Studies on design of single domain antibodies by Alpaca VHH phage library and high throughput sequencing to construct Fab antibody purification system

(Fab抗体精製システム構築のための高速配列解析とアルパカVHHファージライブラリを使った単ドメイン抗体の機能的デザインに関する研究)

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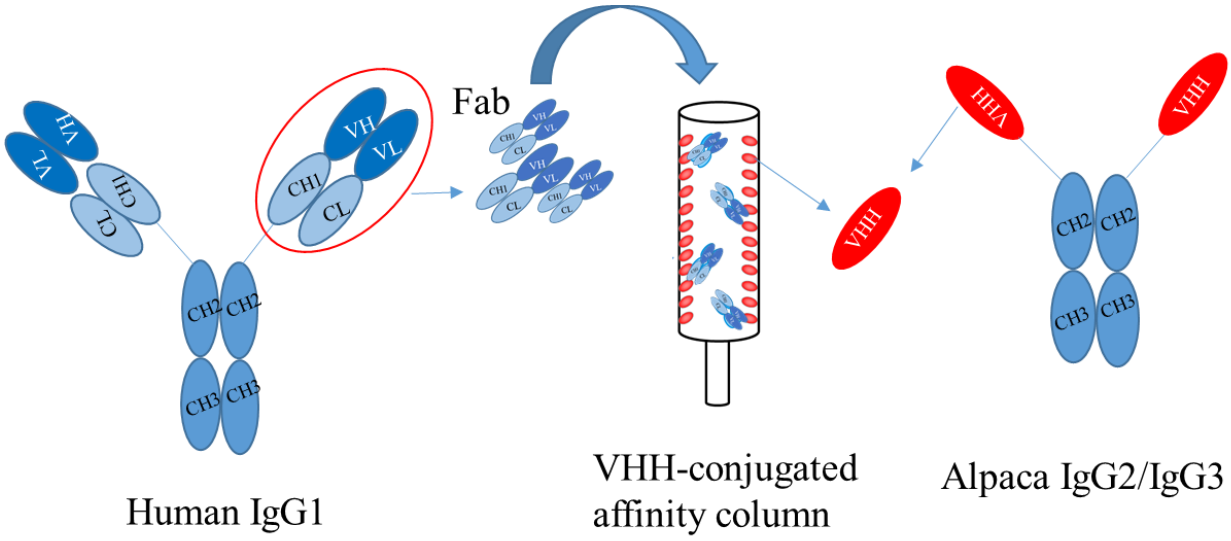
Abstract

To design an affinity ligand for purification of Fab antibody, VHH phage libraries were constructed from Fab-immunized Alpaca and subjected to biopanning against Fabs. We successfully isolated the binding phages specific to Fab by the conventional screening. However, those VHHs could not be used as an affinity ligand due to the lack of resistance against alkaline pH and/or difficulty in acidic elution from the affinity column. To find the other candidates of specific binders, we applied high-throughput sequencing (HTS) analysis of the VHH sequences was done using the NGS. The sequences with high amplification factor were aligned for construction of the phylogenetic tree. VHHs were grouped into five groups. The major three groups among them were identical with the VHHs isolated by the conventional screening. We further focused on the other two groups with minor populations. One of them harbored Cys residue in CDR3 of VHH, which is not suitable for affinity ligand. So, five clones from the other groups were selected and subjected to the binding test. Only one clone showed the binding ability with low affinity against Fabs. So, we improved its affinity tenfold more by affinity maturation through error-prone PCR library techniques. The final designed VHH showed highly alkaline-pH resistance and easy acidic elution together with high affinity to Fabs. These results suggest that our techniques are useful for designing the affinity ligands from the minor population of the VHH sequences using phage library.

Purpose of this study



Schematic representation of the development of human Fab antibody purification column using VHH affinity ligand. Column was conjugated with VHH affinity ligand belongs to Alpaca IgG2/IgG3 that are isolated against Fab fragments. So, that Fab drugs are trapped with VHH ligand during column purification.

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Chapter 1

General Introduction

1.1 Background

The creation of monoclonal antibodies (Abs) using hybridoma technology was a great invention in 1975 by G. Kohler and C. Milstein [**1**]. In the late 1980s, the first murine monoclonal antibody, muromonab (OKT3) approved by FDA for using the treatment of human organ transplant rejection [**2**]. Over the last 30 years, monoclonal antibodies were used as a powerful weapon for human indications. Recently, more than 70 therapeutic monoclonal antibodies are available in the United States and Europe as a therapeutic, with sales in the US alone reaching approximately $18.5 billion in 2015 [**3**].

Monoclonal antibodies are large (150 kDa) multimeric proteins containing numerous disulfide bonds and require post-translational modifications such as glycosylation. They need a piece of sophisticated eukaryotic machinery to be produced an active form. Moreover, most studies have shown that these molecules have to be injected in large amounts to achieve clinical efficacy (e.g. 8–16 doses of 375 $mg \cdot m^{-2}$, that is, a total amount of 6–12 g) per patient for Rituximab. Consequently, the production of therapeutic antibodies necessitates the use of very large cultures of mammalian cells followed by extensive purification steps, under Good Manufacturing Practice conditions, leading to extremely high production costs and limiting the wide use of these drugs [**4**]. Recently, there are several production systems in microorganisms and plants are being evaluated, those would be significant in the near future [**5**]. While mAbs are effective therapeutic agents when the antigenic target is well-defined and accessible (e.g., intracellular), cryptic, hypoallergenic targets [**6, 7**].

In such circumstances, antibody fragments (Fab) may provide advantages due to their smaller size and structure, and the ease with which they can be produced and genetically modified [**8, 9, 10, 11**]. Much recent interest is also directed towards antibody fragments (Fab). Some mAb therapeutics may be developed into antibody fragments (fab) displaying alternate pharmacokinetics and other desired properties. Many more antibody fragments are under development. [**9, 11, 12**]

Fabs are the oldest class [**13**] and were first generated by cleavage of an intact antibody with an enzyme, such as papain [**14**]. Cleavage yields two monovalent Fab fragments, each composed of one VH and one VL chain linked by disulfide bonds and displaying a single antigen-binding site. Fabs exhibit a number of properties that make them attractive as biotherapeutic agents [**6**]. Fragment antigen-binding (Fab) antibody fragments are one example of these next-generation biologic therapeutics that are emerging as credible alternatives to the widely accepted mAbs. Due to several features , the production of antibody fragments pipeline is expanding with three therapeutic Fabs approved by US Food and Drug Administration those are Abciximab (ReoPro, Eli Lilly), ranibizumab (Ranibizumab, Genentech), and certolizumab pegol (Cimzia, UCB) are manufacture in the periplasm of *E.coli* and many in the active clinical pipeline and preclinical research. [**13, 15, 16**].

1.2 Introduction of conventional antibody and its application

Antibody is a protein that acts to protect living organisms when foreign substances invade the body and has high specificity and affinity for specific antigens. Because of its superior functions, the antibody is used as a medicine with fewer side effects or as a highly functional material. The utilization of antibodies was developed by the establishment of hybridoma technology **[17]**, as the

production of monoclonal antibodies became possible. With the development of genetic recombination technology, artificial antibodies can be produced, and now antibodies against various antigens are produced, and it is used not only as pharmaceuticals, but also various test reagents, research tools, and the like. Some important applications of antibodies in medicine and biomedical research are discussed below:

Diagnosis

(a) Antibodies are highly useful in medical diagnostics. Many biochemical assays enable the detection of specific antibodies for the diagnosis of diseases.

(b) Most immunodiagnostic techniques such as ELISA use multiple antibodies to detect specific antigens capable of causing infectious diseases.

(c) In clinical immunology, levels of different classes of immunoglobulins are helpful in analyzing a patient's antibody profile.

(d) An increase in certain immunoglobulins is also a useful indicator in the diagnosis of many ailments. e.g., the elevation of IgM is an indication of viral hepatitis.

(e) Antibodies that can bind to human chorionic gonadotropin are used in over the counter pregnancy test kits.

(f) Antibodies are used to treat immune deficiencies such as hypogammaglobulinemia. Here, ready-made antibodies are administered to the patient to induce passive immunity.

(g) Monoclonal antibodies are widely used to treat several diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis, and several different cancers including colorectal cancer and breast cancer. About a dozen monoclonal antibodies for cancer treatment have been approved

by the US Food and Drug Administration so far. Clinical trials are on for developing more monoclonal antibodies that can help treat many more types of cancer.

Prenatal therapy

Rho (D) immune globulin antibodies are used in prenatal treatment to prevent the risk for hemolytic disease of the newborn. In the case of an Rh-incompatible fetus and mother, any blood mixing may cause sensitization of Rh- mother to the Rh+ antigen from the child. If the mother is treated with anti-RhD antibodies prior to delivery of trauma, it destroys the Rh antigen from the fetus before the antigen stimulates maternal B cells. Thus, treatment with Rho (D) immune globulin prevents sensitization that can cause Rh disease even in future pregnancies.

Western blotting

In this technique, proteins are electrophoretically separated and then transferred to a blotting paper, which is exposed to labeled antibodies to detect the proteins.

Immunosorbent assays

Enzyme-linked immunosorbent assays (ELISAs) are highly popular techniques used to detect and quantitate a particular antigen in blood serum. These assays exploit the high specificity of antibodies for different target antigens. Direct ELISAs use monoclonal antibodies to detect a specific antigen in a solution. Indirect ELISA uses a primary and secondary antibody to detect the antigen.

Immunohistochemistry / immunocytochemistry

Immunohistochemistry and immunocytochemistry are techniques used for in situ determination of the presence and the location of proteins. In these techniques, primary antibodies are used to bind to target antigens and conjugated secondary antibodies are used to detect the antigen-primary antibody complex.

Immunoprecipitation assays

In immunoprecipitation assays, antibodies help label and precipitate target antigens from an aqueous solution. Agarose beads first bind to the Fc region of the antibody and then allow centrifugal separation of the antibody-antigen complexes.

In vivo applications as a cancer therapy

Antibody-based therapy for cancer has become established over the past 20 years and is now one of the most successful and important strategies for treating patients with hematological malignancies and solid tumors. The killing of tumor cells using monoclonal antibodies (mAbs) can result from direct action of the antibody (through receptor blockade, for example), immune-mediated cell killing mechanisms, payload delivery, and specific effects of an antibody on the tumor vasculature and stroma. Tumor antigens that have been successfully targeted include epidermal growth factor receptor (EGFR), ERBB2, vascular endothelial growth factor (VEGF), cytotoxic T lymphocyte-associated antigen 4 (CTLA4), CD20, CD30 and CD52. Serological, genomic, proteomic and bioinformatics databases have also been used to identify antigens and receptors that are overexpressed in tumor cell populations or that are linked to gene mutations identified as driving cancer cell proliferation.

Flow cytometry

Antibodies are widely used in flow cytometry for intracellular analysis. In this method, single cell suspensions are surface stained with highly specific fluorochrome-tagged antibodies that can be detected easily. Cells can also be labeled with multiple antibodies as advanced flow cytometers are capable of detecting 3 or more fluorochromes at the same time. This can help detect proteins in the cytosol, nucleus, and endosomes.

1.2.1 Conventional antibody structure and its fragments

Antibody is a protein that acts in the immune system which is a self-defense mechanism, and it is involved in the elimination of foreign matter by recognizing foreign matter (antigen) invading from outside the body and specifically binding it. The structure of the conventional antibody is Y-shaped multi domain protein molecules composed of two polypeptide chains called heavy (H) chain (either alpha, gamma, delta, epsilon , or mu) and light (L) chain (either kappa or Lambda).

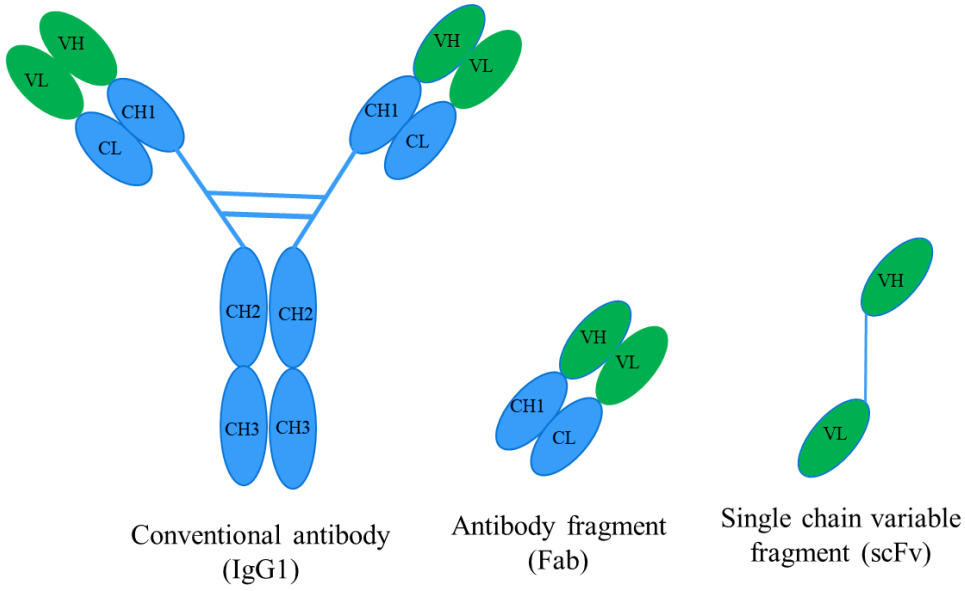

Figure 1. Schematic representation of conventional whole antibody and its fragments.

Each heavy chain contains one V and three C domains, while each light chain displays one V and one L domain (**Fig.1**). The antigen binding specificity of an antibody resides in the V domains located at the N-terminal region of the heavy and light chains is particularly referred to as the variable region (VH, VL). In VH and VL there are hypervariable regions or particularly diverse regions called complementarity determining regions (CDRs), three in each of VH and VL. CDRs are extremely important sites involved in the antigen recognition of antibodies, and by virtue of their diversity, they can bind to various antigens. The remaining areas of the VH and VL domains show less variation and comprise the framework (FR) regions which structurally support the loops. Carboxyl-terminal associated sections of the two H chains comprise the fragment crystallization (Fc) region involved in recruiting effector functions, such as complement fixation or immune cell activation, as well as stabilizing the antibody. Fc promotes enhanced serum half-life, and also binds to protein A. The region other than the variable region is called a constant region, and the amino acid sequence is constant for each antibody class (IgA, IgG, IgD, IgE, and IgM). The constant region is a region necessary for bioactivity such as activation of the body. Variable regions consist with CH1 and CL domain called fragment antigen binding (Fab). VH and VL domain connecting by a linker to call single chain variable fragment (scFv).

1.2.2 Variable domain of heavy chain antibody (VHH)

Camelid animals, including alpacas, produce heavy chain antibodies [**18, 19**]. IgG2 and IgG3 along with conventional antibodies IgG1 (**Fig.2**). It consists a single polypeptide chain lack of CH1 domain. VHH can specifically bind to the antigen, and its affinity is usually the same as that of the antibody. Furthermore, VHH has a small molecular weight with a simple structure, engineering modification is easy and stability is high [**20, 21, 22, 23**] as industrial application is expected.

There are two types of heavy chain antibodies, the hinge region connecting VHH and the constant region, respectively, is called short IgG2 (short hinge) and long IgG3 (long hinge) in the hinge region. In addition, it is reported that about 50% of the antibodies present in the blood are heavy chain antibodies in alpacas which are camelid animals.

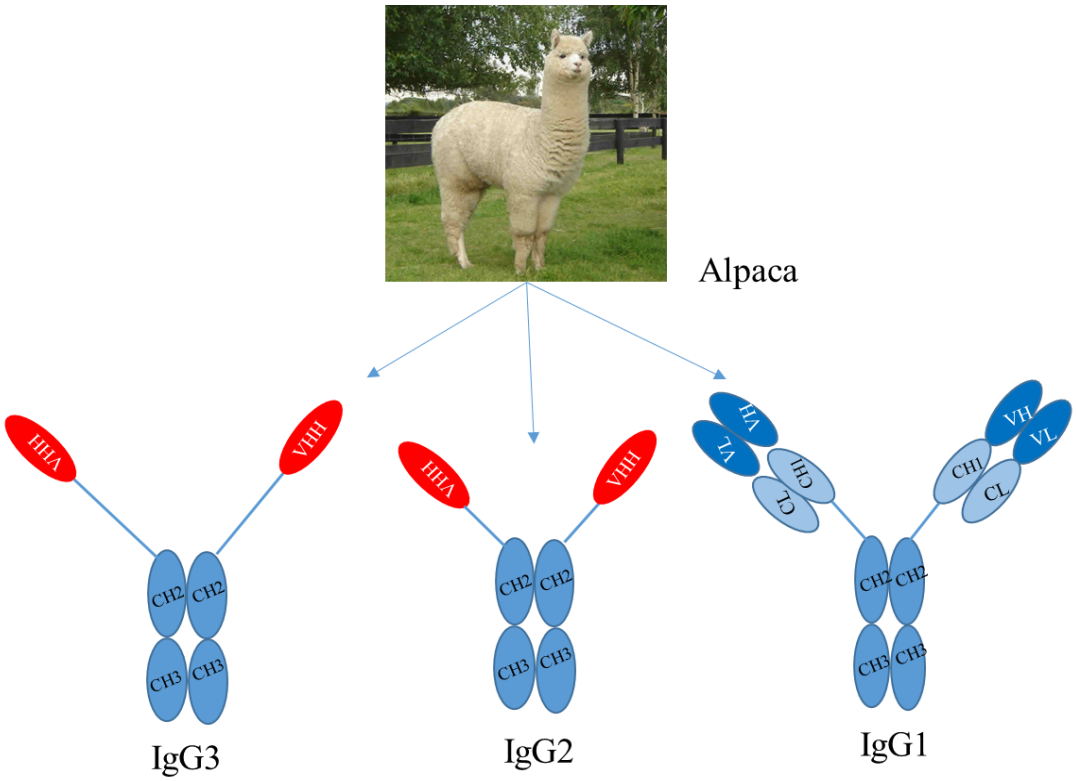

Figure 2. Schematic representation of variable domain of heavy chain antibody. Heavy chain antibody consists single polypeptide without light chain and lack of CH1 constant domain. IgG1 is a conventional antibody, IgG2 a short hinge region containing heavy chain antibody and IgG3 a long hinge region containing heavy chain antibody.

1.2.3 Benefits and limitations of antibody as a therapeutic

Antibody medicine is a medicinal product containing antibody (IgG1) as a main ingredient, and it is a medicine highly expected to be effective from the viewpoint of high affinity and specificity to antigens. The use of conventional antibodies are increasing day by day as a therapeutics. However, conventional antibody drugs have several drawbacks such as high production cost, difficulty in engineering operation due to large size, lower tissue penetration and higher side effects. Consequently, much recent interest is also directed towards antibody fragments (Fab). Some mAb therapeutics may be developed into Ab-fragments displaying alternate pharmacokinetics and other desire properties. Fabs exhibit a number of outstanding properties that make them attractive as biotherapeutic agents such as smaller in size as shown in (**Fig.3**), higher tissue penetration, less side effects, essay to protein engineering and low production cost but the Fab antibody purification is not well established yet whereas conventional antibody purification system is up to date now.

