wash, P: purified anti-PIIINP scFv inclusion body.

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1. Introduction

Doping in athletes is done with from the small molecules, steroid-based drugs to large protein drugs as peptide hormones which are on the prohibited list by World Anti-Doping Agency (WADA). Accordingly, doping control analysis in progress, and the adverse analytical findings (AAFs) have been gradually increased from 1.08% in 2008 to 1.42% in 2018.[1] In addition, the total number of tests has risen dramatically from about 270,000 in 2013 to near 350,000 in 2018.[1] Among these, in the case of small molecule or steroid-based drugs, a method using gas chromatography–tandem mass spectrometry (GC–MS/MS) analysis and liquid chromatography–mass spectrometry (LC–MS) based analysis is well established.[2] However, it is not easy to detect doping in athletes using proteins or peptides like growth hormone (GH), luteinizing hormone (LH), and chorionic gonadotropin (CG), which are analyzed by performing a classical enzyme-linked immunosorbent assay (ELISA) or related immunoassay.[3] Furthermore, these methods have significant disadvantages in that pretreatment takes a long time and requires experts in the analysis method with expensive equipment.

Among these prohibited substances, GH increases body mass by inducing anabolic effect on protein and catabolic effect on fat which eventually promote muscle protein synthesis and connective tissues.[4, 5] From this perspective, GH plays a key drug in abuse increasing athletic strength and sprint capacity that must be banned.[5] Recently, as a GH doping control method, GH biomarker analysis for procollagen type III N-terminal peptide (PIIINP) and insulin-like growth factor-I (IGF-I) has been developed.[4] In particular, a PIIINP is literally a marker of the synthesis of collagen type III which is connective tissue and thus related to GH

concentration in serum.[5] This is evaluated by both competitive radioimmunoassay and a two-site sandwich of chemiluminescent immunoassay in doping control filed. These methods have shortcomings with long analysis time, the need to use radioactive material and multiple reagents, and the need for expensive equipment.[6] Therefore, it is necessary to develop assay methods that can analyze PIIINP efficiently and quickly for the increased number of doping tests.

Herein, we propose a new analysis method for PIIINP in doping control using quenchbody system. The quenchbody is a fluorescence-based antibody sensor which contains fluorescence in itself to validate the antigen concentrations with a single probe.[7] Quenchbody sensor is based-on photoinduced electron transfer (PET) mechanism through interactions between fluorophores and intrinsic tryptophan (Trp) residues of the antibody.[8] A fluorescent dye is conjugated by maleimide–thiol reaction with engineered free cysteine (Cys-tag) in single-chain variable antibody fragment (scFv) (Figure 1. (A), (B)). And this fluorescent molecule interacts with tryptophan residues in the antibody by hydrophobic interaction or π-π stacking.[7, 8] Subsequently, tryptophan continuously act as an quencher for fluorophore preventing fluorescence emission.[9, 10] Because of the Trp residues being highly conserved at the C-terminus boundary of most antibodies' complementary determining regions (CDRs) 1 and CDR3 and not well being presented in the frameworks, the interacting probability between CDRs and fluorescent dye is significantly increased.[11-13] Furthermore, the fluorescence molecule which is quenched upon the antigen binding site can be escape from the antibody fragment when the antigen binds to the antibody, resulting in a strong fluorescence (Figure 1. (C)).[14] Therefore, we aimed to develop a quenchbody that has a short reaction time, simplicity of reagents, and high resolution for PIIINP

quantification.

This study began with finding the sequence of the anti-PIIINP antibody. A hybridoma 35J22 deposited in ATCC which secretes anti-PIIINP antibody was used to obtain the anti-PIIINP antibody sequence, mRNA was extracted from hybridoma 35J22, and cDNA of anti-PIIINP scFv was synthesized from sequencing data.[15] The anti-PIIINP scFv was expressed using the *E. coli* recombinant protein expression system with some soluble expression enhancement strategies, utilizing soluble fusion partner and molecular chaperone co-expression.[16, 17] However, anti-PIIINP scFv was partially soluble and inclusion body refolding was attempted. The refolding process was divided into two step, inclusion body purification and step-wise dialysis refolding against urea concentration. In the dialysis refolding step, the time point of disulfide bond formation and refolding additives for stabilizing the reaction was optimized.[18-22] Following antigen binding ability to PIIINP was confirmed through ELISA. Finally, the quenchbody was developed through fluorescence labeling and the efficacy was evaluated by fluorescence measurements. These features make it suitable for a new doping method as an analytical validation.

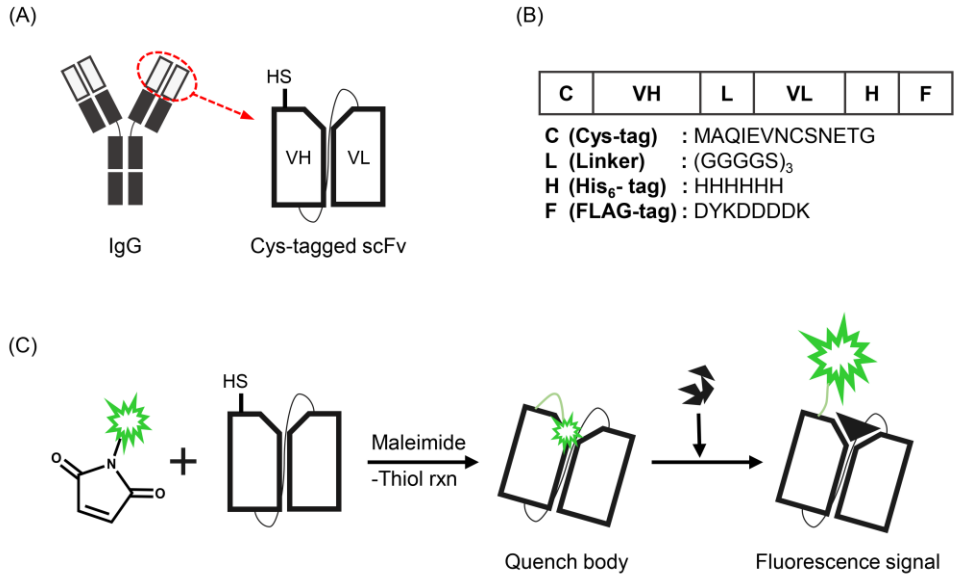


Figure 1. Graphical abstract of our study. (A) Cys-tagged scFv from IgG, (B) anti-PIIINP scFv construction, (C) schematic represent of maleimide–thiol reaction and quenchbody mechanism.

2. Materials and methods

2.1. Chemicals and materials

Orion UniQ PIIINP RIA kit was purchased from AIDIAN (Espoo, Finland). Luria-Bertani (LB) broth and Terrific broth (TB) media were purchased from BD Difco (Franklin Lakes, NJ, USA). FreeStyle 293 Expression Medium was purchased from Gibco (Grand Island, NY, USA). RNeasy mini kit and Ni-NTA Agarose were purchased from QIAGEN (Hilden, Germany). KOD-Plus-Neo DNA polymerase was purchased from TOYOBO (Osaka, Japan). In-Fusion HD Cloning kit and 2×GC I buffer were purchased from Takara Korea Biomedical, lnc (Seoul, South Korea). M-MLV reverse transcriptase and Exonuclease III were purchased from Promega (Madison, WI, USA). T4 RNA ligase I, PEG 8000, and Factor Xa Protease were purchased from New England Biolabs (Ipswich, MA, USA). RNaseA was purchased from BIOFACT Co., Ltd (Daejeon, South Korea). Herculase II Fusion DNA polymerase was purchased from Agilent (Santa Clara, CA, USA). Polyethyleneimine solution was purchased form Alfa Aesar (Ward Hill, MA, USA). L-Arabinose was purchased from Gold Biotechnology (St. Louis, MO, USA), Isopropyl β-D-1-thiogalactopyranoside (IPTG), imidazole, and ampicillin were purchased from Biosesang (Seongnam, South Korea). CAPTIVA protein A affinity resin was purchased from Repligen (Waltham, MA, USA). Immobilized TCEP Disulfide Reducing Gel, TMB substrate solution, and restriction enzymes were purchased from Thermo Scientific (Rockford, IL, USA). Urea was purchased from Daejung (Siheung, South Korea), L-Glutathione oxidized (GSH), L-Glutathione oxidized, and 2-Mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Arginine was purchased from Samchun Pure Chemical Co., Ltd.

(Seoul, South Korea). Tetramethylrhodamine maleimide (TAMRA maleimide) was purchased from Biotium (Hayward, CA, USA). ATTO520-C2-maleimide was from ATTO-TEC (Siegen, Germany). 3K Nanosep Centrifugal Device was purchased from Pall (Ann Arbor, MI ,USA). Recombinant Procollagen III N-Terminal Propeptide (PIIINP) was purchased from Cloud-Cone Corp. (Wuhan, China). For colorimetric absorbance scan, Multiskan Spectrum Microplate Spectrophotometer from ThermoLabsystems (Vantaa, Finland) was used. An instrument used for SDS-PAGE fluorescence detection was ChemiDoc XRS of Bio-Rad (Hercules, CA, USA). For fluorescence scan, Spark 10M multimode microplate reader from TECAN (Männedorf, Switzerland) was used. All DNA oligomers, DNA sysnthesis and sequencing were purchased from Macrogen (Seoul, South Korea). Hybridoma culture was conducted and purchased from A-Frontier (Seoul, South Korea).

2.2. Strains and vectors

Murine hybridoma 35J22 (PTA9525) was purchased from American Type Culture Collection (Manassas, VA, USA) and this cell line used for research has the permission of the patent applicant (US 7,541,149 B1). FreeStyle 293-F (HEK293-F) cell line was purchased from Invitrogen. *E. coli* SHuffle T7 Express LysY strain was purchased from New England Biolabs (Ipswich, MA, USA). *E. coli* BL21(DE3) strain and *E. coli* DH5α strain were in-house prepared. pUC19, pcDNA3.1::human IgG4 CH, pcDNA3.1::human IgG4 CL, pMAL-c2X, and pSQ vectors were in-house prepared. pGro7::groEL::groES (referred to as pGro7), and pKJE7::dnaK::dnaJ::grpE (referred to as pKJE7 were purchased from Takara Korea Biomedical, lnc (Seoul, South Korea).

Primer name	Sequence (5'-3')
kappa circularization	P_{HO}-GATATCTTCCACTTGACATTGATG
IgG1 hinge	P_{HO}-TGCAAGGCTTACAACCACAATCC
CL-EcoF	ATATGAATTCGCTTCTTGAACAACTTCTACC
CL-BamR	AATTGGATCCTTGGTGCAGCATCAGCCCG
CH-EcoF	GATAGAATTCCCAGCGAGACCGTCACCTG
CH-BamR	AATTGGATCCTTGACCAGGCATCCCAGG
pcDNA3.1-VL/VH-IL2-infuR	CGAATTCGTGACAAGTGCAAGACTTAGTGCAATGC AAGACAGGAGTTGCATCCTGTACATTCCCATGGTG GCGGCCGCTC
pcDNA3.1-VH-IL2-infuF	GCTTCTACAAAAGGGCCTAGTGTCTTTCC
pcDNA3.1-VL-IL2-infuF	GCCCCTTCTGTGTTCATTTTCCCACC
PIIINP-VH-infuF	CTTGTCACGAATTCGCAGGTGCAGCTGAAGG
PIIINP-VH-infuR	CCCTTTTGTAGAAGCTGAGGAGACGGTGACTG
PIIINP-VL-infuF	CTTGTCACGAATTCGGATGTTTTGATGACCCAAAG
PIIINP-VL-infuR	GAACACAGAAGGGGCAGCATCAGCCCGTTTG
PIIINP-NdeF	CCTGACCTGAAGGAGATATACATATGG
PIIINP-BamR	GTCGTCCTTGTAGTCGGATCC
PIIINP-NdeR	GTGCCATATGTATATCTCCTTC
PIIINP-BamF	GGTGGATCCGACTACAAGG
InfuF-pMALC2X-XmnI	GGATCGAGGGAAGGATGGCACAGATTGAG
InfuR-pMALC2X-BamH	CGACTCTAGAGGATCTTATTTGTCATCGTCGTC
pMALC2X-BamF	GATCCTCTAGAGTCGACC
pMALC2X-XmnR	ATCCTTCCCTCGATCC

Table 1. List of primers used in this study.