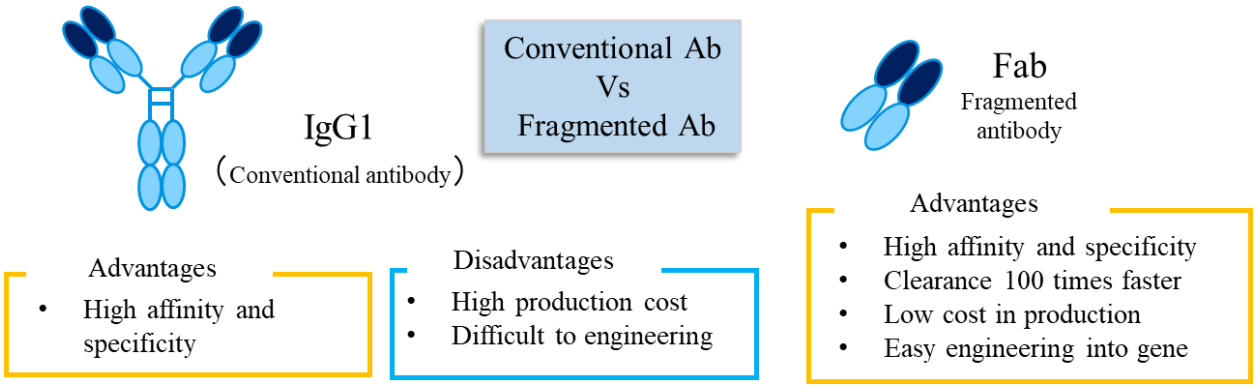

Figure 3. Structural comparison of conventional and fragment antigen binding antibody. Conventional antibody is a whole antibody whereas fragment binding antibody consists only variable and single constant domain of both heavy and light chain.

Fragment antigen-binding (Fab) antibody fragments are one example of these next-generation biologics that are emerging as credible alternatives to the widely accepted mAbs. Due to several features, the production of antibody fragments pipeline is expanding with three

therapeutic Fabs approved by US Food and Drug Administration those are Abciximab (ReoPro, Eli Lilly), ranibizumab (Ranibizumab, Genentech), and certolizumab pegol (Cimzia, UCB).

1.3 Antibody purification techniques

Purification of antibody fragments (Fabs) is more difficult than purification of whole molecule antibody, due to lack of the Fc constant regions of Fabs. The procedure typically employed for Protein-A chromatography, can be successfully used for Fabs containing the Fc region. For next-generation antibody that does not contain an Fc region, already several approaches were utilized as purification technique such as cation exchangers or mixed mode resin in bind/elute mode, anion exchanger or hydrophobic interaction chromatography, (BIO Pharma), affinity column protein G (Fab binding <1%) and Chicken Egg White Lysozyme- CNBr-sepharose (Fab binding 17% and purity was 48%. So, overall outcome was very poor in recovery, efficiency and purity. Though several methods exists for antibody fragment (Fab) purification, there is no particular choice of method have yet emerged (A platform approach to purification of antibody fragments).

However, a best approach, affinity chromatography which separates proteins based on reversible interaction between a protein and its ligand coupled to a chromatography matrix, is successful when an appropriate ligand is accessible for the protein of interest [**24, 25**].

1.4 Phage display technology

The phage display method is a technique for presenting peptides and proteins on the surface of filamentous M13 phage. By presenting the antibody protein on the phage surface, it can be handled as a library, and by binding to the immobilized antigen, antigen specific phage selection (biopanning) becomes possible. Also, by infecting *E. coli* with phage, amplification and isolation

of antibody genes can be easily performed, and they are widely used as a technique for producing antibodies.

The filamentous phage has a circular single-stranded genomic DNA (about 6.4 kb), which is a fibrous elongated cylindrical shell (diameter 7 nm, length 900 to 2000 nm). As a method for allowing foreign protein to be displayed on the phage surface, a method is mainly used in which expression is presented as a fusion protein on the N-terminal side of g3p or g8p protein on the phage surface (**Fig.4**). In the study of ordinary antibodies, a scFv antibody fragment in which VH and VL are linked with a linker is used. Since VH and VL are randomly combined at the time of scFv preparation, there is a problem that a combination which does not exist in nature occurs. However, since the binding domain of the heavy chain antibody used in this study is only VHH, there is an advantage that the combination problem does not occur. By using this phage display technique, it is possible to handle large size library by using phage display antibody library, and selection of antigen specific antibodies can be done by repeating biopanning. Currently, various antibody libraries have been produced all over the world, but mainly due to differences in antibody gene sources. The first one is the immune library. An immune library is a library prepared from a source of antibody genes derived from lymphocytes after raising the antibody titer in blood by inoculating a patient with a disease such as cancer or a specific antigen. It is an effective library for searching for the target antigen because the library contains a lot of antibody genes for specific diseases and antigens used for immunization in the library from the beginning. The second one is a naïve library. A naïve library is a library that is based on antibody genes of healthy human and animal sources. The antibody repertoire is not biased because the antibody gene originally present in the living body is made into a library.

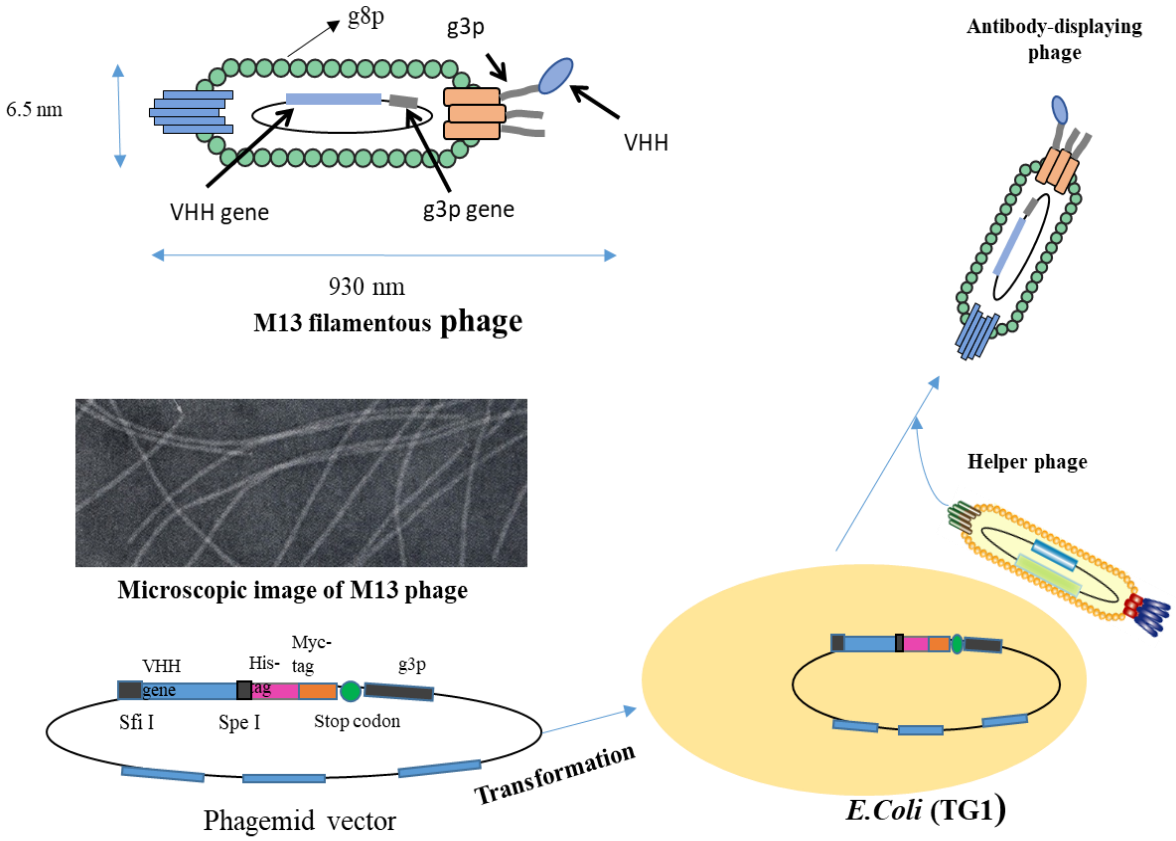

Figure 4. Schematic diagram of phage display technology. M13 phage contains several protein genes, one of them g3p has an ability to bind others foreign protein and represent on their surface.

Unlike the immune library, one library can be used for various antigens, but in order to ensure antibody diversity with few antigen specific clones, it is necessary to construct a large library. The third is a synthetic library in which the CDRs of specific antibody genes and the like are recombined into random amino acid sequences. It is possible to construct a library with high diversity by introducing a random amino acid sequence, and it is expected to obtain highly functional antibodies from the beginning by using antibody genes with high expression efficiency and high stability.

1.5 Next generation sequencing (NGS) and its application

Next-generation sequencing (NGS), also known as high-throughput sequencing, is the catch-all term used to describe a number of different modern sequencing technologies including Illumina (Miseq) sequencing. The isolation of ligands from large combinatorial libraries is a powerful technique allowing the discovery of bioactive molecules with a wide range of applications including diagnostics and therapeutics. This technique is generally carried out within the context of a biological display system, and commonly these include phage, yeast or bacterial cells to display a functional biomolecule. In doing so, the phenotype of the ligand is directly linked to its genotype, and the isolation of an individual clone displaying a particular binding property allows the isolation of the genetic sequence encoding the phenotype of interest (antibody, peptide etc.). This technique has the potential to isolate ligands to a variety of target types from libraries that can contain billions of different specificities. Despite many improvements, the gel-based Sanger sequencing technology still faces drawbacks in the name of cost and low throughput. For achieving high throughput, many commercial companies and scientific labs have come up with different ways of high throughput sequencing with a reasonable cost. The technologies named together as next generation sequencing technologies include sequencing by synthesis developed by 454 Life Science. The major issues to develop the next generation of technology are in read length, sequence quality, high throughput, and low cost.

Next generation sequencing has revolutionary impacts on genetic applications like metagenomics, comparative genomics, high throughput polymorphism detection, analysis of small RNAs, mutation screening, transcriptome profiling, methylation profiling, and chromatin remodeling. The applications of next generation sequencing (NGS) are as follows-

Transcriptome characterization

Currently the most common application of NGS in non-model species is transcriptome characterization. By this we mean generally describing what genes are expressed in a certain tissue, life stage or organism as well as functional characterization of these. Here, cDNA is synthesized by reverse transcription of mRNA and then sequenced. The first study describing the transcriptome in a non-model species through NGS was performed on the wasp Polistes metricus. Here, genome information from the related honeybee was used as template for mapping the reads from 454 sequencing and for downstream analysis. In the Glanville fritillary butterfly (*Melitaea cinxia*), however, the transcriptome was assembled de novo without the help of a closely related reference genome. The fritillary butterfly is a text-book example of a species with complex meta-population dynamics, and the aim of the genomics approaches recently employed in this system is to understand the genetics behind the variation in dispersal and colonization abilities seen between individuals. Since these first ground-breaking studies, sequencing and successful assembly of the transcriptome via 454 sequencing has been performed in a numbers of non-model organisms. The first study to use Illumina/Solexa sequencing data in a non-model species used a combination of de-novo assembly and genomic reference species mapped assembly to study the transcriptome of the polyploid plant Pachycladon enysii. However, since then, complete de-novo assembly of Illumina/Solexa data has also been accomplished. Most studies characterizing transcriptomes so far have been very descriptive by nature, but they provide an important starting point and a valuable resource for further analysis and ecological applications.

Gene expression profiling

In gene expression profiling, the aim is not only to characterize what genes are expressed but also to investigate the specific level (absolute or relative) of gene expression. Traditionally this has been accomplished using micro array and has thus mainly been restricted to model species with

previous genome information. However, as mentioned above, microarrays can now be constructed for non-model species using data from NGS transcriptome profiling or used in a cross-specific way if developed in related genome reference species. Development of microarrays through NGS considerably reduces both the cost and the effort involved.

Candidate gene finding

The candidate gene approach has also been widely used in conservation genetics. With the goal of conserving functionally important genetic information, studies have used genetic structure in ecologically relevant loci to identify taxonomic units of conservation interest. NGS has great potential to open up this approach to conservation genetics to more species and include analyses of a larger number of potentially important genes. The eelpout (*Zoarces viviparous*) is a fish species commonly used in environmental monitoring and ecotoxicological studies. It recently had its transcriptome characterized using 454 sequencing and a number of biomarker genes for ecotoxicology were specifically identified, thus providing an important tool for future studies of the genetic basis for physiological responses to pollutant exposures. When performing transcriptome sequencing with the aim of detecting candidate genes it is important to make sure that RNA from the right tissues and/or life stages are used. Genes under positive selection (therefore likely to be genes of interest) are expressed in a more tissue-specific manner compared with evolutionary conserved genes and could thus easily be missed if coverage is too low.

Targeted sequencing

The NGS applications reviewed so far generate an impressive amount of sequence data, but in terms of population genetics (for example, variation between individuals and populations) the amount of information is limited. For such applications, it is more informative to use NGS to sequence a limited number of targeted loci. By decreasing the number of targets, the coverage is

considerably increased, and consequently more valuable information for population analyses is obtained. Targets for sequencing can be obtained either using PCR or genetic capture techniques before sequencing. The targeted regions can represent individual independent loci (for example, exons) or along stretch of genomic DNA.

Whole genome sequencing (WGS)

An alternative approach to WGS in species without a characterized reference genome may be to use NGS to generate a large amount of sequence data, and to analyze this without attempting a full genome assembly. This approach was taken in a study of mammoth genomics, in which previously generated Sanger sequence data.

1.6 Summary

In the field of monoclonal antibody therapeutics, the popularity of Fab antibody drugs is increasing day by day due to smaller in size (56 kDa), high affinity, specificity, easy engineering modification, low production cost and easy access to the target site. However, the use of Fab antibody drugs is still limited due to Fab purification system has not well established yet.

In this study, we isolated high affinity and specificity containing VHH antibody affinity binders to Ranibizumab and Trastuzumab-Fab from immunized phage display library followed by NGS analysis and affinity maturation was done by error-prone PCR followed by a combination of functional mutation.

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Chapter 2

Isolation and characterization of Fab-specific VHH antibody from immunized Alpaca using phage display technology

2.1 Background

Camelidae species, including alpaca (*Lama pacos*), have dimeric antibodies consisting of two heavy chains (HCs), designated VHH antibodies, in addition to conventional tetrameric antibodies (IgG1) consisting of two heavy and two light chains, as first reported in camels [**1**]. VHH antibodies whose heavy chain does not have the CH1 constant domain are categorized in two classes, IgG2 and IgG3. The former has a short and the latter has a long Pro/Gln-rich hinge region between the variable antigen-binding domain and the CH2 constant domain. VHH antibodies comprise almost 50% of IgG in alpaca serum [**2**] and are considered to play an important role in immunity, but the distinct role of VHH antibodies in humoral immunity remains to be fully elucidated [**3, 4**]. The antigen-binding domain of heavy chain antibodies designated as VHH or nanobody consists of a single immunoglobulin domain generally characterized by a long complementarity determining region (CDR) compared with conventional IgGs [**5, 6, 7**]. Most VHHs are related to the human VH3 family belonging to clan III [**8, 9**], but a recent article reported a novel class of VHHs close to human VH4 belonging to clan II [**10**]. The similarities of VHH sequences to human VH may be advantageous for its clinical application as like Nanobody therapeutics [**11**]. On the other hand, the crucial mutations in framework 2 between VHH and VH are found to be Val37Phe/Tyr, Gly44Glu, Leu45Arg, and Trp47Gly in Kabat numbering [**12**] and possibly contribute to reshape the surface of VH by removing the hydrophobic residues on VH/VL interface. VHHs have several unique advantages for industrial applications [**12, 13**] due to their

superior biochemical and biophysical properties, including smaller in size (15 kDa), high solubility, resistance against aggregation [**14**] and high efficiency in renaturation after thermal denaturation [**15, 16**]. In addition, functional modification of VHHs (e.g. for multi-specificity) can be readily attained by protein engineering like connecting VHHs [**17, 18, 19**]. VHHs also are highly producible in bacteria, yeast, plant and mammalian cells [**20**].

The phage library is a powerful tool to identify the functional binders. Using this system, many antigen specific antibody fragments (including Fab, single chain Fv (scFv) and VHH) have been isolated through a biopanning selection process [**21, 22, 23, 24**]. On the other hand, deep sequencing by high-throughput sequencing (HTS) platform with next generation sequencer (NGS) has been used for genomic and single nucleotide polymorphism analysis and recently been applied to comprehensive analyses of the antibody repertories of B cells to identify antigen-specific antibodies generated after immunizing animals with antigen [**25, 26, 27**].

2.2 Methods and materials

2.2.1 Immunization of Alpaca

An adult male Alpaca (*L.pacos*) was immunized (**Table 1a**)subcutaneously day '0' with 2.8 mg Trastuzumab-Fab conjugate emulsified in Freund complete adjuvant, and days '14' and '28' with 2.8 mg Trastuzumab-Fab conjugate emulsified in Freund incomplete adjuvant. Days '42' and '70' same Alpaca was immunized by 1 mg Ranibizumab conjugate emulsified in Freund incomplete adjuvant and days '56' immunized by 1 mg Human-CL conjugate emulsified in Freund incomplete adjuvant. Serum was collected prior to each injection to check the antibody response. Blood (50 mL) collected on days '21', '63' and '84' (**Table 1b**) was treated with anti-coagulant (0.1% EDTA) and used to isolate peripheral blood mono nuclear cells (PBMC) by Ficoll-plaque method

using Leucosep tubes (Greiner Bio-One). A yield of 5×10^7 cells was obtained, homogenized in RNAiso Plus (Takara Bio) and stored at -80ºC until use. All genetic engineering experiments were performed under the protocol (No. 24027) approved by Gene Recombination Experiment Safety Management Committee in Kagoshima University. All animal studies were performed in accordance with Standard for Proper Conduct of Animal Experiments at Ark Resource Co., Ltd. (Kumamoto, Japan), under the approval (Protocol number: AW-130012) of the company's Institutional Animal Care and Use Committee.

Table 1. Immunization of Alpaca and sample collection.

(a) Condition for immunization

Animal	**Alpaca (Male)**
Antigen	Trastuzumab-Fab (for 1st -3rd times' immunization) Ranibizumab (for 4th and 6th times' immunization) Human CL domain (for 5th times' immunization)
Adjuvant	FCA for the 1st and FIA for the 2nd to sixth immunization

(b) Schedule for immunization

Date	Operation
Day 0 (22-Apr)	1st immunization (Antigen: 2.8 mg),50 mL Blood sampling (1)
Day 8 (30-Apr)	50 mL Blood sampling (2)
Day 15 (7-May)	2nd immunization (Antigen: 2.8 mg), 50 mL Blood sampling (3)
Day 21 (13-May)	50 mL Blood sampling (4)
Day 30 (20-May)	3rd immunization (Antigen : 2.8 mg), 50 mL Blood sampling (5)
Day 37 (27-May	50 mL Blood sampling (6)
Day 43 (3-Jun)	4th immunization (Antigen : 1 mg), 50 mL Blood sampling (7)
Day 50 (10-Jun)	50 mL Blood sampling (8)
Day 57 (17-Jun)	5th immunization (Antigen : 1 mg), 50 mL Blood sampling (9)
Day 64 (24-Jun)	50 mL Blood sampling (10)
Day 71 (1-Jul)	6th immunization (Antigen : 1 mg), Blood sampling (11)
Day 78 (8-Jul)	50 mL Blood sampling (12)
Day 86 (16-Jul)	50 mL Blood sampling (13)
Day 92 (22-Jul)	50 mL Blood sampling (14)
Day 99 (29-Jul)	50 mL Blood sampling (15)

2.2.2 Design of VHH amplification primers.

Primers that amplify the VHH gene were designed as follows.

The first PCR primers:

5´- AGKTGCAGCTCGTGGAGTCNGGNGG-3´ (Forward primer for both IgG2 and IgG3)

5´- GGGGTCTTCGCTGTGGTG- 3´ (Reverse primer for IgG3)

5´- TTGTGGTTTTGGTGTCTTGGG-3´ (Reverse primer for IgG2)

The second PCR primers including Restriction sites (*Sfi I* and *Spe I*):

5´-TGCTCCTCGCGGCCCAGCCGGCCATGGCTCAGGTGCAGCTCGTGGAGTCTGG-3´

(Forward primer for both IgG2 and IgG3)

5´-ATGATGATGTGCACTAGTTTGTGGTTTTGGTGTCTTGGG-3´ (Reverse primer for IgG3)

5´-ATGATGATGTGCACTAGTGGGGTCTTCGCTGTGGTGCG-3´ (Reverse primer for IgG2)

2.2.3 Construction of cDNA from Alpaca lymphocytes

Blood was collected from immunized Alpaca with increased antibody titer in blood by immunization with antigen protein (Ranibizumab, Herceptin Fab, CL domain), a specific gravity solution of lymphocytes was added, PBMCs were separated, RNAiso Plus (TAKARA BIO) was added, and total RNA was recovered. Using the recovered mRNA as a template, cDNA was synthesized by reverse transcription using SuperScript ™ III (Invitrogen) and Oligo (dT) 20.

2.2.4 Preparation of VHH gene libraries from cDNA

VHH gene amplification was carried out using reverse transcribed cDNA as a template using VHH specific same forward primer for both hinge and reverse primer individual for each hinge (described in primer design section). Restriction enzyme sites were added by amplifying the PCR with ***Sfi*** I restriction enzyme site added primers for both hinge and ***Spe*** I restriction enzyme site

added primers individually for each hinge (described in primer design section). Since VHH antibody contains hinge short IgG2 and hinge long IgG3, amplification was separately performed using primers specific for each hinge region (**Fig.5**).

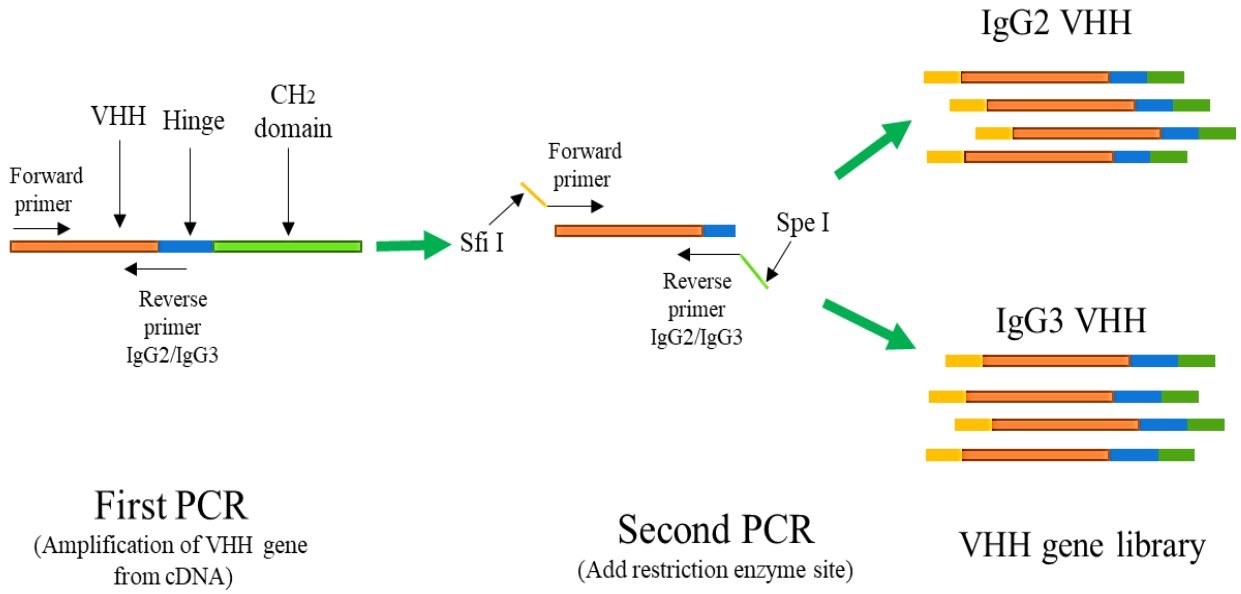

Figure 5. **Synthesis of VHH gene by PCR**. Left panel PCR (First PCR) reactions were short hinge and long hinge specific primers. Middle panel PCR (second PCR) reactions were done by restriction enzyme digestion site containing primers and, right panel were complete length of VHH amplicon (VHH gene libraries).

2.2.5 Construction of phage display library

Total RNA was extracted from a homogenate of alpaca PBMC using RNAiso Plus (Takara Bio) according to the manufacturer's protocol, cDNA was synthesized by reverse transcriptase using Oligo $(DT)_{20}$ primer from 5 µg total RNA by the SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen). VHH gene amplification was carried out using cDNA as a template by PCR. The used primers were the upstream common VHH specific primer: 5´ AGKTGCAGCTCGTGGAGTCNGGNGG-3´) and the down-stream primers: 5´-

GGGGTCTTCGCTGTGGTGCG-3′ for IgG2 and 5′-TTGTGGTTTTGGTGTCTTGGG-3′ for IgG3, respectively. Restriction enzyme sites were added by 2nd PCR with 5′-TGCTCCTCGCGGCCCAGCCGGCCATGGCTCAGGTGCAGCTCGTGGAGTCTGG-3′ (Sfi I restriction enzyme colored in red) as a forward primer and 5′-ATGATGATGTGCACTAGTTTGTGGTTTTGGTGTCTTGGG-3′ (IgG3), 5′-ATGATGATGTGCACTAGTGGGGTCTTCGCTGTGGTGCG-3′ (IgG2) (*Spe* I restriction enzyme colored in red) used as reverse primer as shown in (**Fig.5**). The first PCR was performed in a 50 µL reaction mixture with KOD-Plus-Neo DNA polymerase (Toyobo Co.). The reaction steps were an initial denaturation (94ºC for 2 min) and then 22 repetitions of the three-step cycle: denaturation (98ºC for 10 s), annealing (58ºC for 30 s) and extension (68ºC for 8 s). The second PCR was done with Gene Taq DNA polymerase (Nippon Gene Co., Ltd) using mentioned primers with a 50 µL reaction mixture. The reaction steps were an initial denaturation (95ºC for 2 min) and then 25 repetitions of the three-step cycle: denaturation (98ºC for 30 s), annealing (58ºC for 30 s) and extension (72ºC for 1 min) and final extension (72ºC for 5 mins).

The PCR products purity were checked by agarose gel electrophoresis and purified by column purification. The DNA was digested with *Sfi I* and *Spe I* (New England Biolabs), and ligated to the linearized pKSTV-03 phagemid vector (**Fig.6**). The ligate was treated with phenol/chloroform reagent and precipitated with 70% ethanol. The vector DNA dissolved in water was used for electro-transformation into *E. coli* TG-1 cells (Lucigen Co.). The library size were determined 2.7×10^7 (IgG2) and 5.7×10^7 (IgG3) by estimating the number of transformants on 2TYAG plates [1.5% agar plates containing 2TY medium (16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 L), 2% glucose, and 100 µg/ml ampicillin]. Phage rescued from the transformed *TG-1* cells for the construction of phage library and phage cloning was done.

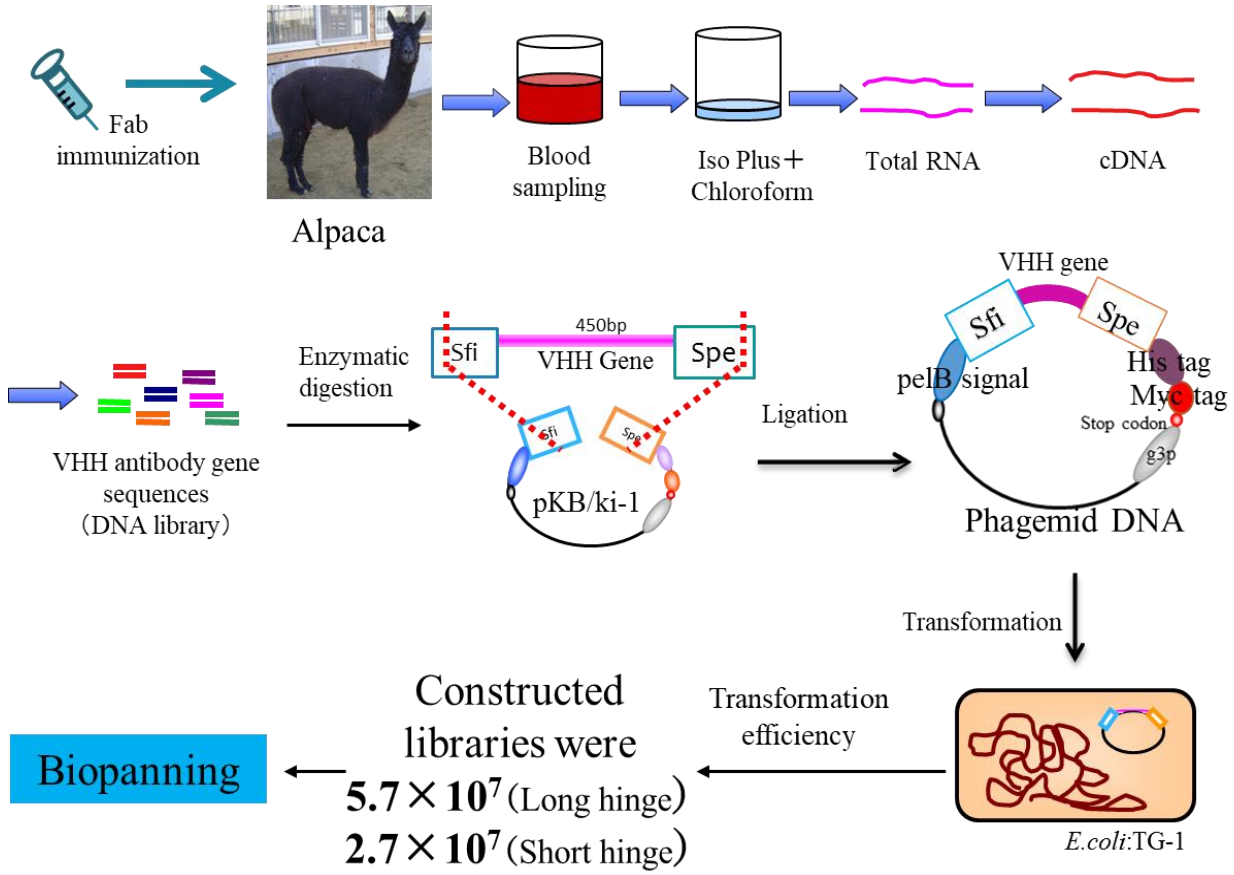

Figure 6. **Schematic representation of M13 phage display library construction**. First immunization of Alpaca by Fabs and collect blood sample to isolate mRNA. VHH gene library is prepared by PCR amplification and construct phagemid vector and transformed to

2.2.5.1 Phage propagation

Phage amplification was carried out in the 500 mL 2TY (Tryptone-Yeast extract) medium containing 100 μg / mL ampicillin, 2% glucose was inoculated with 500 μL of a thawed glycerol stock of *TG1* cells containing the phage library and grown to an OD_{600} of 0.5 . Superinfected by M13KO7 helper phage (Invitrogen) at a multiplicity of infection (MOI) was 20 and cultured at 37 ° C for 30 minutes without shaking followed to 30 minutes at 37 ° C with shaking. After helper phage infection, the bacterial cells were centrifuged at 1500g for 10 minutes, the supernatant

containing excessive uninfected helper phage was discarded, and the *E. coli* pellets infected with the helper phage was inoculated into fresh 2TYAK medium (Tryptone-Yeast medium contained 100 μg / mL ampicillin, 100 μg / mL kanamycin), and cultured at 37 ° C for 16 hours. The culture solution was centrifuged at 8000 rpm for 20 minutes and the phage solution suspended in the supernatant was recovered by 0.2 vol PEG / NaCl which was added for phage precipitation reaction. Finally, centrifuged the supernatant at 10000 rpm for 60 minutes and discarded supernatant. The phage pellets were resuspended by 1 mL of phosphate-buffered saline (PBS), filter sterilized and stored at 4ºC until use.

2.2.5.2 Isolation of Fab specific VHH antibody

To isolate the Fab-specific VHH clones with higher binding affinity, we performed two strategies of biopanning procedure to enrich the pool phage libraries. The applied strategies were-

(A) Biopanning strategy I

In order to obtain phage binding to soluble Fab, phage library was reacted with biotinylated Ranibizumab, Herceptin Fab (**Fig.7**) in solution (blocking agent). Prepared by diluting streptavidin (SA) (500 ng / 200 μL) with PBS in a 96-well microtiter plate (Nunc Thermo Fisher Scientific) and immobilized at room temperature for 2 hours. Blocking agent (0.5% BSA or 5% skim milk) was added and blocking was carried out at room temperature for 2 hours. After washing three times with 0.1% PBST, a mixture of the biotinylated Fab reacted in the solution and the phage library was added and reacted at room temperature for 1 hour to bind the phage by the reaction of SA-biotin. Washing (5 to 20 times) was performed with 0.1% PBST in order to remove nonspecifically bound phage. 0.1 M glycine-HCl (pH 2.2) was added to elute the antigen-specific phage, neutralized with 1 M Tris-HCl (pH 9.1), then infected with Escherichia coli *TG-1* and plated

at 30 ° C for 12 hours. The cultured *TG-1* was heavily infected with M13KO7 helper phage, and the phage displaying antigen-specific VHH recovered by panning was amplified.

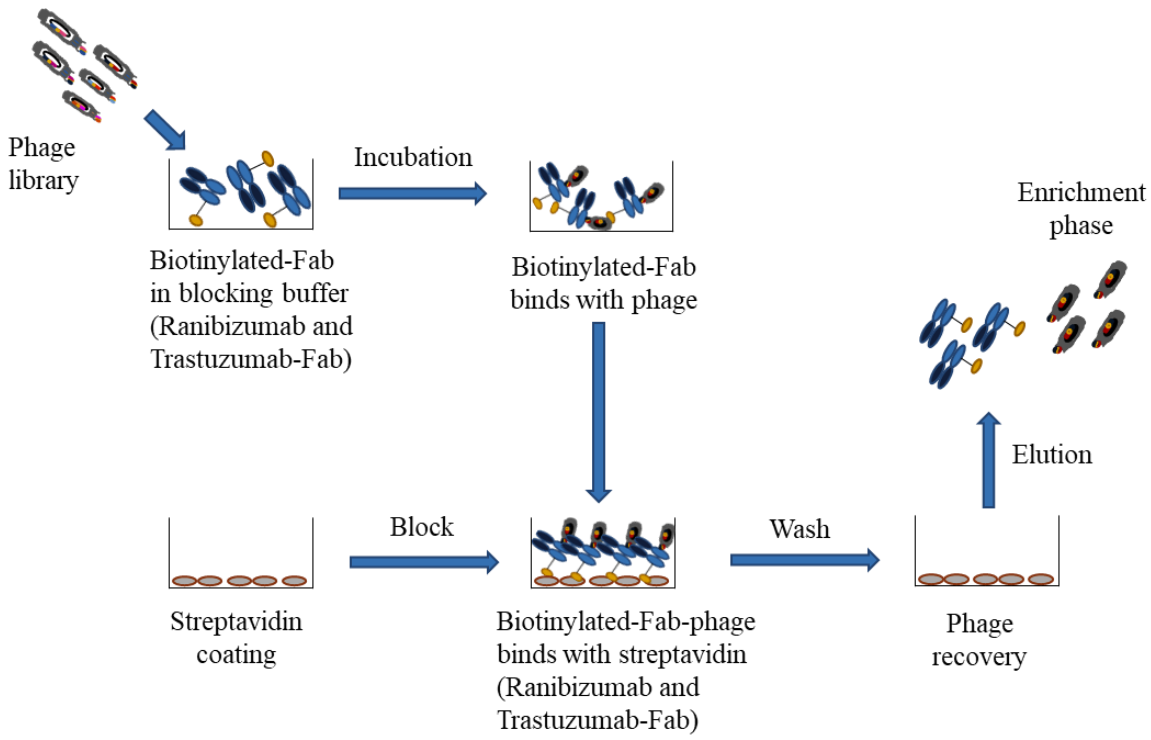

Figure 7. **Schematically representation of biopanning strategy I**. Biotinylated Ranibizumab/ or Trastuzumab-Fab complexed with phages was trapped by streptavidin coated on the well.

(B) Biopanning strategy II

Herceptin was reacted with a phage library in solution (blocking agent) to obtain phage binding to soluble Fab. Her 2-Fc was diluted in PBS with (500 ng / 200 μL) in a 96-well microtiter plate (Nunc Thermo Fisher Scientific) and immobilized at room temperature for 2 hours (**Fig.8**). Blocking agent (0.5% BSA or 5% skim milk) was added and blocking was carried out at room temperature for 2 hours. After washing three times with 0.1% PBST, a mixture of Herceptin

reacted with the solution and phage library was added and allowed to react at room temperature for 1 hour.

Washing (5 to 20 times) was performed with 0.1% PBST in order to remove nonspecifically bound phage. 0.1 M glycine-HCl (pH 2.2) was added to elute the antigen-specific phage, neutralized with 1 M Tris-HCl (pH 9.1), then infected with Escherichia coli TG-1 and plated at 30 ° C. The cultured *TG-1* was heavily infected with M13KO7 helper phage, and the phage displaying antigen-specific VHH recovered by panning was amplified.