Plasmid	Origin	Maker	Descriptions	Ref
pUC19::VH	pMB1	Ap^r	pUC19 with VH from hybridoma 35J22	In this study
pUC19::VL			pUC19 with VL from hybridoma 35J22	
pcDNA3.1::human IgG4 CH	pMB1 f1 SV40	Ap^r Neo^r/ Kan^r	pcDNA3.1 with human IgG4 CH1, CH2, CH3	
pcDNA3.1::human IgG4 CL			pcDNA3.1 with human IgG4 CL	
pcDNA3.1::human IgG4 CH::anti-PIIINP VH			pcDNA3.1::human IgG4 CH with VH from hybridoma 35J22 containing IL-2 signal sequence	
pcDNA3.1::human IgG4 CL::anti-PIIINP VL			pcDNA3.1::human IgG4 C VL from hybridoma 35J22 containing IL-2 signal sequence	
pSQ::anti-PIIINP scFv	pMB1 f1	Ap^r	pSQ with anti-PIIINP scFv	
pMAL-c2X::anti-PIIINP scFv	pMB1 M13	Ap^r	pMAL-c2X with anti-PIIINP scFv	
pGro7	P15A	Cm^r	Molecular chaperone expression vector with groES and groEL	TAKARA
pKJE7			Molecular chaperone expression vector with dnaK, dnaJ, grpE	

Table 2. List of plasmids used in this study.

2.3. DNA construction

2.3.1. ELISA of anti-PIIINP IgG from hybridoma 35J22

Reactivity of anti-PIIINP immunoglobulin (IgG) from hybridoma 35J22 against PIIINP was analyzed by indirect ELISA. PIIINP with 2-fold series dilution from 100 ng to 6.25 ng was prepared into a 96-well plate (n=1). Following 2 hours of antigen binding with 100 μL of phosphate buffer saline (PBS) at 37°C, the plate was blocked by adding 300 μL of blocking solution (2 w/v% bovine serum albumin (BSA) in 0.05 v/v% tween 20 in PBS (PBST)) for an hour at 37°C. For a primary antibody, 100 μL of hybridoma 35J22 cell soup was directly fortified into each well. As a positive control, rabbit anti-PIIINP IgG solution from UniQ PIIINP RIA kit was equally treated. After 2 hours of incubation at 37°C, each well was washed three times with PBST. Subsequently, anti-goat IgG-HRP for the hybridoma 35J22 cell supernatant, and anti-rabbit IgG-HRP for the positive control was 4,000-fold diluted in prep solution (0.5 w/w% BSA in PBST), and each 100 μL of secondary antibody solution was added to each well. After 30 minutes of incubation, each well was washed five times with PBST followed by adding 90 μL of TMB substrate solution. Finally, the reaction was stopped by adding 25 μL of 2 N sulfuric acid, and the absorbance at 450 nm was obtained by a microplate spectrophotometer.

2.3.2. cDNA extraction of anti-PIIINP IgG

The total RNA of hybridoma 35J22 was extracted by using an RNeasy kit following the manufacturer's instructions. For reverse transcription of heavy chain variable region (VH) and light chain variable region (VL), each 25 μL reaction contained 600

ng of extracted total RNA, 1 μL of M-MLV reverse transcriptase, 5 μL of M-MLB 5× Reaction Buffer, 5 μL of 10 mM dNTP mix, and 50 pmoles of 5' phosphorylated IgG1 hinge primer and kappa circularization primer for VH and VL, respectively. Following 42°C for 100 minutes of reaction, the reaction was stopped by heat inactivation with 95°C for 15 minutes and adding RNaseA.

Each cDNA was polymerase chain reaction (PCR) purified, and eluted 20 μL of cDNA was circularized with 12 μL of PEG 8000, 4 μL of T4 RNA ligase buffer, 0.5 μL of 50 uM ATP, 1 μL of T4 RNA ligase I, and upto 20 μL of nuclease free water. The reaction was conducted at 37°C for an hour and stopped by heat inactivation with 95°C for 15 minutes. And linear cDNA was digested by adding Exonuclease III enzyme.

Inverse PCR of circular VH, VL cDNA each was amplified using primers which carrying heavy chain 1 (CH1) or light chain (CL) constant region, a primer set CH-EcoF and CH-BamR for VH gene, and CL-EcoF and CL-BamR primer for VL gene. Subsequently, agarose gel electrophoresis was conducted to extract DNA around its expected size. A piece of gel with band size around 500–600 base pair for VL, 600–700 base pair for VH were isolated and purified. After one more repetition of inverse PCR and agarose gel extraction, each isolated VH and VL gene was cloned into BamHI and EcoRI digested pUC19 vector.

E. coli DH5α competent cells were transformed with pUC19::VH or pUC19::VL plasmid and cultured on LB agar plate with 100 μg/mL ampicillin. Using blue or white screening, white colonies were picked for colony PCR with the same reaction condition to inverse PCR. Followed agarose gel electrophoresis, a single colony which carrying the expected size of VH or VL gene was cultured with LB

media with ampicillin (LBA). Finally, the VH and VL genes of anti-PIIINP was determined by sequencing data analysis with the following considerations: genes that containing the consensus region of CH or CL and without a stop codon in the middle.

2.3.3. Anti-PIIINP IgG4 expression in HEK293-F

Each anti-PIIINP VH and anti-PIIINP VL gene was cloned into pcDNA3.1::human IgG4 CH and pcDNA3.1::human IgG4 CL vector, respectively. pcDNA3.1::human IgG4 CH vector was amplified using Herculase II Fusion DNA polymerase and 2×GC I buffer, and a primer set with pcDNA3.1-VL/VH-IL2-infuR, pcDNA3.1-VH-IL2-infuF. pcDNA3.1::human IgG4 CL was amplified with a primer set of pcDNA3.1-VL/VH-IL2-infuR, pcDNA3.1-VL-IL2-infuF. For insert gene, VH gene was amplified with a primer set of PIIINP-VH-infuF and PIIINP-VH-infuR, VL gene was amplified with a primer set of PIIINP-VL-infuF and PIIINP-VL-infuR. In-Fusion cloning was conducted following the manufacturer's instructions.

Each VH, VL cloned plasmid was amplified in *E. coli* DH5α cells and purified final concentration to 1 μg/μL. For transfection, a DNA solution was prepared by adding 31.25 μg of each amplified plasmid in 2.5 mL of FreeStyle 293 expression media. Besides, polyethyleneimine (PEI) solution was prepared with 187.5 μL polyethyleneimine stock in 2.5 mL of media. The two solutions were combined by adding 0.2 μm membrane filtered DNA solution to polyethyleneimine solution and incubated for 20 minutes at room temperature. The transfection was conducted by adding the DNA–PEI mixture to a 50 mL culture volume of HEK293-F cell with 1×10^6 cells/mL. Further expression of anti-PIIINP IgG was conducted for 7 days at 37°C, 8 % CO_2 concentration and 130 rpm in 50 mL FreeStyle 293

expression media. Subsequently, expressed anti-PIIINP IgG was purified with CAPTIVA protein A affinity resin with the manufacturer's instruction.

2.3.4. ELSIA of anti-PIIINP IgG4 from HEK293-F

Reactivity of anti-PIIINP IgG from HEK293-F was analyzed by indirect ELISA. PIIINP with 2-fold series dilution from 100 ng to 6.25 ng was prepared into 96-well plate (n=1). Following 2 hours of antigen binding with 100 µL of PBS at 37°C, the plate was blocked with 300 µL of blocking solution for an hour at 37°C. For a primary antibody, 100 ng of purified antibody in 100 µL prep solution was added to each well. After 2 hours of incubation at 37°C, each well was washed three times with PBST. Subsequently, 100 µL of 4,000-fold diluted anti-goat IgG-HRP in prep solution was added to each well. After 30 minutes of incubation, each well was washed five times with PBST followed by adding 90 µL of TMB substrate solution. Finally, the reaction was stopped by adding 25 µL of 2 N sulfuric acid, and the absorbance at 450 nm was obtained by microplate spectrophotometer.

2.3.5. Anti-PIIINP scFv construction

VH and VL gene was linked by flexible $(GGGGS)_3$ linker and synthesized after *E. coli* codon optimization. For downstream maleimide–thiol click chemistry, N-terminus of anti-PIIINP scFv gene had a modification with Cys-tag (MAQIEVNCSNTG), and C-terminus of the gene had His_6-tag and FLAG-tag for downstream purification or ELISA. For In-Fusion cloning, the synthesized anti-PIIINP scFv DNA was amplified with a primer set of PIIINP-NdeF and PIIINP-

BamR. Likewise, a cloning vector pSQ was amplified with a primer set of PIIINP-NdeR and PIIINP-BamF. After In-Fusion cloning, a plasmid pSQ::anti-PIIINP scFv was purified by plasmid miniprep kit, and the sequence was confirmed by sequencing analysis.

A fusion partner, maltose binding protein (MBP) was introduced to the anti-PIIINP scFv gene by In-Fusion cloning. Using a primer set of InfuF-pMALC2X-XmnI and InfuR-pMALC2X-BamH, insert DNA was amplified. For the vector, pMAL-c2X was amplified with pMALC2X-BamF and pMALC2X-XmnR primer set. After In-Fusion cloning, a plasmid pMAL-c2X::anti-PIIINP scFv was purified by plasmid miniprep kit, and further sequence was confirmed by sequencing anlaysis.

2.4. Production of anti-PIIINP scFv

2.4.1. Expression optimization

E. coli SHuffle T7 Express LysY cells and *E. coli* BL21(DE3) were transformed with pSQ::anti-PIIINP scFv plasmid and cultured at 30°C for overnight on LBA agar plate. Each of a single colony was used and grown at 30°C for 16 hours with 3 mL of LBA. Scale-up was conducted by inoculating 0.5 mL of culture to 50 mL of fresh TB media containing 100 μg/mL of ampicillin. The cells were cultured at 30°C until OD_{600} reached 0.6–0.7, followed by adding 0.1 mM or 1.0 mM IPTG concentration. Each cell batch was cultured for another 4 hours or 16 hours with the various temperatures at 18°C, 30°C, 37°C.

2.4.2. MBP fusion anti-PIIINP scFv expression

E. coli SHuffle T7 Express LysY cells were transformed with pMAL-c2X::anti-PIIINP scFv plasmid and cultured at 30°C for overnight on LBA agar plate. Each of a single colony was used and grown at 30°C for 16 hours with 2 mL of LBA. Scale-up was conducted by inoculating 1.5 mL of inoculum to 150 mL of fresh TB media containing 100 μg/mL of ampicillin. The cells were cultured at 30°C until OD_{600} reached 0.55, followed by adding 1.0 mM IPTG. The further culture was conducted at 30°C for additional 16 hours.

2.4.3. Molecular chaperone co-expression of anti-PIIINP scFv

Each of the plasmid carrying molecular chaperone—pGro7::groEL::groES, and pKJE7::dnaK::dnaJ::grpE—was co-transformed with pSQ::anti-PIIINP scFv in *E. coli* strain SHuffle T7 Express LysY cells and cultured at 30°C for overnight on LBA agar plate. Each of a single colony was used and grown at 30°C for 16 hours with 2 mL of LBA. Scale-up was conducted by inoculating 0.5 mL of culture to 50 mL of fresh TB media containing 100 μg/mL of ampicillin and 25 μg/mL of chloramphenicol. The cells were cultured at 30°C until OD_{600} reached 0.6-0.7. Each chaperone expression was induced by treating 2 mg/mL of L-arabinose, and anti-PIIINP scFv expression was induced by adding 1 mM of IPTG. Each cell batch was cultured for another 16 hours at 30°C.