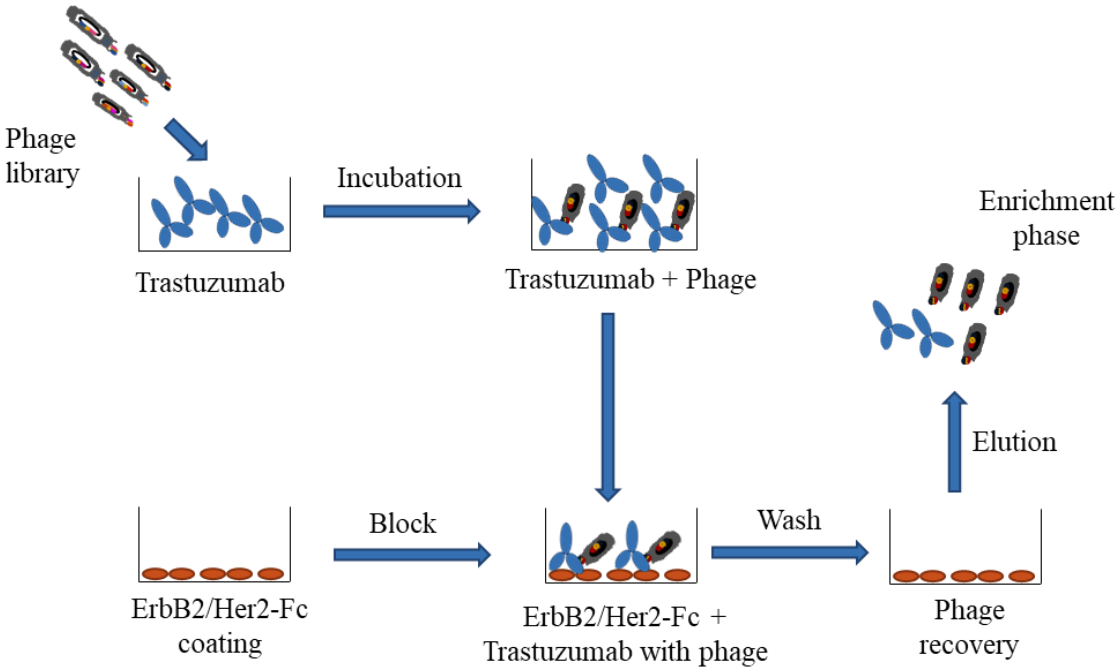

Figure 8. **Schematically representation of biopanning strategy II**. Trastuzumab complexed with phages by incubation was trapped with ErbB2/Her2-Fc coated on the well.

2.2.5.3 Phage ELISA to determine enrichment phage library

Antigen (50 ng / 50 µL) diluted with PBS was immobilized on a 96-well microtiter plate (Nunc Thermo Fisher Scientific) for 2 hours at room temperature and blocking was carried out for 2 hours at room temperature with a blocking agent (0.5% BSA). After washing three times with PBST (T= 0.1% Tween-20), the phage solution (1.0×10^9/50 µL) library phage was added and the reaction was allowed to proceed at room temperature for 1 hour. After washing 5 times with 0.1% PBST, a secondary antibody (biotinylated Mouse anti-M13 antibody (Abcam)) pre-incubated with SA-HRP (VECTOR LABORATORIES) was allowed to react at room temperature for 1 hour. After washing 5 times with 0.1% PBST, a TMB solution (CALBIOCHEM) containing a peroxidase luminescent substrate was added and the reaction was stopped by 1 N HCl by color reaction, and the VHH bound to the antigen protein on the well antibody phage were detected. Detection was carried out by measuring the absorbance at 450 nm using an ELISA plate reader MODEL 680 XR (BIO RAD).

2.2.5.4 Screening of monoclonal phage by ELISA

Individual clones were isolated from panned mutant libraries by monoclonal phage enzyme-linked immunosorbent assay (ELISA). Individual binder clones were selected from FALCON® 96-well tissue culture plates following each round of selection and grown overnight in wells of a 96-well culture plate in 100 µl 2TY containing 100 µg/ml ampicillin and 1% w/v glucose. A small inoculum, about 2 µl, of this overnight culture was used to inoculate identical wells on a second plate containing 200 µl of growing media (2TYAG). After growth at 37°C for 1-1.5 hours with shaking at 200 rpm, 50 µl of 2TY containing 100 µg/ml ampicillin, 1% w/v glucose and 10^9 pfu of M13K07 helper phage was added to each well and incubated without shaking at 37°C for 30 minutes, then grown with shaking for 30 minutes. Next, the plate was centrifuged at 4000 rpm for

10 min, the supernatant was removed and replaced by 250 µl of 2TY containing 100 µg/ml ampicillin and 100 µg/ml kanamycin in each well and incubated overnight at 37°C with shaking at 200 rpm.The following day, the plate was again centrifuged at 4000 rpm for 10 mins and the phage-containing supernatant was used for monoclonal ELISA.

Briefly, wells of a 96-well Nunc MaxiSorp plate were coated with Ranibizumab, Trastuzumab-Fab, IgG-Fc, CL domain and BSA at a concentration of 50 ng/50 µL/well, incubated overnight at 4˚C. Washed three times with PBS containing 0.1% v/v Tween-20. After blocking and washing the wells, 50 µL of monoclonal phage containing supernatant from above was added to each well and incubated for 1 hour at room temperature with shaking. The phage solution was then removed, and the wells were washed three times with PBST to remove unbound phages. Added 50 µL/well of the secondary antibody, horseradish peroxidase (HRP)-streptavidin (Vector Laboratories, Inc. Burlingame, CA) -conjugated with Anti-M13 antibody -Biotin (abcam), was diluted (1: 4000) in 0.5% BSA, and incubated for 60 min with shaking at room temperature. Following incubation, all wells were washed five times with PBST. The Colour was developed by adding 40 µl of TMB substrate (3, 3′, 5, 5′-tetramethylbenzidine) and incubating for 30 mins at room temperature. Color development was stopped with 40 µl of 1 N HCl and the absorbance of the solution in each well was measured. The OD was measured in absorbance at 450 nm. Plasmid DNA was isolated from positive mutants identified by ELISA and sequenced by sequencing primer. Sequences were analyzed using BioEdit Sequence Alignment Software.

2.2.6 Expression of VHH antibody in *E. coli*

Escherichia coli HB2151 cells were infected with cloned phages obtained from Fab-specific immunized library and grown on SOBAG-N plates [1.5% agar plates containing SOB medium (2

g tryptone, 0.5 g yeast extract, 0.01 g NaCl, 0.2 g $MgCl_2$.6H2O), with 2% glucose, 100 mg/ml ampicillin and 100 mg/ml nalidixic acid] overnight at 30ºC. Colonies were picked and transferred to 2TYA (A= ampicillin) medium and incubated at 30ºC overnight. Each culture was diluted 100-fold with 2TYAG and grown to an absorbance of 0.5 at 600 nm. The cultures were then centrifuged and the pellets were suspended in 2TY medium containing 100 mg/ml ampicillin and 1mM isopropyl-b-D-thiogalactopyranoside (IPTG) and incubated at 37ºC for 16 hours with shaking at 200 rpm. The pellets were then collected by centrifugation and treated with 10mM EDTA and 20% sucrose in ice cold water to recover the periplasmic fraction. After dialysis against phosphate buffered saline, the samples were applied to 1 mL HisTrap excel columns (GE Healthcare) to purify VHH antibodies using the added C-terminal His6 sequence.

2.2.7 Purity of VHH antibody by SDS-PAGE

SDS–PAGE was carried out according to Laemmli's method. Proteins were electrophoresed by SuperSep^{TM}Ace 5-20% sodium dodecyl sulfate poly acryl amide gel (Wako) and stained with Coomassic Brilliant Blue R-250 (Bio-Rad laboratories, USA), destained with Coomassic Brilliant Blue R-250 (Bio-Rad laboratories, USA) destaining solution. The protein amount of stained band on the SDS–PAGE was estimated using image of Precision Plus Protein ^{TM} Standards All Blue as a standard. The proteins band was detected by imaging (BIO-RAD, Molecular Imager $^{(R)}$ Chemi Doc ^{TM} XRS+ with Image Lab ^{TM} Software.

2.2.8 Specificity of VHH antibody by ELISA

The wells of a 96 well Microplate (Nunc Maxisorp) were coated with Ranibizumab, Trastuzumab-Fab, Her2-Fc, CL domain, NDOM, Lysozyme, skim milk and BSA (50 ng/50 µL/well) overnight

at 4ºC. After three times washing with 0.1% PBST, blocked for 2 hours at RT with 0.5% BSA in PBS and washed out unbound materials. VHHs (100 ng/well in 0.5% BSA) were added to each well and incubated for 1 hour at RT. After washing the plate, added 50 µL/well pre incubated conjugation of *Anti-His-tag* mAb-*biotin (MBL MEDICAL & BIOLOGICAL LABORATORIES CO., LTD)* and SA-HRP (Vector Laboratories, Inc. Burlingame, CA). After 1hour incubation, five times washed the plates and binding was detected with 40 µL/well TMB (tetramethylbenzidine) reagent. Subsequently, 1N HCl (40 µL/well) was added to stop additional reaction. Finally, the binding was measured by the absorbance at 450 nm in a microplate reader (680XR, Bio-Rad, USA).

2.2.9 Affinity analysis by Biacore T200

K_Ds were determined at 25°C using Biacore T200 (GE Healthcare). To make antigen the surface, 10µM of antigen in 40µl of HBS buffer, pH 4.5, were immobilized on a research grade on CM-5 sensor chips (GE Healthcare) by the amine coupling protocol supplied by the manufacturer. The different concentrations from 100nM to 6.25nM) of solutions in HBS buffer were injected for monitoring association reaction and then eluted with HBS buffer for dissociation reaction. The sensor chips were regenerated by 10 mM glycine-HCl buffer (pH 2.0) containing 0.5 M NaCl. The sensorgrams were analyzed using a 1:1 binding model on BIA evaluation software to determine equilibrium dissociation constants (K_D).

2.2 Results and discussion

We immunized Alpaca by Fab antibody fragments and prepared cDNA from Alpaca lymphocytes. The VHH gene library were constructed through PCR reactions and constructed Fab-specific immuno phage libraries where the library sizes were 2.7×10^7 (IgG2) and 5.7×10^7 (IgG3). First,

we enriched the library phages by conventional biopanning from the plastic plate physically coated with Fabs. However, the VHH clones obtained by that method did not show binding ability toward Fabs solubilized or immobilized on the chip of BIAcore through amine-coupling (data not shown).

Therefore, the panning method was changed into two different strategies, I and II (See details in Materials and Methods). In strategy I, the phage library was mixed with biotinylated-Fab (Ranibizumab or Trastuzumab-Fab) and added to streptavidin coated ELISA plate. In strategy II, the phage library was reacted with Trastuzumab and then the complex was trapped by HER2-Fc coated on ELISA plate (**Fig. 7 & 8**). In both cases, the enrichment of phage libraries were observed in phage ELISA. After getting enriched phage libraries, randomly cloned about 200 clones were tested in the binding to Fab by ELISA showing the specific binding of nine clones (**Fig. 9, right panel**). Nine clones were sequenced their sequences were aligned as shown in **Fig. 10**. Those clones were divided into three different groups (A, B and C) by the sequence differences in their CDR3 region.

Group A, B and C VHHs were expressed in *E.coli* (HB2151) cells infected with cloned phages and purified on HisTrap excel column (**Fig.11A and Table 2**). All groups of VHH showed a clear binding specificity to Ranibizumab and Trastuzumab-Fab by protein ELISA (**Fig. 11B**). Purified VHHs were subjected to Biacore T200 for kinetic analysis. The 6.25-100 nM different concentrations of VHHs solutions were injected to monitor association reaction The SPR analysis indicated that group B and C were strong binders with K_D less than 35 nM but group A is a moderate one with K_D 200 nM as shown in **Fig. 12 and Table 3**.

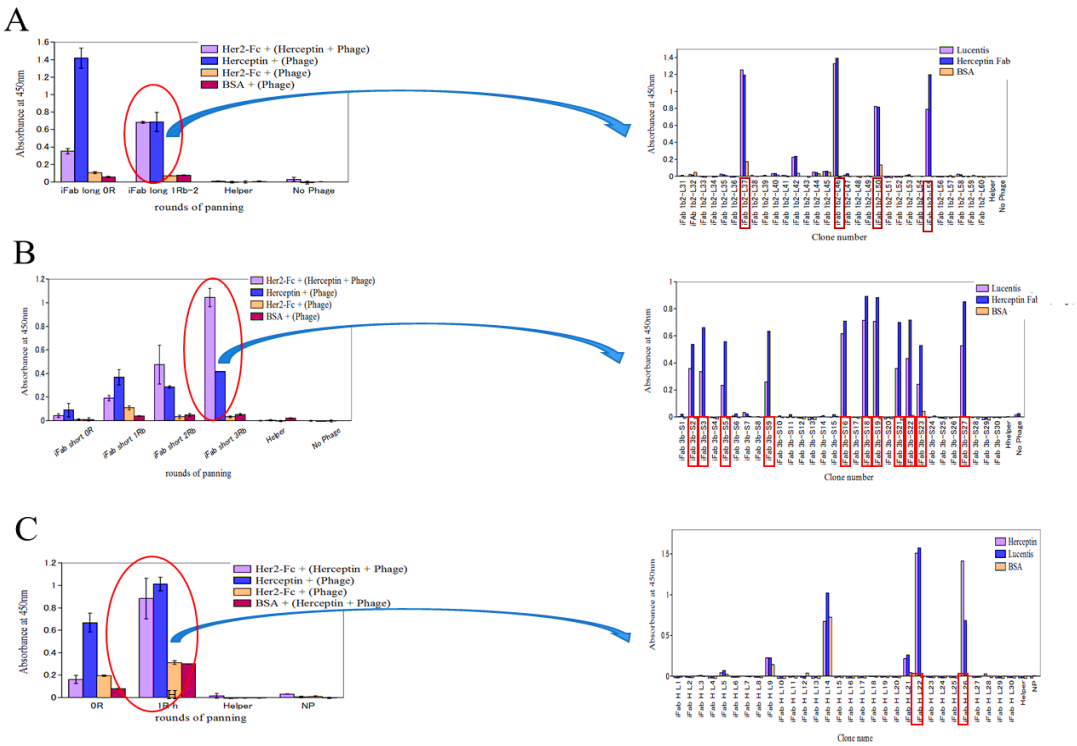

Figure 9. **Phage ELISA to screen highly enrichment pool phage library and binging ability of individual phage**. (A) Left panel polyclonal phage ELISA for 1st-round of short hinge library followed to biopanning Strategy I and right panel, monoclonal phage ELISA for the binding of individual phage. (B) Left panel polyclonal phage ELISA for 3rd –round of short hinge library followed to biopanning Strategy I and right panel, monoclonal phage ELISA for the binding of individual phage. (C) Left panel polyclonal phage ELISA for 1st –round of long hinge library followed to biopanning Strategy II and right panel, monoclonal phage ELISA for the binding of individual phage.

Table 2. Expression of group A, B and C VHH antibodies in *E. coli* (HB2151)

Group name of VHH (Clone name)	Yield [mg/L culture volume]
A (iFab-HL-22)	1.50
B (iFab-3b-s16)	6.5
C (iFab-3b-s3)	2.28

Table 3. Summary of binding affinity parameters of typical VHH clones from groups A, B, and C.

Fab	Binding parameters	Group A VHH	Group B VHH	Group C VHH
Ranibizumab	K_d (nM)	200	24	25
	k_a (/Ms)	1.1×10^5	1.1×10^5	1.5×10^5
	k_d (/s)	0.023	0.003	0.004
Trastuzumab-Fab	K_d (nM)	200	33	35
	k_a (/Ms)	1.0×10^5	7.5×10^4	1.2×10^5
	k_d (/s)	0.021	0.003	0.004

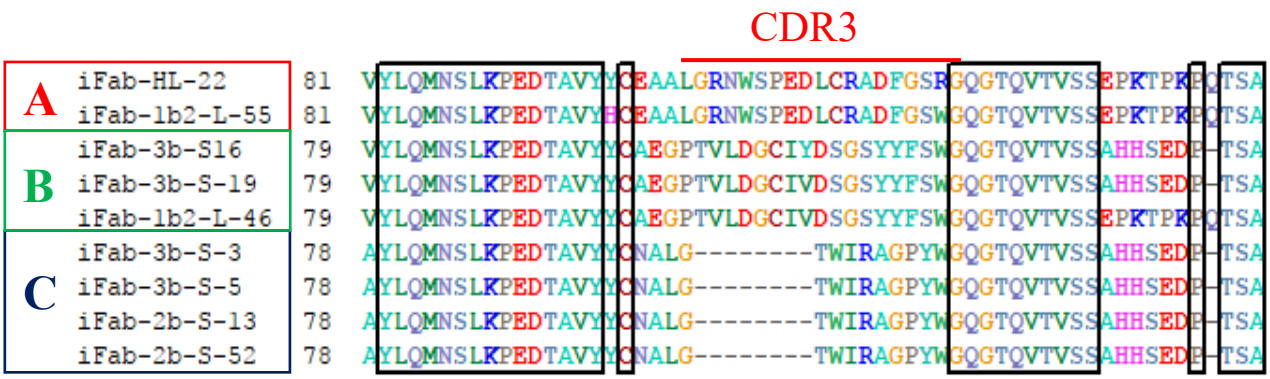

Figure 10. **Amino acid sequences of Fab-specific immunized library isolated VHH clones**. After aligned 9 VHH amino acid sequences, three groups of sequences were generated depending on the difference of CDR3.

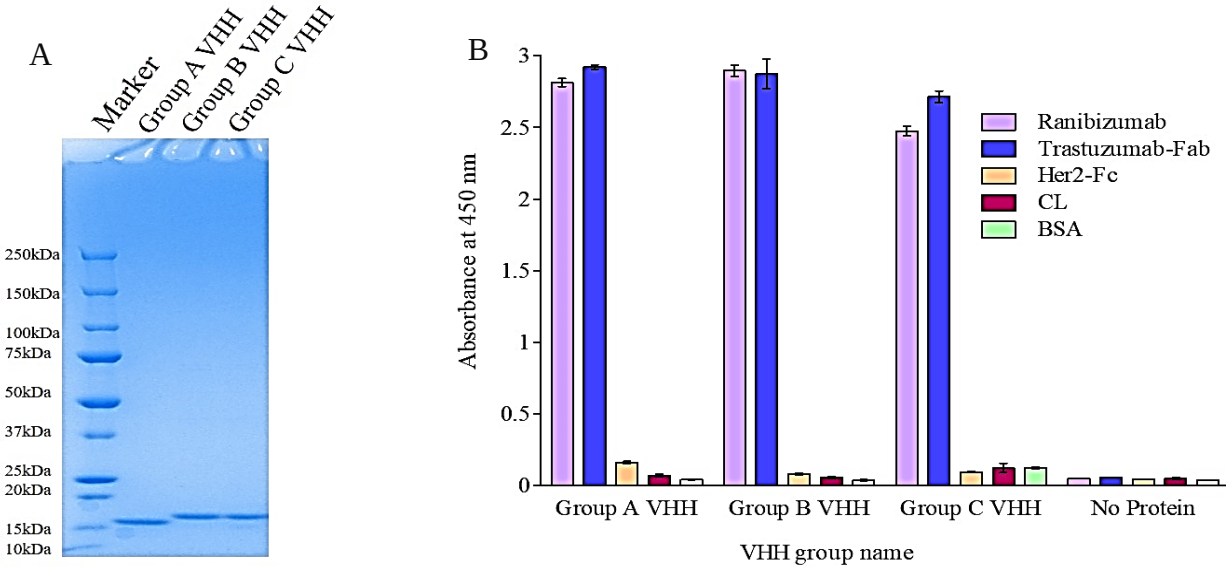

Figure 11. Binding properties of group (A, B, and C) VHH antibodies. (A) SDS-PAGE of purified VHH clones from group A, B and C respectively. (B) Binding specificity of three groups of VHH to Ranibizumab and Trastuzumab-Fab by ELISA.

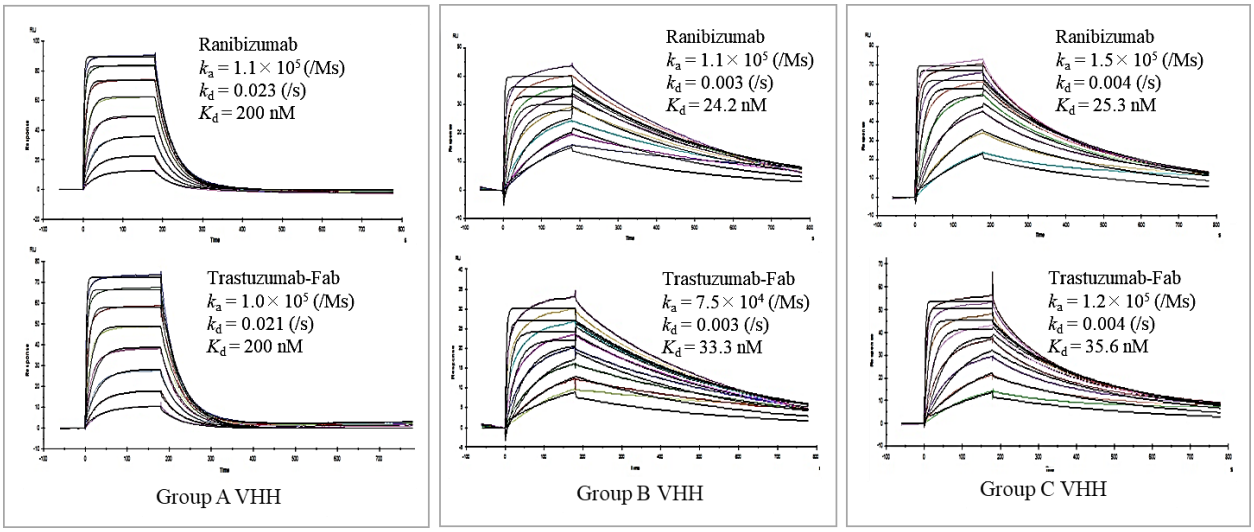

Figure 12. Kinetic binding analysis of typical clones of group A, B and C VHHs to Ranibizumab and Trastuzumab-Fab on BIAcore-T200.

2.4 Summary

In this study, we successfully constructed Fab-specific phage libraries where the library size were 2.7×10^7 (IgG2) and 5.7×10^7 (IgG3). In our first attempt, we failed to isolate functional VHH clones in a conventional biopanning technique where physically plastic plate coated with Fabs. However, the VHH clones obtained by that method did not show binding ability to Fabs immobilized on the chip Biacore through amine coupling. It was considered that probably because the structure of Fab molecule was changed to be unnatural one through the process of its absorption into the surface of the plate. VHHs recognized the neoepitopes newly generated on unnatural VHH structure were enriched preferentially.

Therefore we changed our strategy into I and II for both to enrich the phage libraries (Methods and materials) and, after screening the enriched monoclonal phage (**Fig.9**), we isolated nine functional VHH clones in three different groups (A, B and C) depending on their difference in CDR3 region (**Fig. 10**). All group of VHHs showed a clear binding specificity against Ranibizumab and Trastuzumab-Fab with high purity (**Fig. 11B**), also the affinity of group B and C VHHs about 25 nM for Ranibizumab and 35 nM for Trastuzumab-Fab but group A was 200 nM (**Fig.12** and **Table 3**).

For the commercial development of human Fab antibody purification system using VHHs affinity ligand, we could not be used group A and B VHH as an affinity ligand due to lack of alkaline resistance according to studies those suggested to avoid disulfide bonds or free cysteine to use of protein as a ligand for affinity purification system as a column need to regenerate with alkali pH solution.

We focused on the group C VHH due to without cysteine in CDR3 position and considered it to be an alkaline resistant VHH antibody. Therefore, we evaluated affinity purification with

group C VHH-conjugated column but could not be used as an affinity ligand because the difficulties of acidic elution.

2.5 References

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Chapter 3

Next generation sequencing to expand the diversity of cysteine free VHHs in CDR3

3.1 Background

The most promising therapeutics in the recombinant protein market is the monoclonal antibody, more than 70 therapeutic antibodies are available in US and European market those have been approved for various indications, including cancer and autoimmune diseases [**1**]. In the past few decades, technologies such as transgenic mice encompassing human antibody gene repertoires, and phage display of antibody libraries, have become available, facilitating the rapid flourishing of therapeutic antibodies in the drug discovery field [**2**]

The phage display technology is a powerful technique to isolate and identify the functional binders. Using this technique, many antigen-specific antibody fragments, including Fab, scFv and VHH have been isolated through a biopanning selection process [**3, 4, 5, 6**]. Phage display technology frequently allows the construction of libraries containing up to 10^{11} different variants, which can be used to screen antibody clones by bio-panning [**7**]. Despite the development of alternative display technologies such as bacterial display, yeast display and ribosome display, phage display remains the most widely used display technology due to the robustness of the filamentous bacteriophage M13 [**8**]. Several therapeutic antibodies that are currently either approved or in clinical trials have been developed by phage display technology [**9, 10**].

Though, it has many advantages to identify the targets, but one challenge associated with phage display technology is the identification of potential candidates after the panning process. Traditionally, this step is achieved by randomly picking phage clones for target-binding screening

and Sanger sequencing analysis. This method may not necessarily lead to the isolation of highly potent variants because it is time-consuming and often relies on the development of robust screening assays.

Recently, next generation sequencing (NGS) technologies have been adapted for many aspects of biological research and clinical application [**11, 12**]. Next generation sequencing technology has facilated to discovery of more diverse positive clones from a phage display antibody library at low cost and in a short time frame [**13**].

Several NGS platforms are currently available, with average read lengths of 75–8500 bp and different error rates [**14**]. The CDR3 sequence of the VH and VL genes has been effectively determined by the MiSeq system; [**15**] a single domain antibody gene was successfully determined by the MiSeq system using a 2 × 250 paired-end module; [**16**] and the entire VH gene was successfully sequenced using the 454 pyrosequencing system.[**17**].

Following NGS analysis, the antibody gene is typically cloned and expressed. And the binding reactivity of the antibody to its target as well as its biological activity are tested. However, this may prove to be unproductive when the fraction of positive clones is not high following bio-panning. It has been extensively reported that positive clones tend to be enriched through bio-panning and negative clones show the opposite tendency. Therefore, NGS analysis of clones after each round of bio-panning could provide insights on which clones are more likely to be positive.

In this study, we applied next generation sequencing (NGS) to expand the diversity of Cys-free VHHs in CDR3 using the pooled VHH genes before and after biopanning for both long and short hinge with two different panning strategies I and II.

3.2 Methods and materials

3.2.1 DNA library preparation

Next generation sequence analysis was done by Miseq (Illumina). A total of 5 sets of phagemid DNA including 2nd round of IgG3 and 1st round of IgG2 obtained from biopanning strategy I, 1st round of IgG3 obtained from biopanning strategy II, also 0 round of long and short hinge both were analyzed. DNA sequences in the phage library were analyzed using a MiSeq system (Illumina, Inc., San Diego, CA, USA). The MiSeq library for DNA sequencing was prepared using QIAseq 1-step Amplicon Library kit (QIAGEN) following the protocol provided by the manufacturer. The DNA sequencing library was constructed to use 10 ng DNA samples of the Alpaca VHH phage library using primers for both IgG2 and IgG3 forward primer 5´-CAGGTGCAGCTCGTGGAGTCTGG-3´ and reverse primer 5'-TTGTGGTTTTGGTGTCTTGGGTTC-3´ and 5´-GGGGTCTTCGCTGTGGTGCG-3´ respectively. The final loading concentration was adjusted to 15 pM following the MiSeq loading sequencing reactions. Index sequence and adapter sequences P5 and P7 were added to both ends of the VHH gene protocol as shown in **Fig.13**. The MiSeq reagent kit v3 (Illumina) was used for long paired end (2 ×300 bp).

3.2.2 Cluster generation for NGS sequencing

For cluster generation, the library is loaded into a flow cell where fragments are captured on a lawn of surface-bound oligos complementary to the library adapters. Each fragment is then amplified into distinct, clonal clusters through bridge amplification. When cluster generation is complete, the templates are ready for sequencing (**Fig.14**).

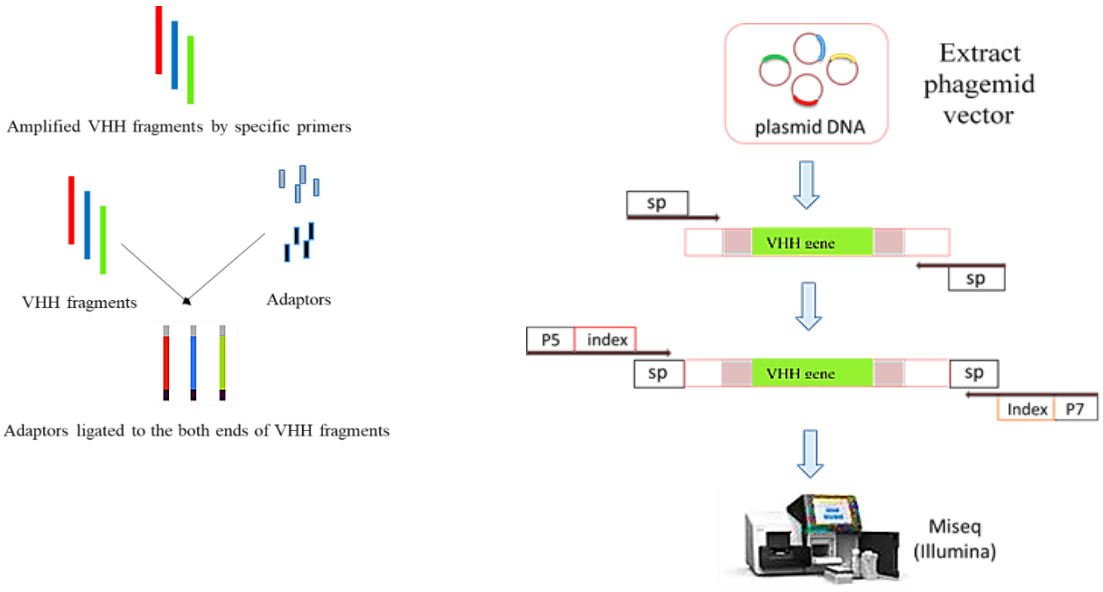

Figure 13. **Schematic representation of sample preparation for NGS**. VHHs amplify using specific primes followed by index and adapter sequences were added with VHH genes using PCR reaction.

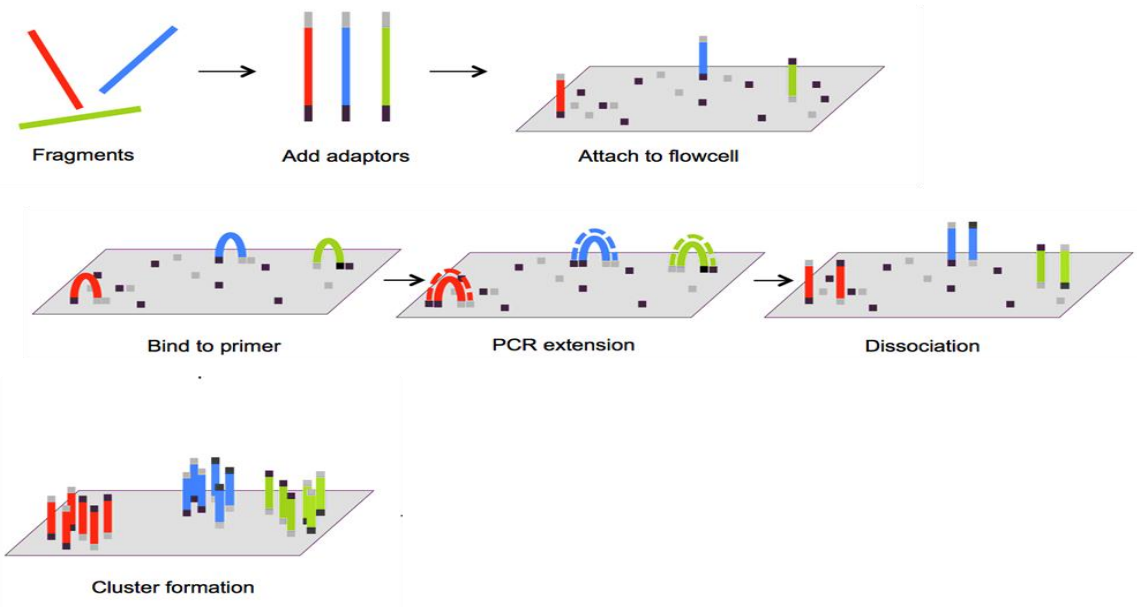

Figure 14. **Cluster formation of VHH genes**. DNA fragments were ligated with index and adaptor then captured on flow cell containing oligos complementary with adaptors.

3.2.3 Sequencing by Synthesis

Illumina SBS technology uses a proprietary reversible terminator-based method that detects single bases as they are incorporated into DNA template strands. As all four reversible terminator-bound dNTPs are present during each sequencing cycle, natural competition minimizes incorporation bias and greatly reduces raw error rates compared to other technologies. The result is highly accurate base-by-base sequencing that virtually eliminates sequence context-specific errors, even within repetitive sequence regions and photopolymers.

3.2.4 Data Collection, Processing, and Analysis

Illumina data collection software enables users to align sequences to a reference in resequencing applications. Developed in collaboration with leading researchers, this software suite includes the full range of data collection, processing, and analysis modules to streamline collection and analysis of data with minimal user intervention. The open format of the software allows easy access to data at various stages of processing and analysis using simple application program interfaces.

Run quality was monitored following the standard Illumina procedure described by service provider. Estimation of the error rate was performed using a control DNA that was sequenced in parallel to the samples. The sequencing reads were assigned to a raw data pool based on a unique 8-bp barcode identifier and generated as a FASTQ file. The raw paired-end nucleotide sequences were merged, filtered, aligned and trimmed by applying USEARCH Ver.8.0 (Edgar, R.C) tool to remove low quality and meaningless short sequences. Subsequently, a custom Perl script was used to summarize, and count sequence reads of the Alpaca VHH phage display library.

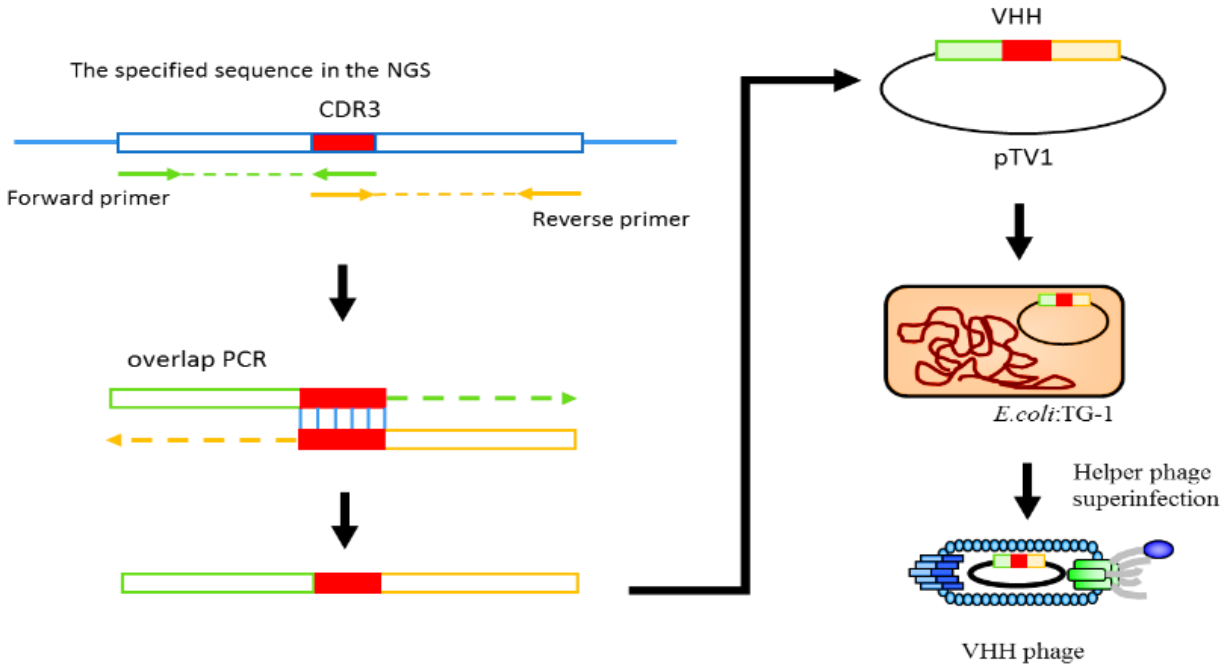

Figure 15. **Reconstruction of NGS identified group Y VHH.** Target VHH genes were amplified by PCR reactions using CDR3 specific primers. Construction of phagemid and transfected to *E.coli*

3.2.5 Reconstruction of NGS identified group Y VHH gene

Regarding the target clones, the VHH gene was reconstructed by making CDR3 specific primers for each clone. By PCR upstream of VHH gene and CDR3 specific primer to amplify upstream side of VHH DNA, downstream of VHH gene and CDR3 specific primer to amplify downstream side of VHH DNA, both fragments were ligated by overlapping PCR and reassembled as VHH phage by incorporation into phagemid vectors. (**Fig.15**)

3.2.6 Phage propagation of group Y VHH clones

Phage amplification was carried out in the 500 mL 2TY (Tryptone-Yeast extract) medium containing 100 μg / mL ampicillin, 2% glucose was inoculated with 500 μL of a thawed glycerol

stock of TG1 cells containing the phage library and grown to an OD_{600} of 0.5. Superinfected by M13KO7 helper phage (Invitrogen) at a multiplicity of infection (MOI) was 20 and cultured at 37 ° C for 30 minutes without shaking followed to 30 minutes at 37 ° C with shaking. After helper phage infection, the bacterial cells were centrifuged at 1500g for 10 minutes, the supernatant containing excessive uninfected helper phage was discarded, and the *E. coli* pellets infected with the helper phage was inoculated into fresh 2 TYAK medium (Tryptone-Yeast medium contained 100 μg / mL ampicillin, 100 μg / mL kanamycin), and cultured at 37 ° C for 16 hours. The culture solution was centrifuged at 8000 rpm for 20 minutes and the phage solution suspended in the supernatant was recovered by 0.2 vol PEG / NaCl which was added for phage precipitation reaction. Finally, centrifuged the supernatant at 10000 rpm for 60 minutes and discarded supernatant. The phage pellets were resuspended by 1 mL of phosphate-buffered saline (PBS), filter sterilized and stored at 4ºC until use.