2.4.4. Native anti-PIIINP scFv purification

For 18°C overnight cultured *E. coli* SHuffle T7 Express LysY cells with 150 mL TB and 1 mM IPTG were harvested after 3,000×g for 15 minutes of centrifugation at 4°C. The pellet was resuspended in 15 mL of Ni-NTA binding buffer (50 mM sodium phosphate, 500 mM sodium chloride, 5 mM imidazole, 0.5 v/v% TritonX-100, pH 7.8) and disrupted by ultrasonication in an ice-cold water for 3 seconds on/off period with a total running time of 10 minutes. Followed by centrifugation at 13,000×g for 15 minutes, the supernatant was incubated with 100 µL of Ni-NTA agarose resin for an hour with gentle agitation. Using a gravity chromatography column, the resins were washed with 50 mL of Ni-NTA wash buffer (50 mM sodium phosphate, 500 mM sodium chloride, 30 mM imidazole, 0.5 v/v% TritonX-100 pH 7.8). The eluent was obtained by adding 2 mL of Ni-NTA elution buffer (50 mM sodium phosphate, 500 mM sodium chloride, 300 mM imidazole, 0.5 v/v% TritonX-100 pH 7.8), and buffer exchange *vs* PBS was conducted with dialysis for overnight at 4°C. All those steps were confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.4.5. MBP cleavage and purification

Cells were harvested after 3,000×g for 15 minutes of centrifugation at 4°C. The pellet was resuspended in 5 mL MBP lysis buffer (50 mM sodium phosphate, 500 mM sodium chloride, 10 mM imidazole pH 7.8) and sonicated for 3 seconds on/off period with total running time of 10 minutes in an ice-cold water bath. Followed by centrifugation at 13,000×g for 15 minutes, the supernatant was incubated with 300 µL of Ni-NTA agarose resin for an hour with gentle agitation. Using a gravity

chromatography column, the resins were washed with 50 mL of MBP wash buffer (50 mM sodium phosphate, 500 mM sodium chloride, 40 mM imidazole, pH 7.8). The eluent was obtained by adding 2 mL of Ni-NTA elution buffer, and buffer exchange *vs* Xa buffer (20 mM Tris-HCl, 100 mM sodium chloride, 2 mM calcium chloride, pH 8) was conducted with 3K Nanosep Centrifugal Device.

For 200 µg of MBP fusion scFv, 4 µg of Factor Xa Protease was added and incubated for 4 hours at room temperature with 10 rpm of 360° angle rotation. To purify the anti-PIIINP scFv, the enzyme reaction solution was incubated with 300 µL of Ni-NTA agarose resin for an hour with gentle agitation. Using a gravity chromatography column, the resins were washed with 20 mL of MBP wash buffer. The eluent was obtained by adding 2 mL of Ni-NTA elution buffer, and buffer exchange *vs* PBS was conducted with 3K Nanosep Centrifugal Device.

2.5. Refolding of anti-PIIINP scFv

2.5.1. Inclusion body isolation

E. coli SHuffle T7 Express LysY cells was transformed with pSQ::anti-PIIINP scFv plasmid and cultured at 30°C for overnight on LBA agar plate. Each of a single colony was used and grown at 30°C for 16 hours with 2 mL of LBA. Scale-up was conducted by inoculating 0.5 mL of culture to 50 mL of fresh TB media containing 100 µg/mL of ampicillin. The cells were cultured at 30°C until OD_{600} reached 0.6–0.7, followed by adding 1.0 mM IPTG concentration. Further culture was conducted at 30°C for additional 16 hours.

For 30°C overnight cultured *E. coli* SHuffle T7 Express LysY cells with

150 mL TB were harvested after 3,000×g for 15 minutes centrifugation at 4°C. The pellet was resuspended in 15 mL of lysis buffer (50 mM Tris-HCl, 500 mM sodium chloride, pH 8.0) and sonicated for 3 seconds on/off period with total running time of 10 minutes in an ice-cold water bath. Followed by centrifugation at 18,000×g for 15 minutes, the pellet was recovered with 30 mL of pellet wash buffer (2 M urea, 50 mM Tris-HCl, 500 mM sodium chloride, 2 v/v% TritonX-100, pH 8.0). After one more centrifugation and resuspension, followed centrifugation at 18,000×g for 15 minutes resulted in a clear-yellow pellet. The pellet was resuspended in 20 mL of denature buffer (8 M urea, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl, 500 mM sodium chloride, 5 mM imidazole pH 8.0) and incubated for 3 hours at room temperature with 100 rpm agitation. The solubilized pellet turned into clear solution and filtered through a 0.22 μm cellulose acetate filter.

2.5.2. Optimization of disulfide bond formation

Solubilized inclusion body solution was incubated with 1200 μL of Ni-NTA agarose resin for 30 minutes at room temperature with 10 rpm rotation speed. Subsequently, the resin was divided into 400 μL each, and loaded into three gravity empty columns. The packed columns were washed with 10 mL of 8 M urea refolding wash buffer (8 M urea, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl, 500 mM sodium chloride, 20 mM imidazole, pH 8.0), followed by washing 10 mL of 6 M, 4 M, 3 M , 2 M, 1 M urea refolding wash buffer (urea with 50 mM Tris-HCl, 500 mM sodium chloride, 20 mM imidazole, pH 8.0). Each of the three columns was washed with a solution containing 2 mM oxidized glutathione (GSSG) from the concentration of 3 M Urea, 2 M urea, and 1 M urea refolding washing step, respectively. Refolded anti-PIIINP

scFv was eluted with 2 mL of elution buffer (50 m Tris-HCl, 500 mM sodium chloride, 400 mM imidazole, pH 8.0).

Each 100 ng of refolded scFv which had different disulfide bond formation point was coated on 96-well plate at 4°C for overnight (n=1). The plate was blocked by adding 300 µL of blocking solution and was incubated for an hour at 37°C. Subsequently, 20 ng of PIIINP in 100 µL in blocking solution and without PIIINP for negative control were prepared into a 96-well plate, after three times of plate wash with PBST. For 2 hours of antigen binding at 37°C, the plate was washed two times with PBST. For primary antibody, 100 µL of rabbit anti-PIIINP IgG from UniQ PIIINP RIA kit was treated and incubated for 2 hours at 37°C, followed by five times of plate wash. As a detection antibody, 100 µL of 3,000-fold diluted anti-rabbit IgG-HRP in blocking solution was fortified into each well and incubated for 30 minutes at 37°C, followed by five times of plate wash. Finally, 90 µL of TMB substrate solution was added into each well, and the reaction was stopped by adding 25 µL of 2 N sulfuric acid when the color was near deep blue. The absorbance at 450 nm was obtained by microplate spectrophotometer and recorded.

2.5.3. Optimization of refolding additives

The anti-PIIINP scFv inclusion body bound Ni-NTA resin were prepared in six different gravity empty columns. The scFv was refolded by refolding wash buffer without 2-mercaptoethanol from 8 M urea to 3 M. From the 2 M urea to 0 M refolding step, the refolding additives combinations were tested. In order, combinations of 2 mM L-cysteine or GSSG, 400 mM L-arginine, and 500 mM sucrose were tested. The refolded anti-PIIINP scFv was eluted with 2 mL of elution

buffer.

Each 100 ng of refolded scFv which had different disulfide bond formation point was coated on 96-well plate at 4°C for overnight (n=1). The plate was blocked by adding 300 µL of blocking solution and was incubated for an hour at 37°C. Subsequently, 20 ng of PIIINP in 100 µL in blocking solution and without PIIINP for negative control were prepared into a 96-well plate, after three times of plate wash with PBST. For 2 hours of antigen binding at 37°C, the plate was washed two times with PBST. For primary antibody, 100 µL of rabbit anti-PIIINP IgG from UniQ PIIINP RIA kit was treated and incubated for 2 hours at 37°C, followed by five times of plate wash. As a detection antibody, 100 µL of 3,000-fold diluted anti-rabbit IgG-HRP in blocking solution was fortified into each well and incubated for 30 minutes at 37°C, followed by five times of plate wash. Finally, 90 µL of TMB substrate solution was added into each well, and the reaction was stopped by adding 25 µL of 2 N sulfuric acid when the color was near deep blue. The absorbance at 450 nm was obtained by microplate spectrophotometer and recorded.

2.5.4. Refolding scale-up: step-wise dialysis refolding

The solubilized inclusion body solution from 50 mL culture scale was incubated with 400 µL of Ni-NTA agarose resin for 30 minutes at room temperature with 10 rpm rotation speed. Subsequently, the resin packed columns were washed with 30 mL of 8 M urea refolding wash buffer (8 M urea, 10 mM 2-mercaptoethanol, 50 mM Tris-HCl, 500 mM sodium chloride, 20 mM imidazole, pH 8.0), followed by elution with 2 mL of elution buffer (8M urea, 50 mM Tris-HCl, 500 mM sodium chloride, 400 mM imidazole, pH 8.0). The concentration of purified inclusion body was adjusted

to 150 μg/mL using the denature buffer and put into a 7K MWCO dialysis bag. Refolding was started with dialyzing *vs* 6 M urea refolding buffer based on 50 mM Tris-HCl, 500 mM sodium chloride, pH 8.0 buffer solution. Following urea gradient was 4, 2, 1, 0.5 M urea with one day interval period. All dialysis step was conducted at 4°C, and 400 mM L-arginine was introduced from the 2 M urea dialysis step to prevent protein aggregation. For disulfide bond re-bridging, 2 mM L-cysteine was introduced first in 2 M urea dialysis step. Final buffer exchange *vs* PBS was conducted for 2 days at 4°C.

2.6. ELISA of native and refolded anti-PIIINP scFv

Reactivity of native and refolded anti-PIIINP scFv against PIIINP were analyzed by sandwich ELISA. As a capture antibody, 100 ng of anti-PIIINP scFv in 100 μL blocking solution was added into each well and incubated for 2 hours at 37°C (n=3). The plate was blocked by adding 300 μL of blocking solution and was incubated for an hour at 37°C. The antigen, 100 μL of PIIINP with 3-fold series dilution from 100 nM to 0.1 nM in blocking solution and negative control were prepared into a 96-well plate, after three times of plate wash with PBST. For 2 hours of antigen binding at 37°C, the plate was washed two times with PBST. For primary antibody, 100 μL of rabbit anti-PIIINP IgG from UniQ PIIINP RIA kit was treated and incubated for 2 hours at 37°C, followed by five times of plate wash. As a detection antibody, 100 μL of 3,000-fold diluted anti-rabbit IgG-HRP in blocking solution was fortified into each well and incubated for 30 minutes at 37°C, followed by five times of plate wash. Finally, 90 μL of TMB substrate solution was added into each well, and the reaction was stopped by adding 25 μL of 2 N sulfuric acid when the color was near deep blue.

The absorbance at 450 nm was obtained by microplate spectrophotometer and recorded.

2.7. Fluorescence labeling and purification

Anti-PIIINP scFv—native and refolded scFv— was concentrated into 0.8 mg/mL by using a 3K Nanosep Centrifugal Device. 100 µL of each anti-PIIINP scFv was reduced by mixing with an equivalent volume of pre-washed Immobilized TCEP Disulfide Reducing Gel. The N-terminal Cys-tag reduction was conducted for 45 minutes at room temperature and 10 rpm of wheel rotation. The reduced protein was isolated by using a gravity chromatography column. Each fluorescence dye with 10-fold to the protein molarity—ATTO520-maleimide and TAMRA-maleimide in dimethyl sulfoxide—was added to each half of the reduced protein. The reaction was conducted for 12 hours at 4°C in dark. The fluorescence–protein mixture was added to 50 µL of Ni-NTA agarose resin, followed by an hour incubation at room temperature with 100 rpm agitation in dark. The resins were moved into a gravity chromatography column and washed five times with 0.6 mL of PBS by centrifugation at 500×g and 30 seconds. After adding 50 µL of elution buffer (PBS with 250 mM imidazole, pH 7.4) and 2 minutes of incubation, the followed eluent was collected by 500×g and 30 seconds of centrifugation and two more repetitions of elution step. Finally, removal of unreacted fluorescence dye and buffer exchange *vs* PBS was done by utilizing 3K Nanosep Centrifugal Device and concentrated under volume as 100 µL. All those steps were confirmed by SDS-PAGE.