3.2.7 Phage binding analysis by ELISA

Antigen (50 ng / 50 μL) diluted with PBS was immobilized on a 96-well microtiter plate (Nunc Thermo Fisher Scientific) for 2 hours at room temperature and blocking was carried out for 2 hours at room temperature with a blocking agent (0.5% BSA). After washing three times with 0.1% PBST, the phage solution (1.0×10^9/50 μL) library phage was added and the reaction was allowed to proceed at room temperature for 1 hour. After washing 5 times with PBST (T= 0.1% Tween-20), a secondary antibody (biotinylated Mouse anti-M13 antibody (Abcam)) pre-incubated with SA-HRP (VECTOR LABORATORIES) was allowed to react at room temperature for 1 hour. After washing 5 times with PBST, a TMB solution (CALBIOCHEM) containing a peroxidase luminescent substrate was added and the reaction was stopped by 1 N HCl by color reaction, and

the VHH bound to the antigen protein on the well Antibody phage were detected. Detection was carried out by measuring the absorbance at 450 nm using an ELISA plate reader MODEL 680 XR (BIO RAD).

3.2.8 Expression of LH3 VHH antibody in *E. coli*

Escherichia coli HB2151 cells were infected with cloned phages obtained from Fab-specific immunized library and grown on SOBAG-N plates [1.5% agar plates containing SOB medium (2 g tryptone, 0.5 g yeast extract, 0.01 g NaCl, 0.2 g $MgCl_2$.6H2O), with 2% glucose, 100 mg/ml ampicillin and 100 mg/ml nalidixic acid] overnight at 30ºC. Colonies were picked and transferred to 2TY-A medium and incubated at 30ºC overnight. Each culture was diluted 100-fold with 2TY-AG and grown to an absorbance of 0.5 at 600 nm. The cultures were then centrifuged, and the pellets were suspended in 2TY medium containing 100 mg/ml ampicillin and 1mM isopropyl-b-D-thiogalactopyranoside (IPTG) and incubated at 37ºC for 16 hours with shaking at 200 rpm. The pellets were then collected by centrifugation and treated with 10mM EDTA and 20% sucrose in ice cold water to recover the periplasmic fraction (31). After dialysis against PBS, the samples were applied to HisTrap excel columns (GE Healthcare) to purify VHH antibodies using the added C-terminal His6 sequence.

3.2.9 Affinity analysis by Biacore T200

K_Ds were determined at 25°C using Biacore T200 (GE Healthcare). To make antigen the surface, 10µM of antigen in 40µl of HBS buffer, pH 4.5, were immobilized on a research grade on CM-5 sensor chips (GE Healthcare) by the amine coupling protocol supplied by the manufacturer. The different concentrations of LH3 VHH antibody from 100 nM to 6.25 nM of solutions in HBS

buffer were injected for monitoring association reaction and then eluted with HBS buffer for dissociation reaction. The sensor chips were regenerated by 10 mM glycine-HCl buffer (pH 2.0) containing 0.5 M NaCl. The sensorgrams were analyzed using a 1:1 binding model on BIA evaluation software to determine equilibrium dissociation constants (K_D).

3.3 Results and discussion

To prepare the VHH gene library for next generation sequencing NGS), we extracted phagemid DNA from the enriched phage libraries by the repeated biopanning as shown in **Fig. 9.** VHH genes were amplified by PCR and ligated at both ends with adapters DNA including and index sequences. DNA sequences of VHH genes in the phage library were analyzed using MiSeq system. The summary of NGS data was listed in **Table 3 and 4.** To identify the sequences of VHHs binding to Fab, the frequencies (%) of each read of the VHH genes in the phage libraries were compared before and after biopanning, and the changes of the frequencies of every VHH sequence through biopanning were calculated and assigned as amplification factor (**Table 5**). This change of the frequency, named as amplification factor (folds) can be considered to reflect the efficiency in increase of the number of individual VHH clones enriched through biopanning. Therefore, it can be a good indicator to identify good binders. The top 18 VHHs showing amplification factor more than 20 in each round of biopanning were selected and aligned (**Fig.16**), and the phylogenetic tree was constructed based on the sequences (**Fig. 17**) using neighbour joining method. The tree indicated three highly homologous groups (groups A, B, and C) showing more than 92% identity and sharing the common CDR3 in each group (**Fig.16**). On the other hand, non-homologous sequences can be divided to two groups: one contained Cys-residue in CDR3 (group X) and the other did not (group Y).

As it was found that groups A, B, and C VHHs are not suitable for affinity ligands as described above, we started to find other candidates. We focused on the group Y, excluding group X, because group X also contained Cys residues on CDR3, as did groups A and B. Five genes of VHHs (LH_3, Lb_5, Lb_14, Lb_16, Lb_22) in group Y were reconstructed phagemid DNA by PCR using CDR3-specific primers and transformed into *E.coli* TG1 to prepare the phages. The prepared phages were tested in binding to Ranibizumab and Trastuzumab-Fab by phage ELISA as shown in **Fig. 18A**. As a result, LH3 phage only showed the binding ability to Fabs.

Therefore, the LH3 protein was prepared in *E. coli* (HB2151) cells by infecting the LH3 cloned phage and purified on HisTrap excel column (**Fig. 18B**). We further subjected the purified LH3 VHH for binding analysis on BIAcore T200 with 6.25-100 nM different concentrations in HEPES buffered saline for association reaction, subsequently HEPES buffered saline was loaded for dissociation reaction. The SPR results indicated a clear binding of LH3 VHH to Fabs, but low affinity with K_d 187 nM as shown in **Fig.18C**.

Table 3. **Summary of HTS results for VHH short-hinge library**. The NGS sequence data for VHH short-hinge library were deposited in DDBJ Sequence Read Archive (DRA) under accession number DRA007729 and the NGS sequence data before (S0R) and after (S2Rb) biopanning were assigned as SAMD00155281 and SAMD00155282 in DDBJ BioSample accession number, respectively.

Short-hinge library	Before biopanning (S0R, %)	After 2nd round biopanning of strategy I (S2Rb, %)
Total number of read sequences	1128262	619987
Number of merged sequences	65497 (100%)	30972 (100%)
Single-occurrence sequences [a]	31148 (47.6%)	15562 (50.2%)
Unique sequences	34619 (52.9%)	17258 (55.7%)
Highest frequency	2040 (3.1%)	1937 (6.3%)

[a] Single-occurrence sequences: number of amino acid sequences occurring a single time in the set of unique sequences.

Table 4. **Summary of HTS results for VHH long-hinge library**. The NGS sequence data for VHH long-hinge library before (L0R) and after biopanning (L1RH and L1Rb) were assigned as SAMD00155278, SAMD00155279 and SAMD00155280 in DDBJ BioSample accession number, respectively.

Long-hinge library	Before biopanning (L0R, %)	After 1st round biopanning of strategy II (L1RH, %)	After 1st round biopanning of strategy I (L1Rb, %)
Total number of read sequences	1195565	1154461	1199340
Number of merged sequences	57320 (100%)	53972 (100%)	47261 (100%)
Single-occurrence sequences	35699 (62.2%)	34660 (64.2%)	27784 (58.8%)
Unique sequences	38704 (67.5%)	37624 (69.7%)	30514 (64.6%)
Highest frequency	1387 (2.4%)	685 (1.3%)	783 (1.7%)

Table 5. Summary of frequencies (%) for HTS−identified clones through biopanning. Sb, Lb and LH series of clones were isolated from biopanning strategy I for short hinge library, biopanning strategy I and II for long hinge library, respectively.

Clone name	Frequency before biopanning (%)	Frequency after biopanning (%)	Amplification factor
Sb_1	0.0034	6.3647	1871.12
Sb_2	0.0034	3.3311	979.29
Sb_3	0.0034	1.4531	427.19
Sb_4	0.0076	2.8722	379.98
Lb_4	0.0017	0.3078	175.81
LH_4	0.0017	0.2564	146.5
Sb_5	0.0034	0.3569	104.92
Sb_7	0.0038	0.2804	74.19
Sb_9	0.0038	0.2549	67.45
LH-3	0.0016	0.0725	46
LH_1	0.007	0.3103	44.32
Lb-5	0.0016	0.0594	37.73
Lb_1	0.0016	0.0552	35.03
Lb_9	0.0017	0.0573	32.74
Lb-14	0.0017	0.0488	27.89
Lb_7	0.0016	0.0431	27.48
Lb-16	0.0016	0.0403	25.6
Lb-22	0.0016	0.0339	21.56

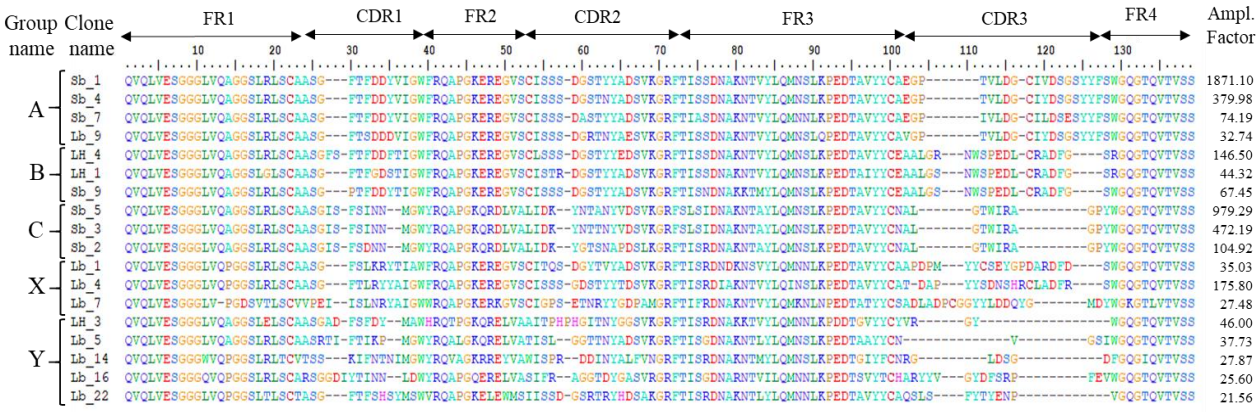

Figure 16. **Amino acid sequences of NGS identified top 18 clones**. According to high amplification factor the 18 sequences were divided into five groups with or without cysteine in CDR3. Cysteine in CDR3 sequences were belonged in a group but without cysteine in CDR3 were unique group of sequence.

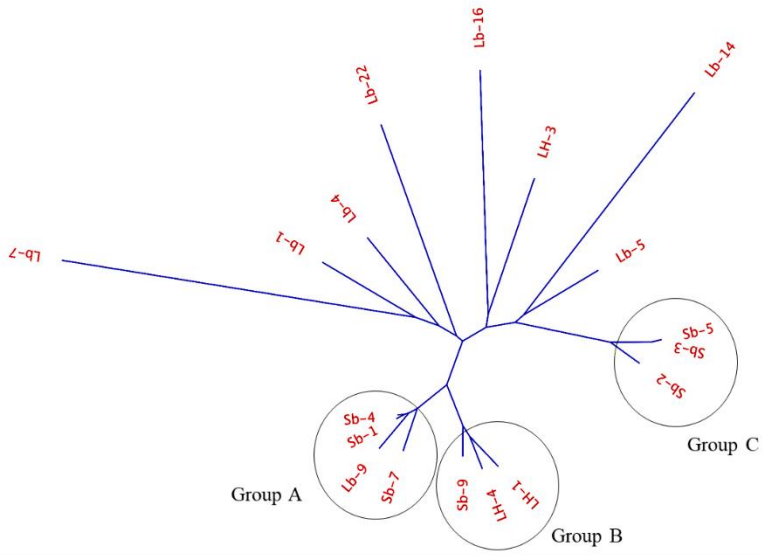

Figure 17. **Phylogenetic tree was constructed by neighbor - joining method among top 18 VHH clones NGS analysis**. Group A, B & C VHH clones were identical with conventionally isolated clones. Group X & Y newly identified VHH clones where group X contained Cys in CDR3 and group Y without Cys in CDR3.

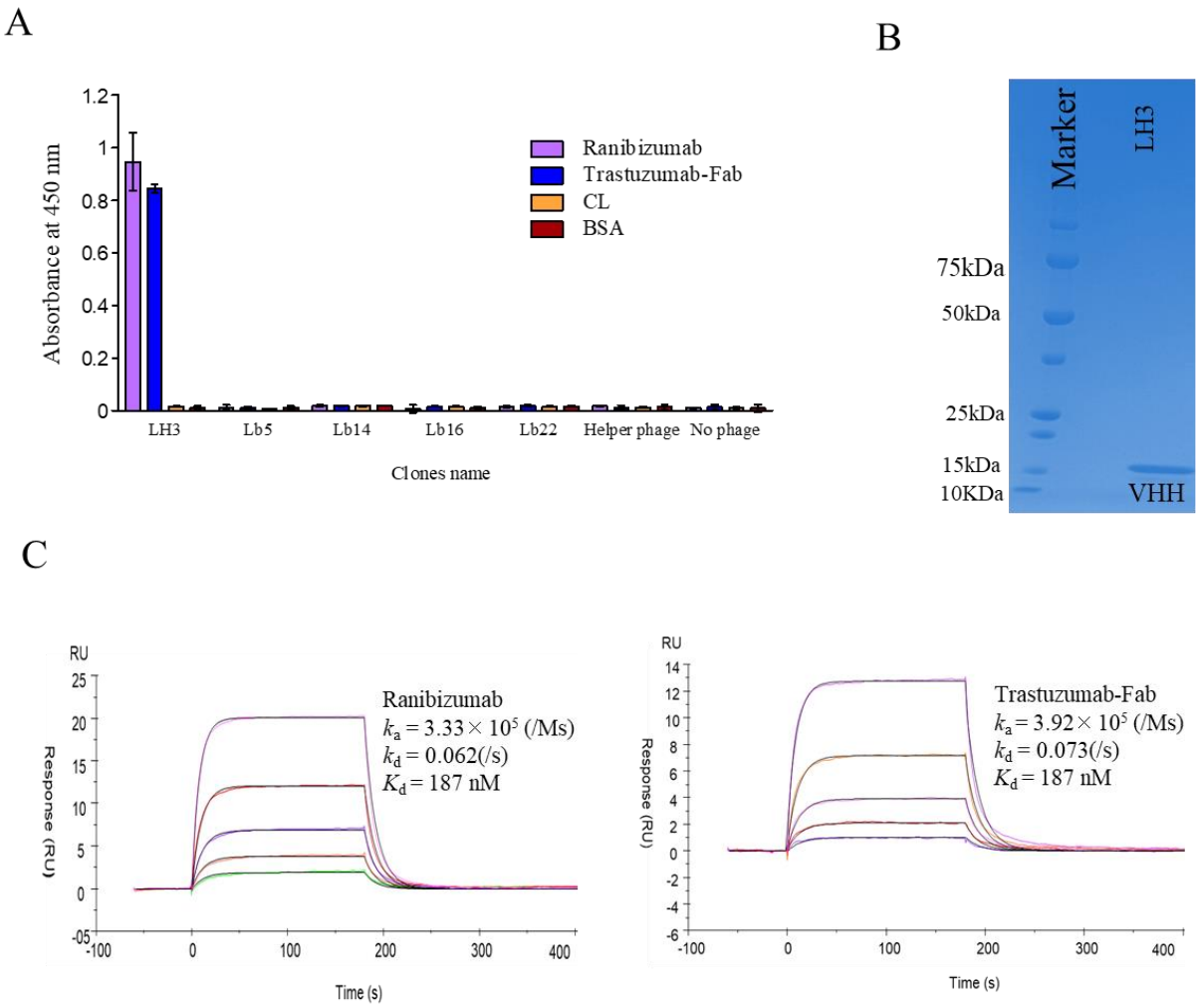

Figure 18. **Binding analysis of group Y VHHs.** (A) ELISA of five VHH phage clones from group Y to Ranibizumab and Trastuzumab-Fab. (B) SDS-PAGE of purified LH3 VHH antibody. (C) Kinetic binding analysis of LH3 VHH antibody to Ranibizumab and Trastuzumab-Fab by SPR on BIAcore-T200.

3.4 Summary

For the commercial development of a Fab antibody purification system using VHHs as a ligand for affinity purification, several studies suggested the avoidance of disulphide bonds or free cysteines in protein ligand that will be used as a ligand for affinity purification system, as a column need to regenerate with alkali pH solution.

Our isolated group (A, B and C) VHH antibodies from Fab-specific immuno phage library had the same epitope mapping recognition sequences in CDR3 for each group and group A and B contained cysteine in CDR3. Only group C considered to be alkaline resistant due to without cysteine in CDR3. To expand the diversity of VHH clones, we aimed to perform next generation sequencing using Illumina. After successfully carrying out the NGS analysis, LH3 clone without cysteine in CDR3 was identified with binding ability and specificity to Ranibizumab and Trastuzumab-Fab, also K_D was 187 nM for both Ranibizumab and Trastuzumab-Fab that were lower than group-C VHH.

Finally, we successfully identified the specific binders Cys-free in CDR3. Our results suggest that biopanning followed by NGS analysis is a robust technique for expanding repertoire of functional antibodies to obtain the purposeful antibody clones.

3.5 References

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Chapter 4

Affinity maturation of NGS identified LH3 VHH antibody through error-prone PCR library technique

4.1 Background

Affinity maturation is the process by which B cells produce antibodies with increased affinity for antigen during the course of an immune response. The first exposure to a given antigen entices clones of B cells displaying antigen-specific antibodies to undergo a rapid phase of multiplication and mutation resulting in an expanded series of B cell clones displaying antibodies more specific and with higher affinity for the antigen. With repeated exposures to the same antigen, a host will produce antibodies of successively higher affinities. A secondary response can elicit antibodies with higher affinity than in a primary response. The main principles of the *in vivo* affinity maturation, namely somatic hyper mutation and antigen selection of high-affinity clones are utilized for the biotechnological approach of the *in vitro* affinity maturation.

Improving the affinity of protein-protein interaction as a therapeutic and diagnostic biomolecule is a challenging problem [**1**]. Several approaches have been developed for affinity maturation of antibodies derived from the original libraries, but most fall into site-directed and/or random mutagenesis [**2**]. Error-prone polymerase chain reaction (PCR) is one of the most popular random mutagenesis methods, where point mutations are randomly introduced into a gene [**3**]. Random mutagenesis, coupled with genetic selection or high-throughput screening, is a technique for developing enzymes with novel properties, including altered substrate specificity, enantioselectivity, stability and reaction specificity [**4, 5**]. This technique has the advantage of enabling the development of new enzymatic properties without a structural understanding of the

targeted enzyme, and often yields unique mutations that could not have been predicted. In addition, further improvements can be expected by repeating the mutagenesis and selection (screening) processes in a manner mimicking Darwinian evolution. This approach, called directed evolution, is a principle method for biomolecular engineering [**4, 5, 6**].

The most commonly used random mutagenesis method is error-prone PCR [**7**], which introduces random mutations during PCR by reducing the fidelity of DNA polymerase. The fidelity of DNA polymerase can be reduced by adding manganese ions or by biasing the dNTP concentration. Use of the compromised DNA polymerase causes mis-incorporation of incorrect nucleotides during the PCR reaction, yielding randomly mutated products. To convert the product to a suitable form for transformation of a host strain, at least three steps are required: digestion of the product with restriction enzymes, separation of the fragments by agarose gel electrophoresis and ligation into a vector. Although these steps do not constitute special techniques, they require almost an entire day of handling time. Further, the ligation step can sometimes be troublesome because low ligation efficiency can cause loss of the library. For these reasons, it is desirable to simplify these steps.

Another useful random mutagenesis method is the bacterial mutator strain method [**8**]. The most popular mutator strain is *Escherichia coli* XL1-Red (Stratagene, La Jolla, CA), which lacks three of the primary DNA repair pathways, *MutS*, *MutD* and *MutT*, resulting in a random mutation rate ~5000-fold higher than in wild type. The protocol for using the mutator strain is composed of two steps: transformation of the mutator strain and recovery of the mutant from the transformant. This protocol is much simpler than error-prone PCR, and a ligation step is unnecessary. However, the mutation frequency is low under the standard conditions (0.5 mutations per kilobase) [8], and a cultivation period longer than 24 h is often required for introducing multiple mutations.

Phage-display technology with mutagenesis methods can be used for affinity maturation of parent peptides from the random libraries. After generation of the second phage-display library for affinity maturation, the most common selecting method is enrichment of the phage clones by biopanning process [**9]**. In our previous work [**10**], antigen-specific alpaca (Lama pacos) VHH antibodies by biopanning followed by high-Throughput sequencing

In the present study, in an attempt to increase affinity of human Fab-specific VHH isolated from an immune phage display library, we constructed a VHH mutant phage display library using error-prone PCR reaction followed by the combination of single point mutation into parent VHH gene.

4.2 Methods and materials

4.2.1 Sample preparation by error-prone PCR

For construction of mutant phage display library by error-prone PCR technique, GeneMorph II Random Mutagenesis Kit (Agilent) was used to amplify 1ng of LH3 VHH gene using a set of primers, forward 5'-TGCTCCTCGCGGCCCAGCCGGCCATGGCTCAGGTGCAGCTCGTGGAGTCTGG-3' and reverse primer: 5'-ATGATGATGTGCACTAGTTTGTGGTTTTGGTGTCTTGGG -3'. Forward primer matched with framework 1 containing *Sfi* I restriction site and reverse primer matched with framework 4 containing *Spe* I restriction site. Error-prone reaction was carried out according to **Table 6**. The mutagenic reaction was done with the following thermal conditions: Initial denaturation at 96ºC for 5 minutes, then 35 cycles of 96ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 1 minute, followed by a 72ºC incubation period for 10 minutes.

Table 6. Conditions of error-prone PCR reaction

Template DNA (1 ng)	1µl
Forward primer **(**10 µM)	1µl
Reverse primer (10 µM)	1µl
10× Mutazyme reaction buffer	5µl
40 mM dNTP mix	1µl
Mutazyme II DNA polymerase	1µl
Ultra-pure water	40µl
Total volume	50µl

4.2.2 Construction of mutant phage library

The mutated VHH gene fragments (400 bp) with the restriction enzyme sites and the phagemid vector pKSTV03 (TAKARA BIO) were treated with restriction enzymes *Sfi* I and *Spe* I (NEW ENGLAND BioLabs $_{Inc.}$) under respective optimum conditions and then performed gel cut to purify the digested fragments. The vector and the insert were mixed at 1: 2, and the ligation reaction was carried out at 16 ° C. for 12 hours using T4 DNA ligase (WAKO, Nippon gene). The ligation product was purified by phenol: chloroform extraction method. 500 ng of purified ligation products were electrophoresed into *E. coli* TG-1 cells (Lucigen Co) by electroporation. A part of the transformation product was used for titer check to measure the gene diversity, and the rest were subjected to plate culture (2 TYAG at 30 ° C for 12 hours) for stock. Colony PCR was performed to determine the presence of VHHs in the vector. The culture served as the Fab-specific VHH random mutagenesis library.

4.2.2.1 Phage propagation

Phage amplification was carried out in the 500 mL 2TY (Tryptone-Yeast extract) medium containing 100 μg / mL ampicillin, 2% glucose was inoculated with 500 μL of a thawed glycerol stock of *TG1* cells containing the phage library and grown to an OD_{600} of 0.5. Superinfected by M13KO7 helper phage (Invitrogen) at a multiplicity of infection (MOI) was 20 and cultured at 37 ° C for 30 minutes without shaking followed to 30 minutes at 37 ° C with shaking. After helper phage infection, the bacterial cells were centrifuged at 1500g for 10 minutes, the supernatant containing excessive uninfected helper phage was discarded, and the *E. coli* pellets infected with the helper phage was inoculated into fresh 2 TYAK medium (Tryptone-Yeast medium contained 100 μg / mL ampicillin, 100 μg / mL kanamycin), and cultured at 37 ° C for 16 hours. The culture solution was centrifuged at 8000 rpm for 20 minutes and the phage containing supernatant suspended by 0.2 vol PEG / NaCl which was used for phage precipitation reaction. Finally, centrifuged the supernatant at 10000 rpm for 60 minutes and discarded supernatant. The phage pellets were resuspended by 1 mL of phosphate-buffered saline (PBS), filter sterilized and stored at 4ºC until use.

4.2.2.2 Phage library enrichment through biopanning

Immuno tube was immobilized with Ranibizumab or Trastuzumab-Fab (5 μg to 100 ng / 1 mL) at room temperature for 2 hours (the coating amount of antigen was decreased each time rounds were applied, and panning conditions were tightened). Wash 5 times with PBST (T= 0.1% Tween-20), Blocked with 3% skim milk and incubated at room temperature for 2 hours. After washing 5 times with PBST (T= 0.1% Tween-20), the phage library was added and, the incubation was carried out at room temperature for 1 hour. In order to remove nonspecifically bound phage, washing was carried out with PBST (T= 0.1% Tween-20) for 1.5 hours. Antigen-specific phage were eluted

with 1.5 mL of 0.1 M glycine-HCl at pH 2.2, neutralized the reaction with 100 µL of 1 M Tris-HCl (pH 9.0). And then rescued phage were infected into precultured *Escherichia coli TG-1* (**Fig.19**) Checked titer by serial dilution and made 2TYAG plate for library stock. These procedures were repeated three times as shown in **Table 7**.

Table 7. Summary of biopanning procedures of mutant phage display library

Panning conditions	1st round	2nd round	3rd round
Coating	Ranibizumab 5µg/ml	Trastuzumab-Fab 500ng/ml	Her2-Fc 100ng/ml
Phage	0R phage 5.0×10^{10} / 3ml	1R phage 5.0×10^{10} / 3ml	2R2 phage (3.0×10^{10}) with Trastuzumab-Fab (100ng) in 3% skim milk (3ml), incubation at RT 1 h.
Washing	30 min with 0.1% PBST	24 hours with 0.1% PBST	24 hours with 0.1% PBST

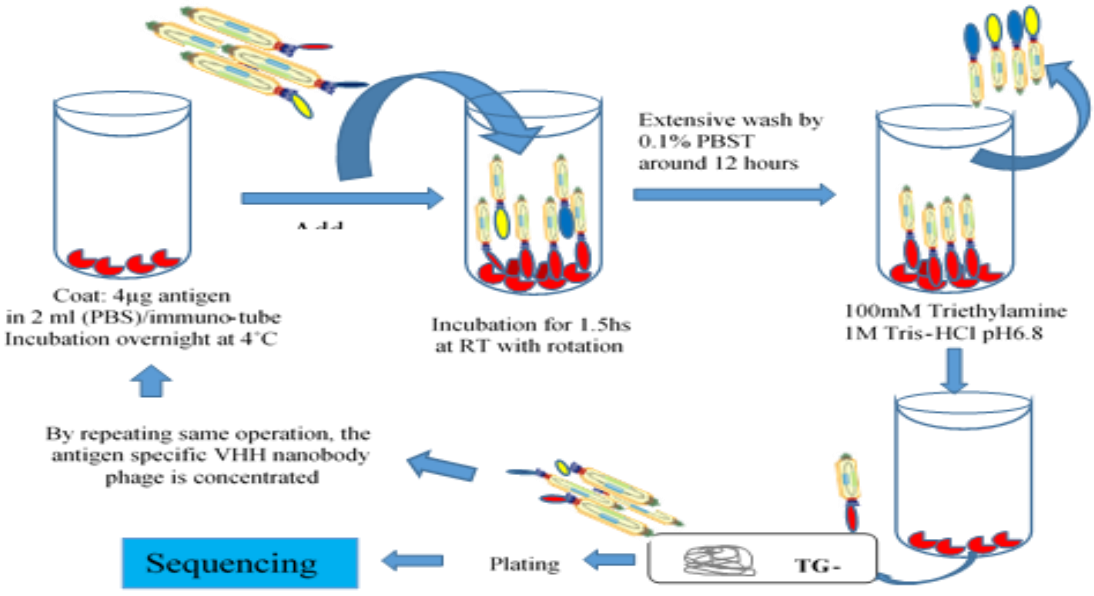

Figure 19. Overview of biopanning procedures in an imunotube strategy to screen out highly enrichment pooled phage.

4.2.2.3 Phage ELISA to determine enrichment phage library

Antigen (50 ng / 50 μL) diluted with PBS was immobilized on a 96-well microtiter plate (Nunc Thermo Fisher Scientific) for 2 hours at room temperature and blocking was carried out for 2 hours at room temperature with a blocking agent (0.5% BSA). After washing three times with PBST (T= 0.1% Tween-20), the phage solution (1.0×10^9/50 μL) library phage was added and the reaction was allowed to proceed at room temperature for 1 hour. After washing 5 times with 0.1% PBST, a secondary antibody (biotinylated Mouse anti-M13 antibody (Abcam)) pre-incubated with SA-HRP (VECTOR LABORATORIES) was allowed to react at room temperature for 1 hour. After washing 5 times with 0.1% PBST, a TMB solution (CALBIOCHEM) containing a peroxidase luminescent substrate was added and the reaction was stopped by 1 N HCl by color reaction, and the VHH bound to the antigen protein on the well Antibody phage were detected. Detection was carried out by measuring the absorbance at 450 nm using an ELISA plate reader MODEL 680 XR (BIO RAD).

4.2.2.4 Screening of monoclonal phage by ELISA

Briefly, wells of a 96-well Nunc MaxiSorp plate were coated with Ranibizumab and Trastuzumab-Fab at a concentration of 50ng/50μl/well, incubated overnight at 4˚C. Washed three times with PBS containing 0.1% Tween-20. Following blocking of the wells, 50 μl of monoclonal phage containing supernatant from above was added to each well and incubated for 60 min at room temperature with shaking. The phage solution was then removed, and the wells were washed three times with PBST. Added 50 ul of the secondary antibody, horseradish peroxidase (HRP)-conjugated with anti-M13, was diluted (1: 4000) in 0.5% BSA and incubated for 60 min with shaking at room temperature. Following incubation, all wells were washed five times with PBST.

The Colour was developed by adding 40 µl of TMB substrate (3, 3′, 5, 5′-tetramethylbenzidine) and incubating for 30 mins at room temperature. Color development was stopped with 40 µl of 1 N HCl and the absorbance of the solution in each well was measured. The OD was measured in absorbance at 450 nm.

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4.2.3 Expression of Mutant VHH antibodies in *E. coli*

E. coli HB 2151 with VHH DNA incorporated therein was cultured in 2 TYAG 10 ml at 37 ° C. overnight, 500 µL of the resulting culture was added to 500 ml of fresh medium (2 TYA) and cultured at 37 ° C. until OD 600 = 0.6-0.8 . The cells were collected by centrifugation, suspended in 2 TYAI (2 TY medium, 100 µg / ml ampicillin, 1 mM IPTG), and cultured at 30 ° C. for 10 hours. The culture solution was separated into supernatant and cells by centrifugation, suspended in 10 ml of TES buffer (0.2 M Tris-base, 0.5 mM EDTA, 0.5 M sucrose) and left to stand on ice for 2 hours. Add 20 ml of 1/4 TES buffer and resuspended it and leave on ice for 1 hour. Centrifugation was performed and the supernatant was collected (periplasm). Soluble VHH was purified from the supernatant using an affinity column (His trap excel, GE Healthcare).

4.2.4 Purity of mutant VHH antibodies by SDS-PAGE

SDS–PAGE was carried out according to Laemmli's method (8). Proteins were electrophoresed by SuperSep^{TM}Ace 5-20% sodium dodecyl sulfate poly acryl amide gel (Wako) and stained with Coomassic Brilliant Blue R-250. The protein amount of stained band on the SDS–PAGE was estimated using image of Precision Plus Protein ^{TM} Standards All Blue as a standard. The proteins band was detected by imaging (BIO-RAD, Molecular Imager $^{(R)}$ Chemi Doc ^{TM} XRS+ with Image Lab ^{TM} Software.

4.2.5 Specificity of mutant VHH antibodies by ELISA

The wells of a 96 well Microplate (Nunc Maxisorp) were coated with Ranibizumab, Trastuzumab-Fab, Her2-Fc, CL domain, NDOM, Lysozyme, skim milk and BSA (50 ng/50 µL/well) overnight at 4°C. After three times washing with 0.1% PBST, blocked for 2 hours at RT with 0.5% BSA in PBS and washed out unbound materials. VHHs (100 ng/well in 0.5% BSA) were added to each well and incubated for 1 hour at RT. After washing the plate, added 50 µL/well pre incubated conjugation *of* Anti-His-tag *mAb*-biotin (MBL MEDICAL & BIOLOGICAL LABORATORIES CO., LTD) and SA-HRP (Vector Laboratories, Inc. Burlingame, CA). After 1-hour incubation, five times washed the plates and binding was detected with 40 µL/well TMB (tetramethylbenzidine) reagent. Subsequently, 1N HCl (40 µL/well) was added to stop additional reaction. Finally, the binding was measured by the absorbance at 450 nm in a microplate reader (680XR, Bio-Rad, USA).

4.2.6 Affinity analysis by SPR

K_Ds were determined at 25°C using Biacore T200 (GE Healthcare). To make antigen the surface, 10µM of antigen in 40µl of HBS buffer, pH 4.5, were immobilized on a research grade on CM-5 sensor chips (GE Healthcare) by the amine coupling protocol supplied by the manufacturer. The different concentrations from 100nM to 6.25nM) of solutions in HBS buffer were injected for monitoring association reaction and then eluted with HBS buffer for dissociation reaction. The sensor chips were regenerated by 10 mM glycine-HCl buffer (pH 2.0) containing 0.5 M NaCl. The sensorgrams were analyzed using a 1:1 binding model on BIA evaluation software to determine equilibrium dissociation constants (K_D).

4.2.7 Preparation of double and triple mutant VHH antibodies

The single-point mutation VHH antibody obtained from mutant library with higher affinity, among them 3 VHH clones had 2.5-3 folds more affinity than parent VHH those were considered as functional mutation analyzed by Biacore and free energy change of antigen-antibody binding. Therefore, we prepared double and triple mutant VHH by overlapping PCR using LH3-Q39E as a template.

4.2.7.1 Design of point mutation primers

For each mutation we designated forward and reverse primer to overlap the mutation point.

S30N: Forward primer 5'-GAGCCGACTTCA**A**TTTCGATTATATG-3'

Reverse primer 5'-CATATAATCGAAATTGAAGTCGGCTC-3'

H56Y: Forward primer 5'-TACTCCTCATCCT**T**ATGGTATTACAAAC-3'

Reverse primer 5'-GTTTGTAATACCATAAGGATGAGGAGTA-3'

4.2.7.2 Overlapping PCR

Overlapping PCR was done by using mutation designated forward and reverse primer. Prime START Max (TAKARA bio) were used as the DNA polymerase in the PCR reaction (**Fig.20**). 1 ng of plasmid DNA was used as a template with 0.5 µM primer of each. The thermal cycler condition was 96ºC for 3 minutes in 1 cycle, 96ºC for 30 seconds 58ºC for 30 seconds and 72ºC for 1 minute in 25 cycles finally 72ºc for 10 minutes in 1 cycle.

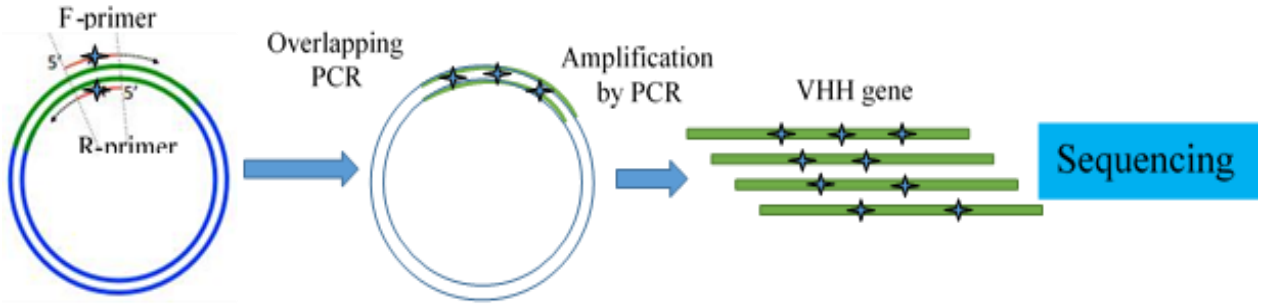

Figure 20. Representation of overlapping PCR to combine single-point mutations into LH3 VHH gene

4.2.8 Calculation of free energy change of antigen-antibody binding

The antigen-antibody reaction is widely used in laboratory diagnostics, including immunoassay. It is a reversible chemical reaction:

Antigen + antibody $\rightleftharpoons$ antigen – antibody complex ……. (1)

At the beginning, a chemical reaction proceeds predominantly in one direction, but the reverse rate progressively increases until the forward and reverse speeds are equal. At this point, the reaction is said to have reached its equilibrium.

According to the law of mass action:

$$\frac{[complex]}{[antigen]\times[antibody]} = \frac{ka}{kd} = K_{eq} \quad \text{..... (2)}$$

at the equilibrium, the ratio between the concentrations of the products ([complex]) and the reactants ([antigen] and

[antibody]) is constant. K_{eq} is called the equilibrium constant and is equal to the ratio between the association (k_a) and the dissociation (k_d) rate constant.

In order to improve antibody detection, the ratio between bound and free antigen ($\frac{[complex]}{[antigen]}$) should be increased as much as possible. Rearranging equation (1):

$$\frac{[complex]}{[antigen]} = K_{eq} \times [antibody]$$

We note that this can be obtained in two ways: increasing either the equilibrium constant or the antibody concentration [2].