2.8. ELISA of quenchbody

As a capture antibody, 100 ng of quenchbody in 100 µL blocking solution was added into each well and incubated for 2 hours at 37°C (n=3). The plate was blocked by adding 300 µL of blocking solution and was incubated for an hour at 37°C. The antigen, 20 ng of PIIINP in 100 µL in blocking solution and negative control without antigen were prepared into a 96-well plate, after three times of plate wash with PBST. For 2 hours of antigen binding at 37°C, the plate was washed two times with PBST. For primary antibody, 100 µL of rabbit anti-PIIINP IgG from UniQ PIIINP RIA kit was treated and incubated for 2 hours at 37°C, followed by five times of plate wash. As a detection antibody, 100 µL of 3,000-fold diluted anti-rabbit IgG-HRP in blocking solution was fortified into each well and incubated for 30 minutes at 37°C, followed by five times of plate wash. Finally, 90 µL of TMB substrate solution was added into each well, and the reaction was stopped by adding 25 µL of 2 N sulfuric acid when the color was near deep blue. The absorbance at 450 nm was obtained by microplate spectrophotometer and recorded.

2.9. Fluorescence measurement

To confirm the quenching state of the quenchbody, denaturant solution (7 M guanidine hydrochloride (GdnHCl), 100 mM dithiothreitol) was mixed with 2 nM of quenchbody and incubated 5 minutes in a 96-well plate before the measurement. Labeling efficiency was calculated by measuring the fluorescence intensity of the fluorescence standard in the same denaturant and comparing it to the signal of the denatured quenchbody. For ATTO520-labeled quenchbody, excitation wavelength at

485 nm (±20 nm bandwidth) was used. Whereas TAMRA-labeled quenchbody, 535 nm (±25 nm bandwidth) of excitation wavelength was used for measurement, and emission wavelength with 5 nm of step size was recorded.

Antigen reactivity of the quenchbodies were analyzed on a 96-well plate, with 2 nM of quenchbody in PBST. Each PIIINP concentration from 0.1 nM to 100 nM was added into the plate and incubated for 5 minutes before fluorescence measurement. Each fluorescence intensity (FI) curve with a broad emission wavelength range was acquired by OriginPro2021. And dose-response curves with maximum FI at which emission wavelength 545 nm for ATTO520-labeled quenchbody, 585 nm for TAMRA-labeled quenchbody, were drawn in sigmoidal curves by OriginPro2021. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated from these sigmoidal curves with a five-point logistic equation. The LOD values were calculated as mean blank FI plus three times of the standard deviation, and LOQ values were calculated as mean blank FI plus ten times of the standard deviation. All experiment was conducted in thrice replication (n=3) and at a temperature of 24°C–27°C.

3. Results

3.1. ELISA of anti-PIIINP IgG from hybridoma 35J22

Because the anti-PIIINP antibody sequences were not disclosed, we had to find an available anti-PIIINP antibody source from an early developed hybridoma cell. [11] The patent applicants described that hybridoma 35J22 produced a monoclonal antibody to PIIINP and the antibody had no reactivity with procollagen type III C-terminal peptide (PIIICP) (Figure 2).Therefore, the developed hybridoma cell line deposited in ATCC was imported and antigen binding affinity had to be evaluated and confirmed before further research. Compared to the positive control in Figure 3, the hybridoma antibody had a binding affinity to PIIINP. We could not compare the absolute binding affinity due to unknown concentrations of anti-PIIINP IgG and to the different secondary antibodies. However, the anti-PIIINP IgG from the hybridoma was undoubtedly confirmed to be having a binding affinity to PIIINP.

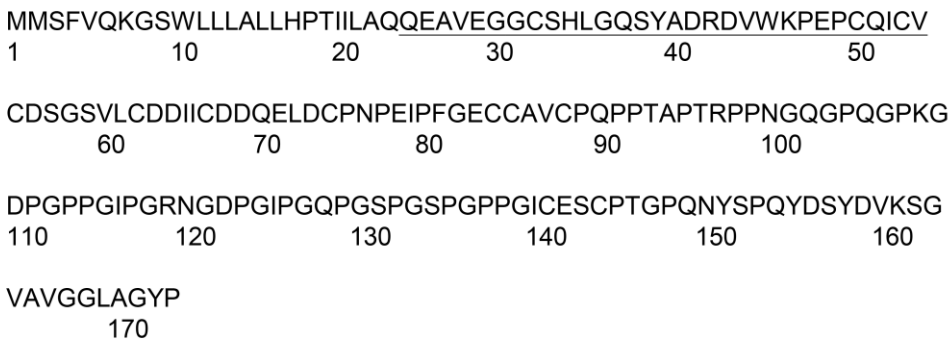


Figure 2. PIIINP Col I domain sequence. The underlined amino acid sequence is the epitope of the anti-PIIINP antibody from hybridoma 35J22.

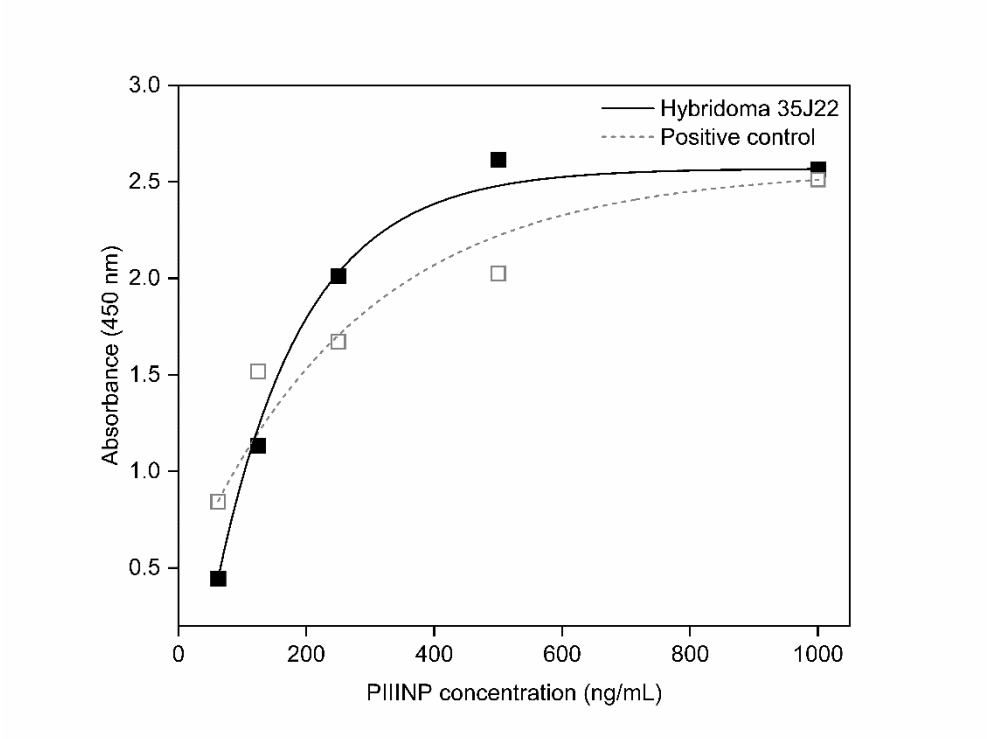


Figure 3. Indirect ELISA result against PIIINP with antibody from hybridoma 35J22.

3.2. ELISA of anti-PIIINP IgG4 from HEK293-F

Before the synthesis of the anti-PIIINP scFv gene, it was necessary to confirm that the anti-PIIINP VH and VL genes extracted from hybridoma 35J22 were correctly selected. Therefore, we expressed a form of human IgG4 modified anti-PIIINP VH and VL in an animal cell line HEK293-F. In the gene cloning step, the modified human interleukin-2 (IL-2) signal sequence (MYRMQLLSCIALSLALVTNS) was attached to the N-terminus of the recombinant antibody to induce the antibody secretion. As the result, Figure 4 showed that purified anti-PIIINP IgG4 was confirmed to be having a binding affinity to PIIINP, which means the anti-PIIINP antibody sequence was correctly screened.

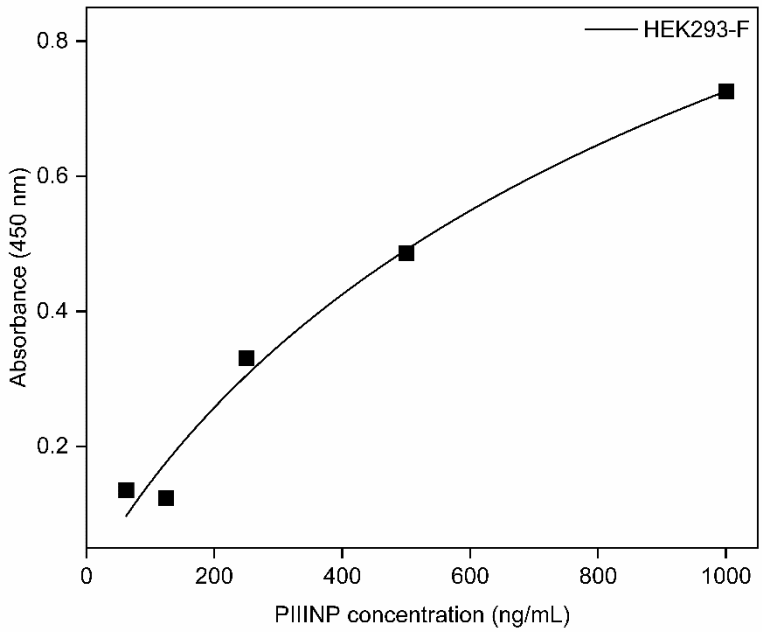


Figure 4. Indirect ELISA result against PIIINP with anti-PIIINP IgG4 antibody from HEK293-F.

3.3. Production of anti-PIIINP scFv

3.3.1. Native anti-PIIINP scFv

In Figure 5, despite various expression conditions, anti-PIIINP scFv was not soluble in the *E. coli* expression system. Nonetheless, 18°C, 16 hours culture with 1 mM IPTG culture condition was selected for downstream work because the soluble protein to total cell lysate ratio at this condition showed the highest level. In addition, because the scFv contains two intra-disulfide bonds, we selected the *E. coli* SHuffle T7 Express LysY as a host strain that has an oxidative condition in cytoplasm adequate for disulfide bond formation.[23] With this culture condition, the purified protein is shown at Figure 6. The band representing anti-PIIINP scFv around 29 kDa appeared on the SDS-PAGE. And the expression yield after purification was calculated as 5.3 mg/L with Bradford assay and ImageJ analysis.

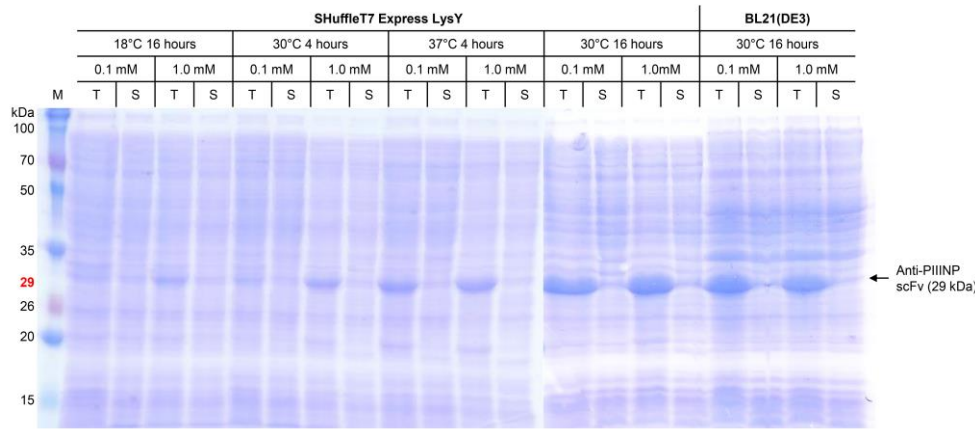


Figure 5. SDS-PAGE analysis of anti-PIIINP scFv expression in *E. coli*. M: marker, T: total fraction of cell lysate, S: supernatant, soluble fraction of cell lysate.

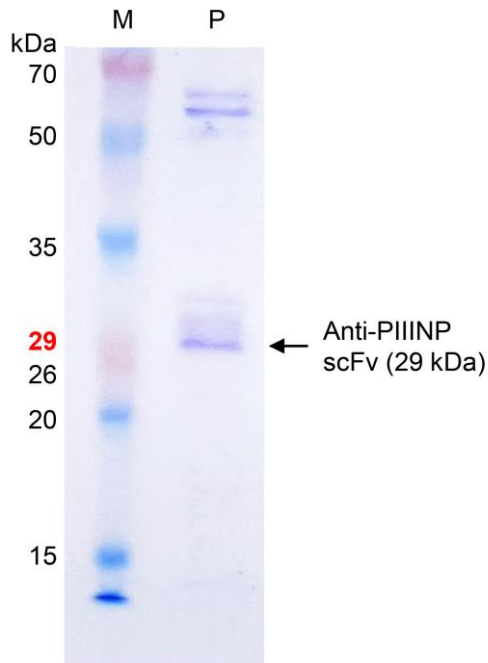


Figure 6. SDS-PAGE analysis of purified anti-PIIINP scFv. M: marker, P: purified anti-PIIINP scFv.

3.3.2. MBP fusion anti-PIIINP scFv

To overcome the low solubility issue in our scFv, we adopted fusion protein MBP.[16] The anti-PIIINP scFv was cloned into the C-terminus of MBP and expressed in SHuffle T7 Express LysY. In Figure 7, the solubility and expression of MBP fusion anti-PIIINP showed to be greatly enhanced. Furthermore, His_6-tag purification of MBP fusion protein was also successful yielding as 8.05 mg/L. After the Factor Xa Protease reaction, most of the MBP fusion protein was bisected into MBP and anti-PIIINP scFv. However, the uncleaved MBP fusion protein still remained even after the enzymatic hydrolysis. Besides, purified anti-PIIINP scFv had a low concentration that hardly detected on SDS-PAGE yielding 2.17 mg/L. Therefore, the MBP-fusion partner method has not been used because the yield is less than half that of normal expression.

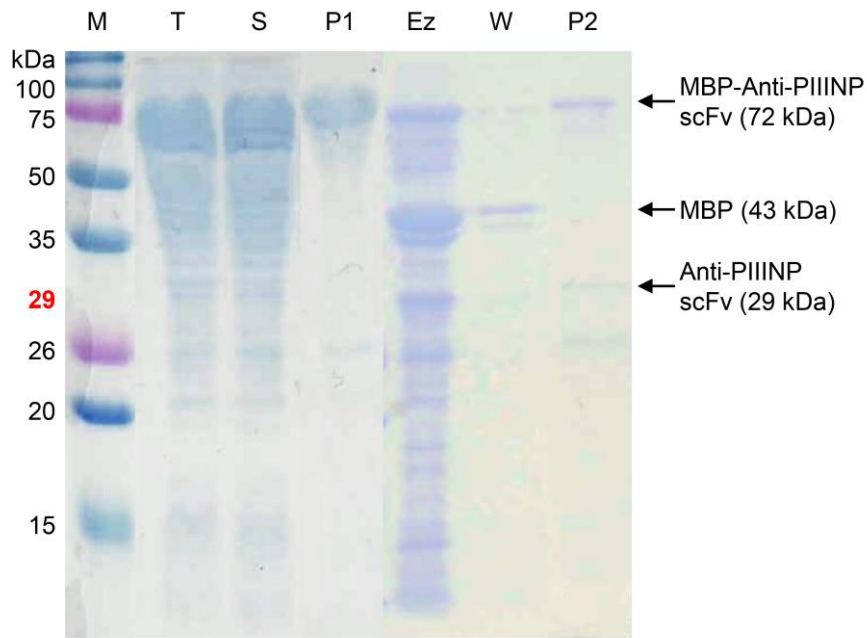


Figure 7. SDS-PAGE analysis of MBP fusion anti-PIIINP scFv. M: marker, T: total fraction of cell lysate, S: supernatant, soluble fraction of cell lysate, P1: purified MBP fusion anti-PIIINP scFv, Ez: after Factor Xa Protease reaction, W: Ni-NTA wash, P2: purified anti-PIIINP scFv.

3.3.3. Molecular chaperone co-expression

Promoting the correct folding of scFv, molecular chaperone co-expression could be an alternative method to increase solubility.[17] Molecular chaperone with pGro7 showed a distinct band of groEL, whereas groES (10 kDa) was a small protein hardly marked on SDS-PAGE (Figure 8). Likewise, co-expression with pKJE7 exhibited bands around the molecular weight of dnaK and dnaJ. Despite these chaperone co-expression, there was no dramatic solubility improvement in scFv expression, resulting in 4.0 mg/mL productivity. Therefore, this method also not used further because the yield is less than that of normal expression.

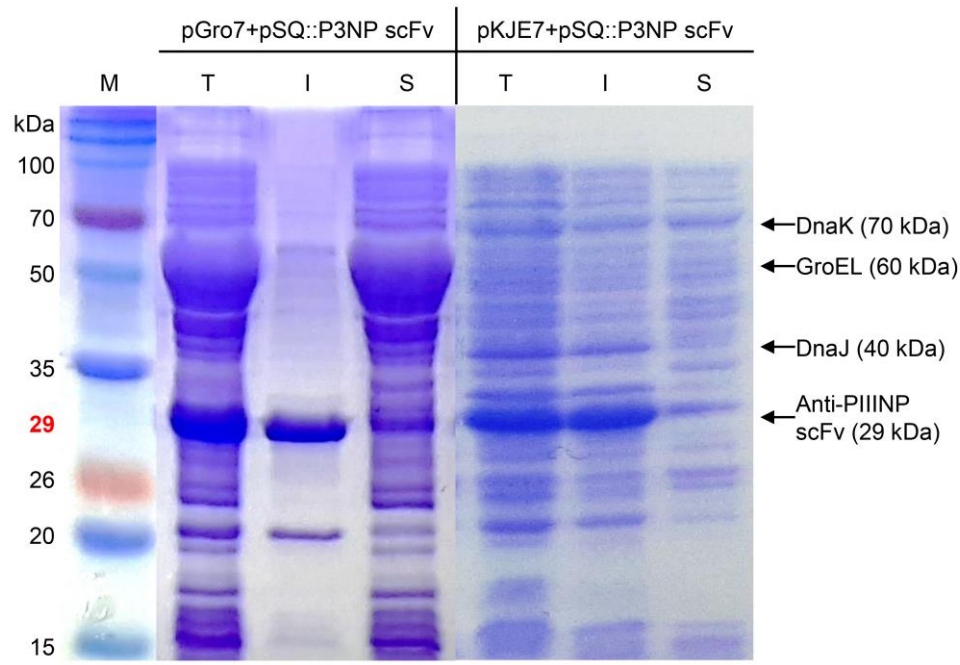


Figure 8. SDS-PAGE analysis of molecular chaperone co-expression with anti-PIIINP scFv. M: marker, T: total fraction of cell lysate, I: insoluble fraction, S: supernatant, soluble fraction of cell lysate.

3.4. Refolding of anti-PIIINP scFv

3.4.1. Inclusion body isolation

Since the MBP fusion partner or molecular chaperone co-expression did not show significant productivity improvement rather decreased yield, refolding was attempted as a method to obtain a more amount of protein. For the refolding of anti-PIIINP scFv, inclusion bodies were linearly released through a high chaotropic agent concentration with 8 M of urea and a reducing agent as 10 mM of 2-mercaptoethanol used for disulfide bond cleavage. After utilizing protein purification under denature conditions, the overexpressed inclusion body was obtained with extra-high purity (Figure 9).[24, 25] High purity of the refolded scFv was supposed to be derived from the denatured scFv, which could give a high degree of freedom to His_6-tag, resulted in a strong bind to the Ni-NTA resin. This point gives great advantages in productivity of anti-PIIINP scFv.

3.4.2. Optimization of disulfide bond formation

The most critical consideration in antibody refolding is that the scFv type antibody has four cysteine residues which form two respective intra-disulfide bonds. During refolding, it is important to form disulfide bonds by matching the correct pairing of these cysteines.[18] In detail, if an oxidizing agent is added to re-bridging the cysteines in the denatured state, the probability of forming a disulfide bond between unpaired cysteine residues increases due to the randomness of the linearly arranged poly-peptides. Therefore, inducing a disulfide bond when the native cysteine pairs are close to each other after the secondary structure of the protein is formed will be

a way to increase the success rate of refolding.[18, 21] In this regard, we attempted to optimize the point of disulfide bond formation as the concentration of urea which could represent the protein folded/unfolded state. In this screening stage, solid-phase refolding was performed for simultaneous comparison and short process time.[22]. The denatured inclusion bodies were bound to Ni-NTA resin, and a urea gradient was treated. Disulfide bond formation was induced by adding 2 mM GSSG in each of the 3 M, 2 M, and 1 M urea gradient steps, which concentrations were considered to be medium–low that could bring on the protein nearly folded state.

As a result, Figure 10 showed that oxidizing agent treatment when the scFv was in the unfolded/folded state at a concentration of 2 M urea had the best activity recovery. Although there is not much difference between the three, we further treated the oxidizing agent in a 2 M urea gradient step.

3.4.3. Optimization of refolding additives

Next, refolding additives such as the types of oxidizing agents and some stabilizers were investigated. Several papers stated that using refolding additives can improve refolding stability and increase recovery by preventing aggregation of the final product.[18, 19, 21, 24, 26] Therefore, the use of L-cysteine or GSSG as an oxidizing agent was set as the first variable. Second, experiments with L-arginine and sucrose as an aggregation inhibitor and stabilizer were conducted. Although the exact mechanism of L-arginine as a refolding aggregation inhibitor has not been clarified, it is known to prevent aggregation by affecting the surface tension of the refolded protein due to its high charge.[26] As a consequence, in Figure 11, experiments have shown that using L-cysteine as an oxidizing agent and L-arginine alone as an

adjuvant is better than none or combined with sucrose. On the other hand, when GSSG was treated for an oxidizing agent, the refolding efficiency was decreased whenever additives were added. When L-cysteine was used instead of GSSG, the absorbance was higher in all cases than in the GSSG treated group, which means that L-cysteine was a more effective disulfide bond accelerator than GSSG. Furthermore, it was found that L-arginine and sucrose showed a synergistic effect when used together with L-cysteine. However, refolding additives with L-arginine and L-cysteine combination resulted the highest refolding efficiency, and this condition was chosen for further research.

3.4.4. Refolding scale-up: step-wise dialysis refolding

The optimized refolding additive combination was scaled-up to step-wise dialysis refolding. Compared to the solid-phase refolding using Ni-NTA resin, the dialysis method can give some advantages for a high-throughput and more progressive gradient.[22] Furthermore, refolding buffer with L-arginine for Ni-NTA resin will result in drastic loss of protein yield due to electrically charged L-arginine is not compatible with Ni-NTA resin. After step-wise dialysis refolding, the calculated yield of the purified inclusion body based on the culture volume was 41.6 mg/L, and the recovered refolded anti-PIIINP scFv was 19.7 mg/L, and the productivity of the anti-PIIINP scFv was the highest.

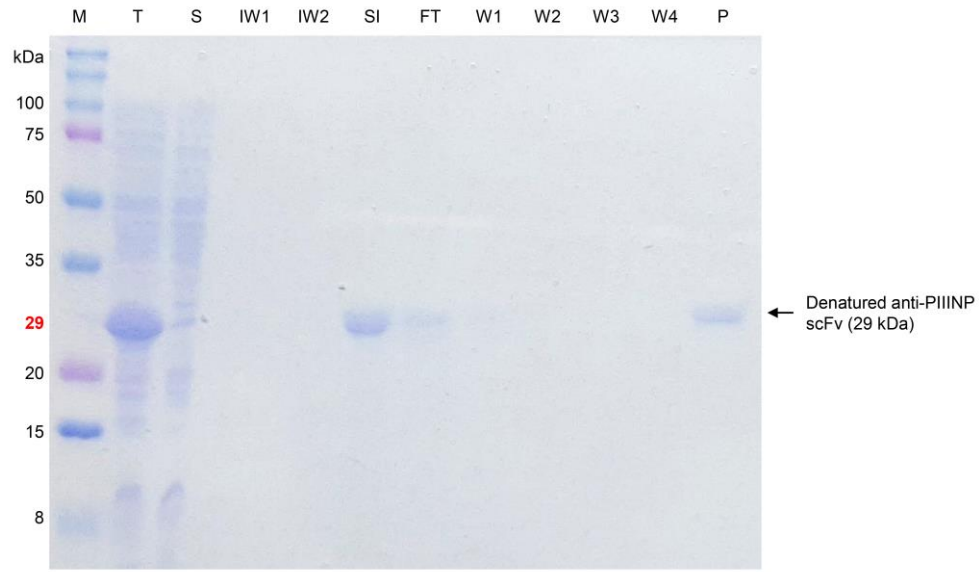


Figure 9. SDS-PAGE analysis of inclusion body purification process. M: marker, T: total fraction of cell lysate, S: supernatant, soluble fraction of cell lysate, IW: Ni-NTA wash of inclusion body, SI: solubilized inclusion body, FT: Ni-NTA flow through, W: Ni-NTA wash, P: purified anti-PIIINP scFv inclusion body.

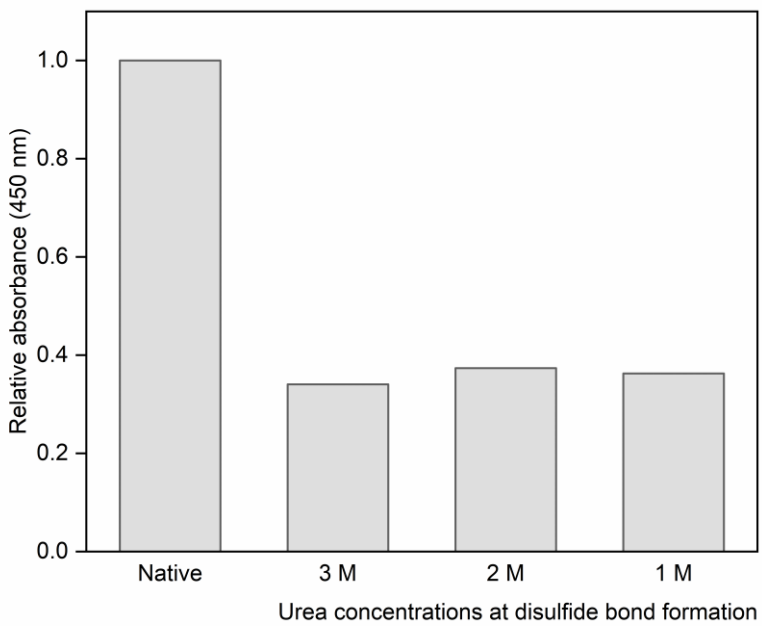
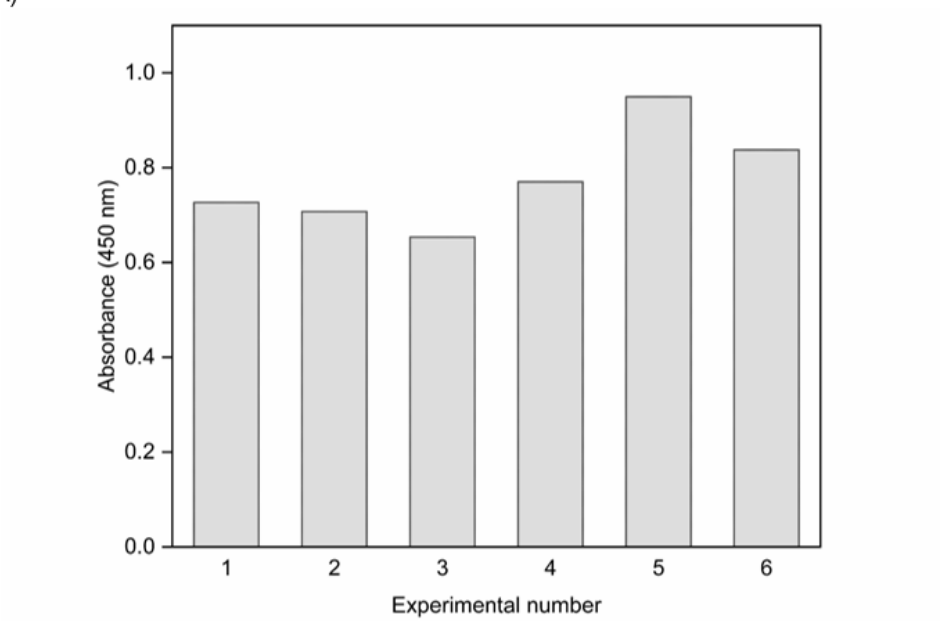


Figure 10. Sandwich ELISA result of refolded anti-PIIINP scFv with different disulfide bond formation point.

(A)



(B)

	Disulfide bond accelerator		Aggregation inhibitor	Stabilizer
No.	GSSG (2 mM)	Cys (2 mM)	Arg (400 mM)	Sucrose (500 mM)
1	+	-	-	-
2	+	-	+	-
3	+	-	+	+
4	-	+	-	-
5	-	+	+	-
6	-	+	+	+

Figure 11. (A) Sandwich ELISA result of refolded anti-PIIINP scFv with various refolding additives. (B) Types of additives and concentrations used in (A).

3.5. ELISA of native and refolded anti-PIIINP scFv

The result revealed that both native and refolded anti-PIIINP scFv had a binding affinity to its antigen linear range between from 0.1 nM up to 42-50 nM (Figure 12). Furthermore, the refolded scFv result demonstrated that an active form of anti-PIIINP scFv was made through the successful refolding process. Notably, refolded anti-PIIINP scFv showed little higher signal amplification in the ELISA compare to the native scFv. This consequence was considered because of the low purity of native anti-PIIINP scFv, which resulted in co-absorption of scFv with impurities in the ELISA plate as well as occurring errors in scFv quantification. Subsequently, less amount of scFv would be bound compared to the highly purified refolded scFv.

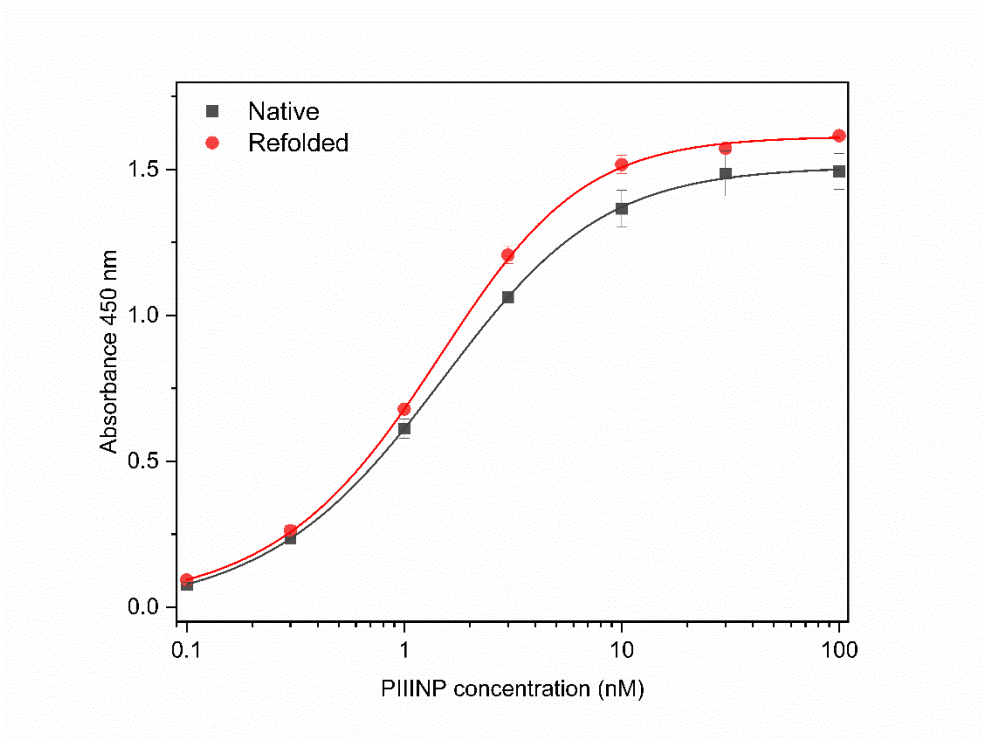


Figure 12. Sandwich ELISA result of native and refolded anti-PIIINP scFv.

3.6. Fluorescence labeling and purification

Fluorescent dye conjugations to native and refolded anti-PIIINP scFv were confirmed by SDS-PAGE analysis on UV light. In Figure 13, only a single band around 29 kDa was labeled with a fluorescence dye, green with ATTO520 and orange-red with TAMRA, demonstrating that maleimide–thiol click chemistry was successful.

The quenching efficiency of the anti-PIIINP quenchbody was evaluated by comparing the fluorescence intensity between the denatured state and the native state (Figure 14). The quenching capacity of ATTO520-labeled quenchbody was 1.90-fold with native scFv and 1.05-fold with refolded scFv, and TAMRA-labeled quenchbody resulted 2.47-fold with native scFv and 2.73-fold with refolded scFv. From these results, we could figure out that TAMRA dye was more suitable for quenchbody than ATTO520 dye. Interestingly, ATTO520-labeled refolded scFv recorded near 1-fold of quenching capacity which meant there were no differences in denatured state and native state of quenchbody. In short, most of the conjugated fluorescent dye was exposure to the surface, and did not quenched. Furthermore, the different quenching capacity between native and refolded scFv was observed in TAMRA labeled quenchbodies. The varying quenching capacity for the same fluorophore could not be precisely explained, but by inequivalent folding structures of the scFv between native and refolded scFv might be a key. Despite the optimized refolding process was performed, the protein structure could not be exactly equal to the naturally produced scFv.[27, 28] Therefore, little different folding structures consequently resulted in different distances between Cys-tag and tryptophan residues and quenching reaction would be significantly influenced. [29, 30]

Labeling efficiency was calculated based on the fluorescence intensity of each dye standard. And corresponding dye concentration at measured fluorescence intensity was divided into 2 nM of denatured quenchbody (Figure 15). Consequently, 46.5 % of scFv was labeled with ATTO520 and 52.8 % with TAMRA-labeled quenchbody with native anti-PIIINP scFv, which was approximately 50 % of labeling efficiency in our process.

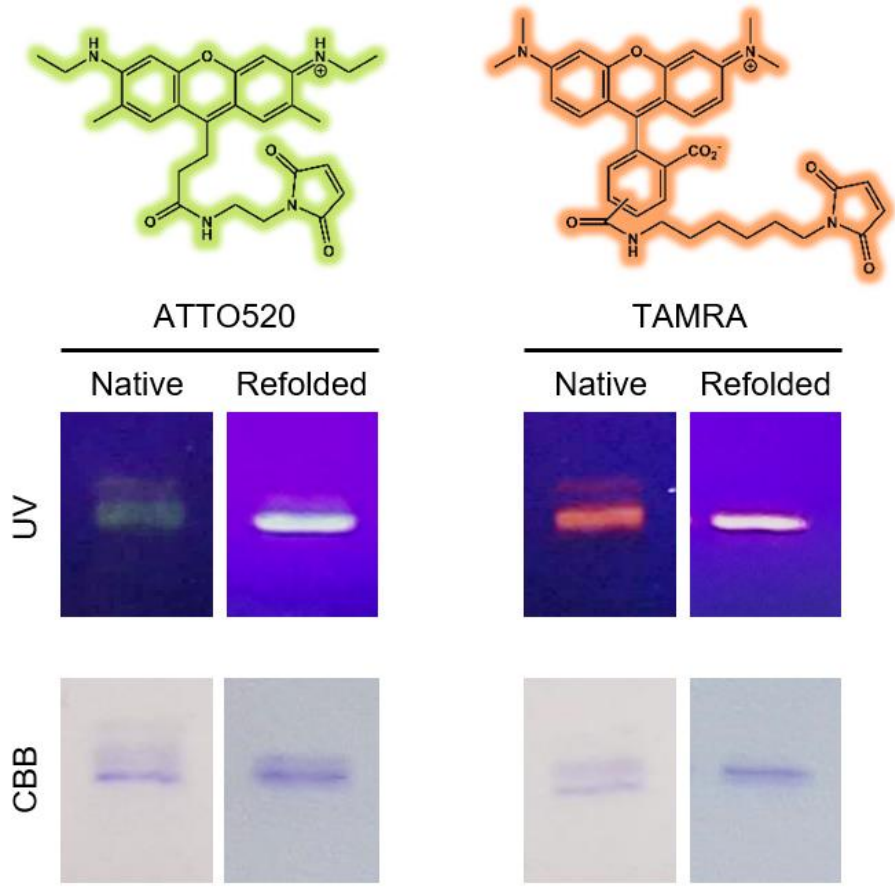


Figure 13. SDS-PAGE analysis of fluorescence labeling procedure. The molecular structures of two maleimide-fluorescent dyes are shown above, and the fluorescent (UV) and coomassie blue (CBB) stained bands of quenchbody are shown below.

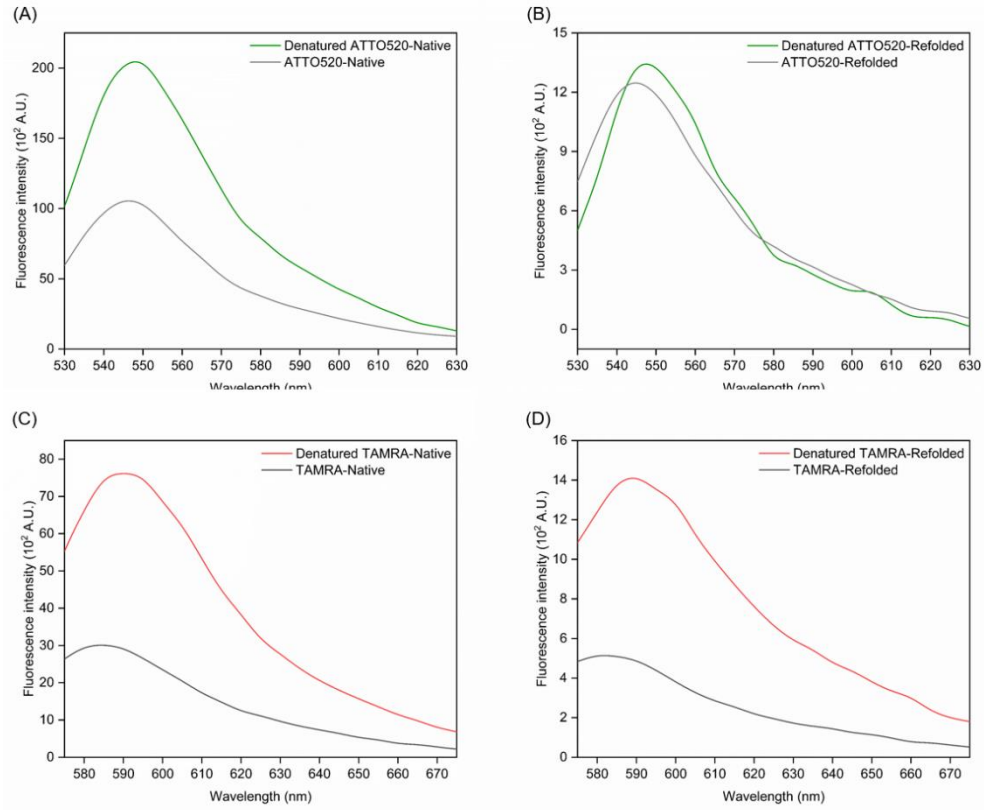


Figure 14. Fluorescence spectra of quenchbody in denaturant (green or red) or PBST (gray). (A) ATTO520-Native, (B) ATTO520-Refolded, (C) TAMRA-Native, (D) TAMRA-Refolded.

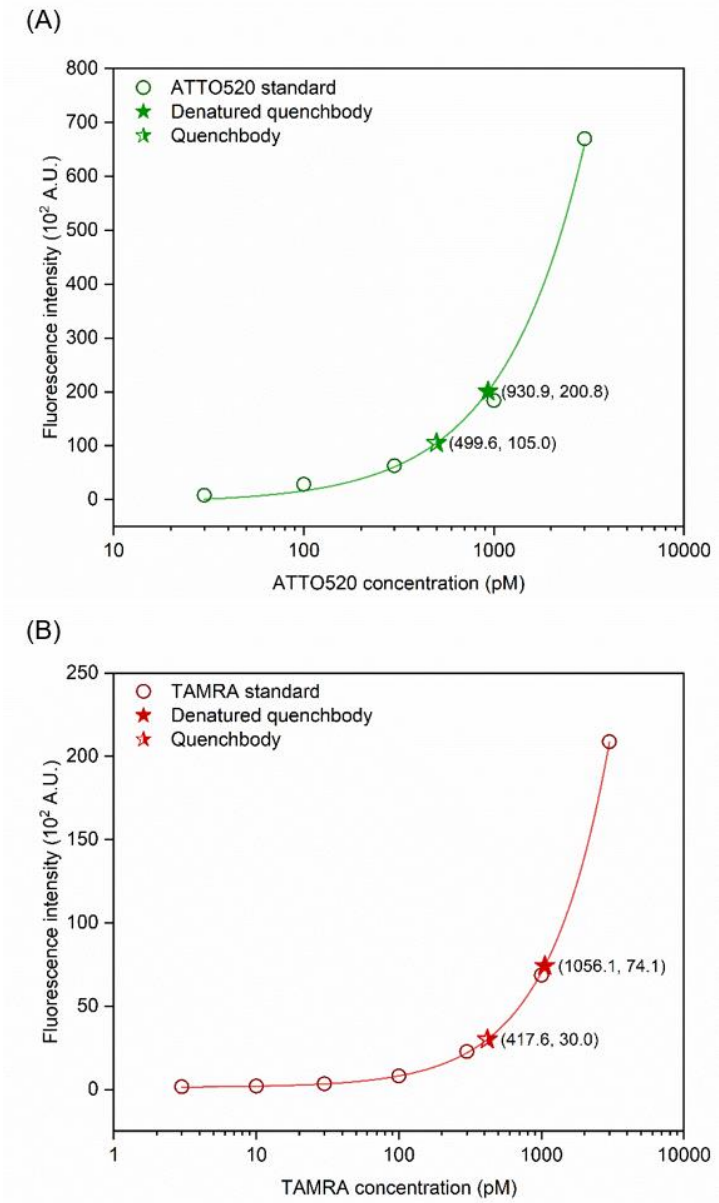


Figure 15. Fluorescence spectra of fluorescence dye standard and 2 nM of quenchbody. (A) ATTO520-labeled quenchbody, (B) TAMRA-labeled quenchbody.

3.7. ELISA of quenchbody

The binding affinity analysis of quenchbody was performed by sandwich ELISA (Figure 16). Native and refolded anti-PIIINP scFv exhibited similar antigen binding corresponding to the previous result in Figure 12. Moreover, it was confirmed that the absorbance of each fluorescence-labeled scFv was slightly decreased compared to its original form, but it was negligible because the difference was small. In short, the binding affinity of scFv was not remarkably influenced after the fluorescence labeling reaction, including Cys-tag reduction, maleimide–thiol reaction, and additional purification step. Although the antigen binding properties of the quenchbody were stable, the reason for the different quenching efficiency could only be explained by the fact that the factors affecting quenching were differed between native and refolded scFv. Since these factors are the structural positions of the Cys-tag and tryptophan residues to which the fluorescent dye is attached, it is considered that the detailed structural difference occurring before and after refolding has a decisive effect on the quenchbody.

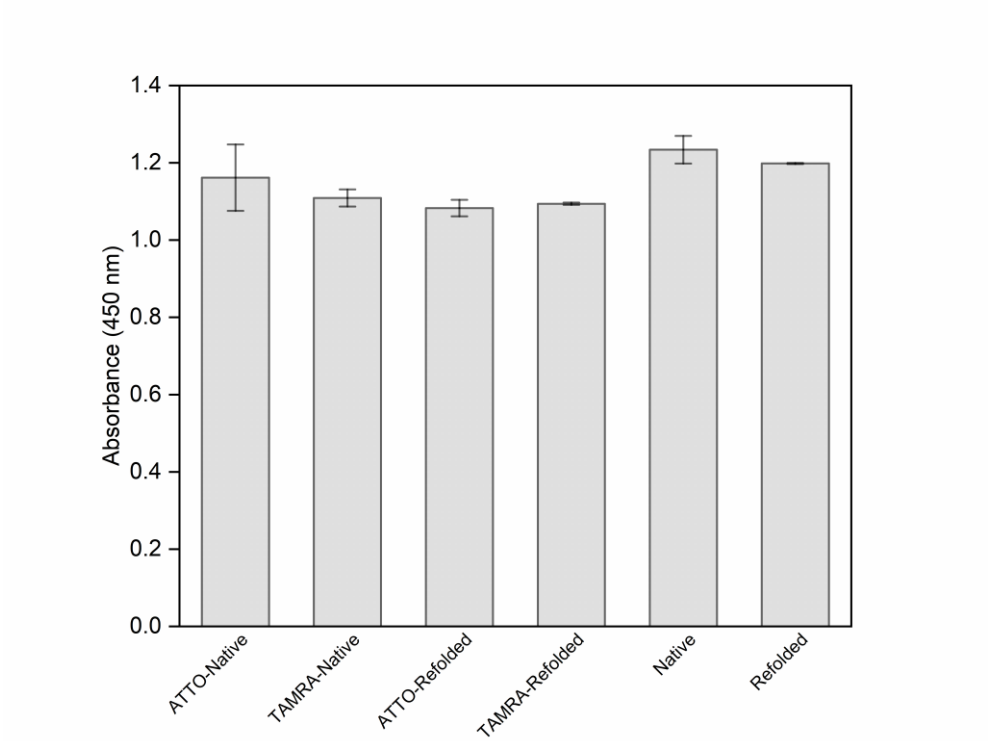


Figure 16. Sandwich ELISA result of quenchbodies.

3.8. Dose–dependent fluorescent response

In Figure 17, the dose-dependent fluorescence response of PIIINP was recorded by fluorescence spectrophotometer, starting from 0.1 nM to 100 nM of PIIINP. The fluorescence intensity gradually increased by the PIIINP concentration and saturated around 30 nM of antigen in the case of the quenchbody with the native scFv. However, irregular tendency in fluorescence intensity was observed in ATTO520 labeled refolded scFv as the dye was not quenched.

In Figure 18, for quenchbody with native scFv, the sigmoidal dose-response curves at a single emission wavelength were fitted into five-point logistic curves with a high regression, implying that the quenchbody signal was highly dependent on the antigen concentrations. With these fitted dose-response curves, LOD and LOQ of each quenchbody were calculated as 1.74 nM and 4.95 nM in ATTO-labeled quenchbody with 1.33-fold maximum fluorescence intensity, and 1.64 nM and 3.89 nM in TAMRA-labeled quenchbody with 1.48-fold maximum fluorescence intensity (Table 3).

In contrast, TAMRA-labeled quenchbody with refolded scFv which had the highest quenching capacity as 2.73-fold, showed a low level of signal amplification with 1.27-fold. We have already confirmed that the antigen binding ability and the action of the quenchbody are separated issues. Additionally, data on Figure 18 (B) demonstrates that the fluorescence motion in the quenchbody was also independent of the quenching capacity. For example, we can expect that high quenching capacity gives more signal amplitude. However, if the fluorescence molecule is quenched to tryptophan residue irrelevant with CDR in scFv, this is useless and is only an antibody hiding the fluorescence. Accordingly, the subtle structural difference

caused by the refolding process seemed not to affect the antigen binding affinity but had a critical effect on the very detailed quenching mechanism by distracting florescence and quencher as tryptophan.

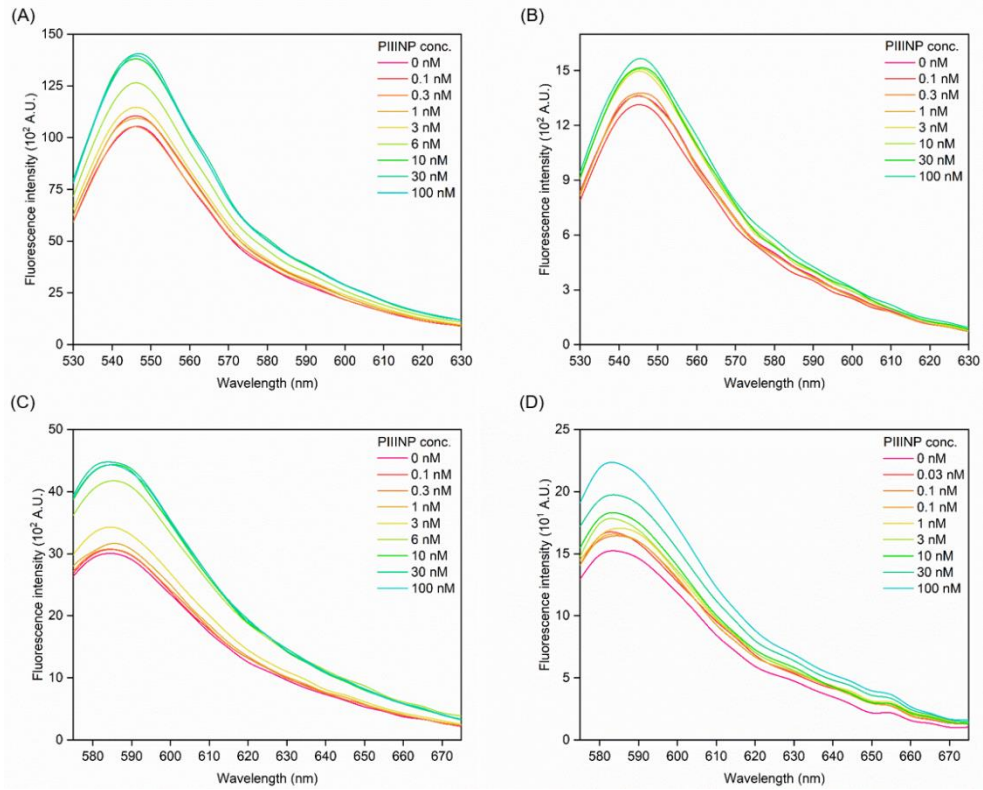


Figure 17. Fluorescence spectra of quenchbody to a series dilution of PIIINP. (A) ATTO520-Native, (B) ATTO520-Refolded, (C) TAMRA-Native, (D) TAMRA-Refolded.

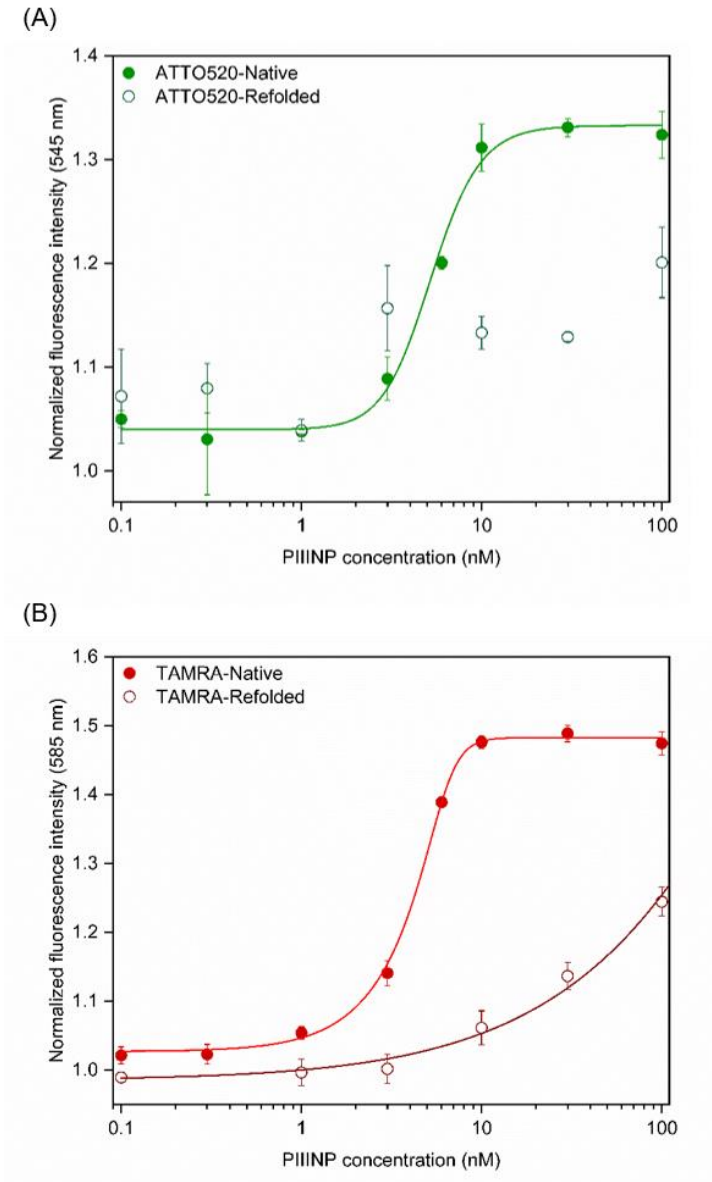


Figure 18. Dose-response curve of quenchbody to PIIINP. (A) ATTO520-labeled quenchbody, (B) TAMRA-labeled quenchbody.

	ATTO520-labeled quenchbody	TAMRA-labeled quenchbody
LOD (nM)	1.74	1.64
LOQ (nM)	4.95	3.89
R^2 (COD)	0.9936	0.9992
Equation (x refers PIIINP concentration)	$1.039 + \frac{1.333 - 1.039}{\left[1 + (\frac{4.833}{x})^{3.04}\right]^{1.20}}$	$1.027 + \frac{1.483 - 1.027}{\left[1 + (\frac{6.38}{x})^{6.72}\right]^{0.251}}$

Table 3. LOD and LOQ, and regression equation of quenchbodies.

4. Discussion and conclusion

Since the scFv form is the smallest and effective unit of an antibody, it has several advantages not only in antigen binding but also in *E. coli* expression. Moreover, as the number of disulfide bonds can be minimized, therefore, it was considered that a large amount of protein could be obtained by short and simple culture of *E. coli*. However, anti-PIIINP scFv was found to be insoluble in cytoplasmic expression. Besides, the MBP fusion partner and molecular chaperone co-expression, which were performed to increase solubility resulted in insufficient amounts in the final product. Other attempts were made to induce periplasmic expression by attaching the pelB sequence to the N-terminus of the anti-PIIINP scFv, but no significant change in the expression profile was observed (data not shown). Instead, refolding was performed as the last method. A series of refolding optimization revealed that addition of 2 mM of L-cysteine and 400 mM of L-arginine, starting from the 2 M urea concentration in step-wide dialysis refolding was the best condition for refolding. And the 19.7 mg/L of refolded anti-PIIINP scFv having activity was successfully obtained.

Because it was not known which fluorescent dye would give better results for this scFv, ATTO520-maleimide and TAMRA-maleimide fluorescence dye were used for simultaneous comparison. Labeling via maleimide–thiol reaction revealed that fluorescent dye was specifically conjugated to scFv regardless of the impurities, and the removal of unreacted dye in the purified quenchbody was confirmed by SDS-PAGE. Further investigation shows that labeling efficiency was about 50 %. In terms of fluorescence values to antigen concentration, TAMRA-labeled quenchbody with native anti-PIIINP scFv showed better results in quenching capacity, maximum

fluorescence intensity, LOD, and LOQ than ATTO520-labeled quenchbody. These differences were considered to be the different linker length between fluorescence dye and maleimide moiety. As the linker of TAMRA-C_6-maleimide is relatively longer than that of ATTO520-C_2-maleimide. TAMRA had merit for quenching that it could be located closer to the tryptophan, which is an amino acid involved in quenching.[31, 32] Therefore, our results suggest that the TAMRA-labeled quenchbody is more suitable for PIIINP detection and validation. However, in the case of refolded scFv, TARMA-labeled quenchbody which exhibited the highest quenching efficiency did not follow this principle. It is thought that some structural differences between native and refolded anti-PIIINP scFvs affected quenching action.

Indeed, there are still many tasks to be solved in order to use quenchbody for doping control analysis of PIIINP. According to the guidelines of the WADA, the LOQ acceptance criterion for PIIINP analysis is 0.023 nM, whereas for TAMRA-labeled quenchbody is 3.89 nM.[33] To overcome this problem, the improvement of the fluorescence intensity in quenchbody should be followed. As TAMRA-labeled quenchbody has a difference in signal due to the longer carbon linker length, it seems that it is possible to amplify fluorescence signal through linker length manipulation. These correspond to the linker length in the fluorescence dye itself, but it also includes adjusting the distance between the Cys-tag and VH with a Gly-Ser linker.[34] In addition, improving labeling efficiency should be accompanied by controlling the maleimide–thiol reaction time and temperature, the molar ratio between dye and scFv, and concentrations. Therefore, these can be added to enhance quality and signal in the next studies.

Our study aimed to develop an analysis system that can be used in the doping control field with a short analysis time and a reagentless sensor. Accordingly,

a quenchbody capable of validating the PIIINP within 30 minutes was made, and its performance was shown to be LOD 1.64 nM and LOQ 3.89 nM in the case of TMARA-labeled quenchbody. As quenchbody employs antibody-antigen binding affinity, the sensor has powerful features in protein analysis and can be applied diagnosis field as well, which can be achieved by simply changing the antibody. Therefore, we expect to be able to address areas in need of improvement through continuous research and hope that this technology will be able to benefit not only doping analysis but also clinical diagnosis.

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국문초록

프로콜라겐 III 형 N-말단 펩타이드는 성장 호르몬의 주요한 바이오마커로서 성장호르몬은 세계반도핑기구의 금지약물목록에 올라와 있다. 이에 따라, 근래에 개발된 방사면역측정법이나 형광기반의 샌드위치 효소결합 면역흡착검사와 같은 방법으로 프로콜라겐 III N-말단 펩타이드를 검출하고 있으나, 이들 방법은 방사능 안전성, 긴 분석 소요시간, 값비싼 장비의 사용과 같은 문제가 있다. 그러므로 기존 분석법의 문제를 해결할 수 있는 새로운 방법의 개발이 요구되고 있는 상황이다. 따라서 본 연구에서는 형광기반의 항체 센서인 퀜치바디를 만들어 고속 도핑 분석법을 개발하고자 하였다. 퀜치바디는 단일 쇄 가변 단편 형태로 만들어진 항체와, 항원의 유무에 따라 형광을 방출하는 형광 분자로 이루어진다. 항프로콜라겐 III 형 N-말단 펩다이드의 단일 쇄 가변 단편의 서열은 하이브리도마 세포로부터 얻어졌으며 재조합된 단백질은 대장균에서 발현되었다. 많은 양의 단일 쇄 가변 단편을 얻기 위해 봉입체 재접힘이 시도되었으며, 항원 결합성이 확인된 천연 및 재접힘 단일 쇄 가변 단편에 형광을 부착하였다. 마지막으로 형광광도계를 이용하여 퀜치바디의 항원 농도에 따른 형광 세기의 증가를 확인하였다. 그 결과로 5 개 변수 로시스틱 곡선 회귀 분석에 따라 TAMRA-라벨된 천연 단일 쇄 가변 단편을 사용한 퀜치바디가 각각 1.64 nM 과 3.89 nM 의 검출한계와 정량한계를 가져 가장 우수한 것으로 나타났다. 또한, 이는 2 nM 의 퀜치바디를 이용해 실험 준비부터 결과 검증까지 30 분 이내에 분석을 진행할 수 있었다. 따라서 우리는

새로운 도핑 분석법에 필요한 신속성과 고감도성을 퀜치바디에서 확인할 수 있었다.

주요어: 도핑 콘트롤, 프로콜라겐 III 형 N-말단 펩타이드, 형광 기반 항체 센서, 퀜치바디

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