The greater the strength of the bond, the higher its equilibrium constant. This relationship is expressed by the following formula:

$$In(Keq) = -\frac{\Delta G}{R \times T}$$

Where In means the natural logarithm, ΔG is the change in free energy, R is the universal gas constant (8.314472 $JK^{-1}.mol^{-1}$) and T is the absolute temperature (298K at 25°C).

4.2.9 Measuring the melting temperature

Melting temperature also were determined by binding of a fluorescent dye during protein unfolding**.** DSF is a rapid and inexpensive screening method to identify low-molecular-weight ligands that bind and stabilize purified proteins. The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye with affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds.

Thermal stability and refolding capability of shark derived single domain antibodies). Protein Thermal Shift Dye kit (Applied Biosystems by life technologie**s**) was diluted (1000×) to 8× with PBS. Final protein concentration was 1mg/ml. Total reaction volume was 20 µl (5 µl Protein Thermal Shift Buffer, 12.5 µl protein and Diluted Protein Thermal Shift Dye (8×) 2.5 µl. The experiments were carried out with a StepOne Real-Time PCR system (Applied Biosystems). The proteins were treated from 25 to 99˚C at a rate of 1% (2˚C/min) with continuous monitoring of fluorescence using the ROX channel. First derivatives of the data were calculated by manufacturer's software and the melting temperature was taken to be the peck of the derivatives data. All measurements were in four replicates.

4.2.10 Irreversible thermal stability assay

VHH nanobody were prepared at a concentration of 1.0 mg/ml and incubated at various temperature range for 5 minutes and then cooled to room temperature and centrifuged to avoid precipitated pellets. The OD_{280} for each was measured, as well as binding activity was measured by ELISA.

4.3 Results and discussion

We tried to improve the affinity of NGS-identified LH3 VHH antibody using error-prone PCR library technique. The error-prone PCR was carried out using GeneMorph II Random mutagenesis Kit. The mutated VHH gene fragments were digested with *Sfi* I and *Spe* I restriction enzyme and inserted to linearized phagemid vector pKSTV-02. The ligation DNA transfected into *E. coli* TG1 to construct mutant library. The mutant library was successfully constructed with 1.4×10^8 unique members.

We subjected phage library to biopanning against Ranibizumab and Trastuzumab-Fab for enrichment of phage library in immuno tube panning methods. After repeating these procedures several times under the strict conditions, 3rd round of panning showed the higher enrichment against Fabs (**Fig.21**). The ninety-six phages were randomly cloned from the 3rd round phage library to analyze Fab binding in ELISA (**Fig.22**) and sequenced for the all positive clones. After sequencing, we aligned the sequences where seven clones harboring a single-point mutation were successfully isolated (**Fig.23**).

The mutated VHH proteins were expressed in *E. coli* (HB2151) cells by infecting cloned specific phages (**Fig.24A**) and purified on HisTrap excel column. The purified VHHs showed highly specific to Ranibizumab and Trastuzumab-Fab by protein ELISA (**Fig.24B**) and subjected to affinity measurement on Biacore T200 in the 6.25-100 nM different concentrations with HEPES buffered saline. The affinity parameters of these mutants are summarized in **Table 8**. The affinities of the mutated clones were 1-2.5 folds higher than parent clone and three of them LH3-S30N, LH3 -Q39E and LH3-H56Y showed 1.5–2.5-fold-higher binding affinities than parent LH3 VHH (**Fig.25**), indicating these mutations can contribute to increase the affinity (**Table 8**).

Therefore, we combined these mutations to make stronger binders. Double and triple mutations at the numbers 30, 39, and 56 were introduced onto the LH3 sequence by overlapping PCR using mutation-specific primers. Furthermore, we prepared three double and one triple mutant VHHs in *E. coli* (HB2151) cells and purified on HisTrap excel column (**Fig26A**). The purified double and triple mutant VHHs were subjected to protein ELISA to confirm the binding specificity to Ranibizumab and Trastuzumab-Fab (**Fig.26B**). Therefore, SPR analysis was done on Biacore T200 using similar concentration like the case in single point mutant VHH. The SPR analysis indicated that the double mutants clearly enhanced their affinity 3–6 folds and the triple mutant LH3-S30N-Q39E-H56Y VHH showed more than ten folds higher affinity (K_d: 18 nM) than the parent VHH (**Fig. 25**). The binding free energies of the mutants are summarized in (**Table 8),** demonstrating the additivity of the effects on the mutations in affinity.

In addition, we determined the melting temperature using differential scanning fluorimetry (DSF) technique by RT-PCR. The observed chromatograph indicated that the triple mutant VHH had 4^{o}C higher melting temperature than parent VHH as shown in **Fig.27 and Table 9**.

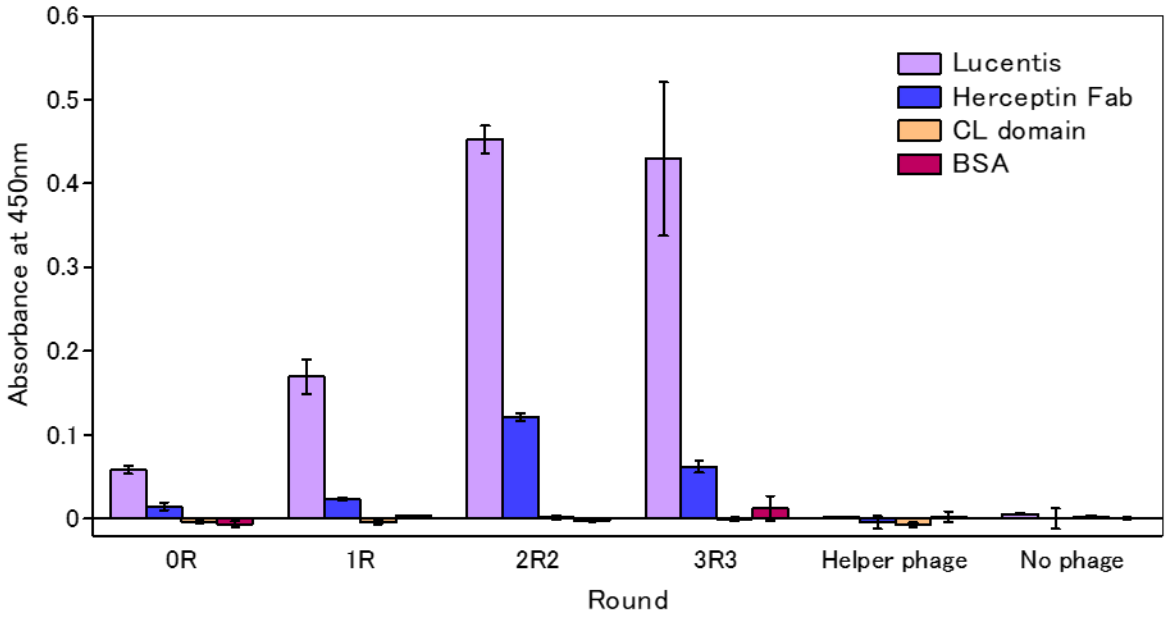

Figure 21. Polyclonal phage ELISA to determine human Fab-specific enrichment of pooled phage library after successive round of panning.

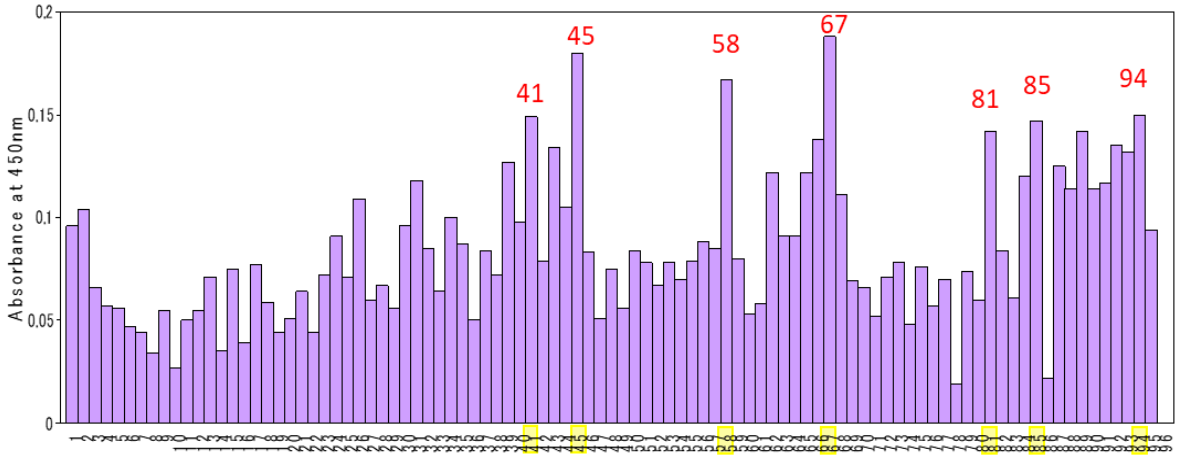

Figure 22. Monoclonal phage ELISA to determine human Fab-specific enrichment phage from 3rd round of panning.

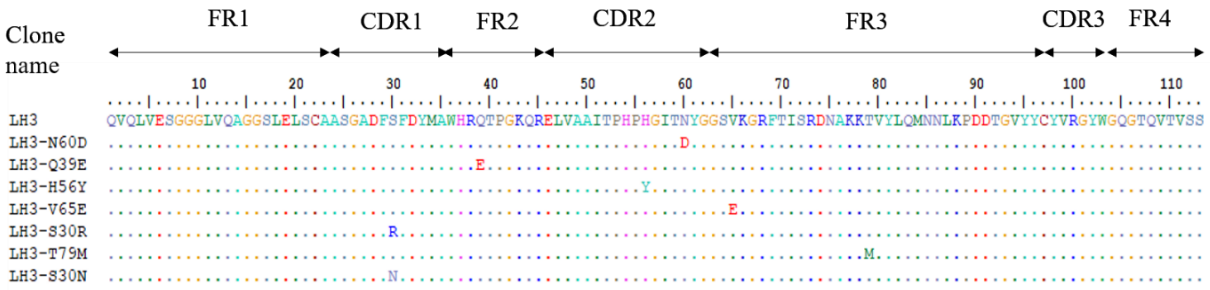

Figure 23. Amino acid sequences of VHHs with single-point mutation obtained from error-prone PCR library.

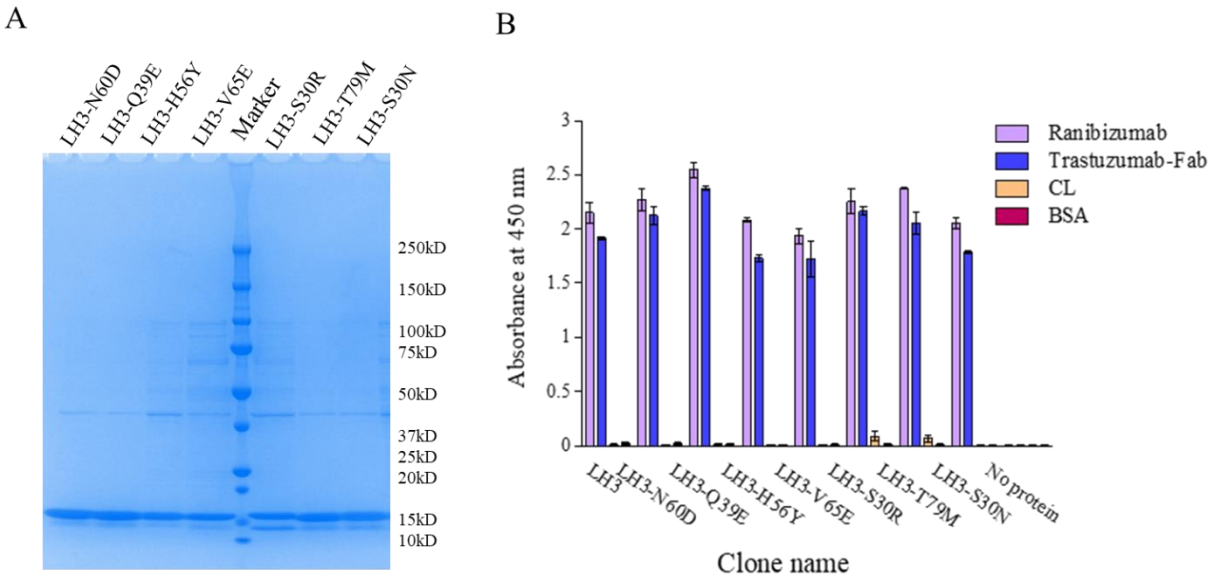

Figure 24. Binding properties of LH3-S30N-Q39E-H56Y VHH antibodies. (A) SDS-PAGE of purified VHH clones from mutant library. (B) Binding specificity of single-point mutant of VHH to Ranibizumab and Trastuzumab-Fab by ELISA.

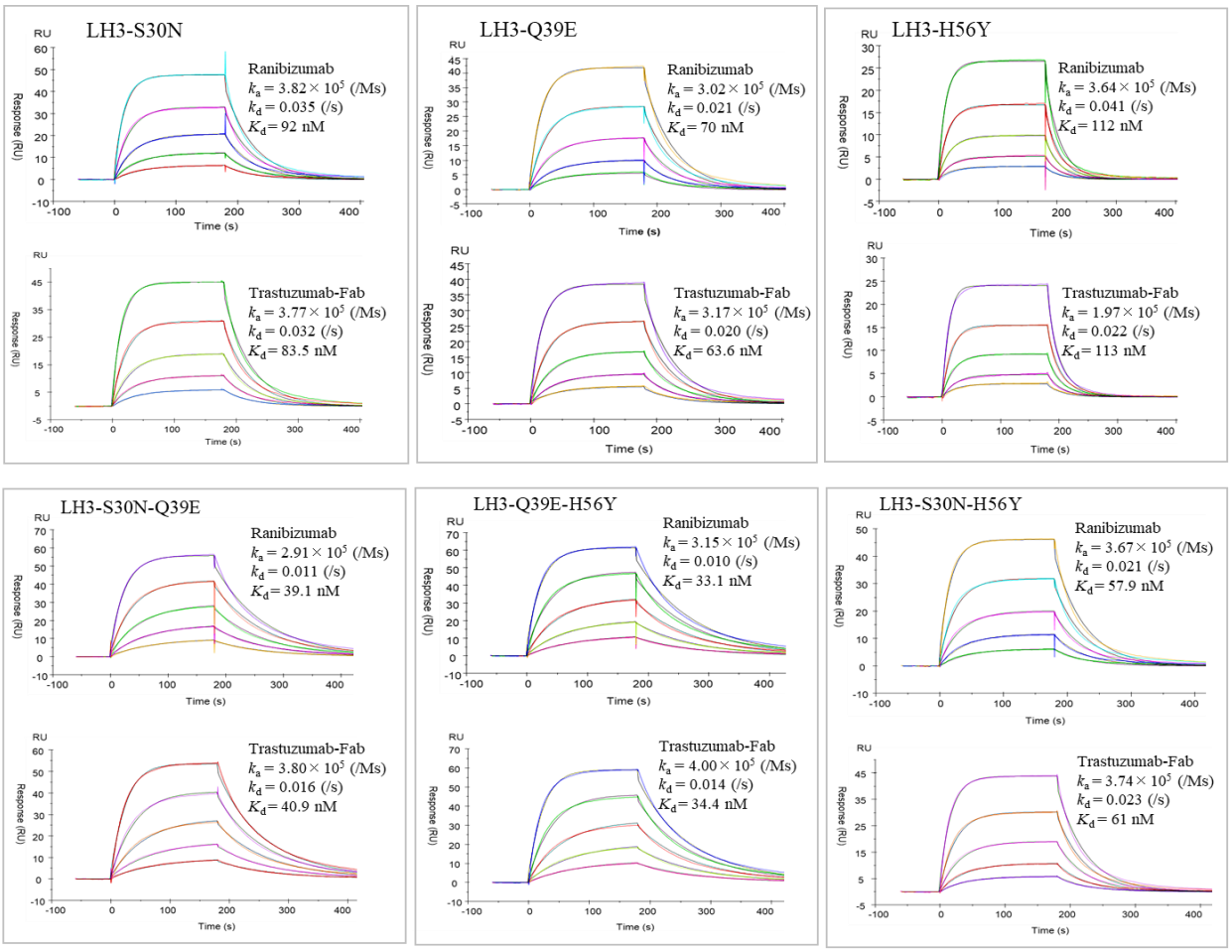

Figure 25. Representative SPR sensorgram binding profiles for affinity maturation. Single point mutants LH3-S30N, Q39E and H56Y, the lead double mutants LH3-S30N-Q39E, Q39E-H56Y and S30N-H56Y, and the lead triple mutant LH3-S30N-Q39E-H56Y. The 6.25–100 nM different concentrations of VHH range used in each experiment. The sensorgrams were analyzed using a 1:1 binding model on BIA evaluation software to determine equilibrium dissociation constants (K_d) and kinetic parameters (k_a and k_d).

Table 8. Summary of binding affinity parameters and differences in binding free energies (*ΔΔ*G) of single-point, double and triple mutant VHHs derived from the parent LH3 clone

VHH	Ranibizumab				Trastuzumab-Fab			
	k_a ($\times 10^5$/Ms)	k_d (/s)	K_d (nM)	*ΔΔ*G (kcal/mol)*	k_a ($\times 10^5$/Ms)	k_d (/s)	K_d (nM)	*ΔΔ*G (kcal/mol)*
Parent LH3	3.3	0.062	187	-	3.9	0.073	187	-
Single-point mutants								
LH3-N60D	4	0.075	188	0	4.8	0.082	170.5	-0.05
LH3-Q39E	3	0.021	70	-0.57	3.2	0.020	63.6	-0.62
LH3-H56Y	3.6	0.041	112.3	-0.31	2.0	0.022	113.2	-0.29
LH3-V65E	8.6	0.120	140	-0.17	9.9	0.130	135	-0.19
LH3-S30R	3.4	0.049	145.3	-0.14	3.7	0.052	138	-0.17
LH3-T79M	4.5	0.067	150	-0.13	5.0	0.076	151.2	-0.1
LH3-S30N	3.8	0.035	92	-0.38	3.8	0.032	83.5	-0.48
Double mutants								
LH3-S30N-Q39E	4.0	0.011	39.1	-0.93 (- 0.95)	4.8	0.082	40.9	-0.91 (- 1.10)
LH3-Q39E-H56Y	3.1	0.010	33.1	-1.03 (- 0.88)	4.0	0.014	34.4	-1.00 (- 0.91)
LH3-S30N-H56Y	3.7	0.021	57.9	-0.69 (- 0.69)	3.7	0.023	61	-0.67 (- 0.77)
Triple mutant								
LH3-S30N-Q39E-H56Y	4.3	0.008	17.6	-1.41 (- 1.26)	4.2	0.008	17.9	-1.39 (- 1.39)

*Differences of experimental values in binding free energies (*ΔΔ*G, kcal/mol) and theoretical values in parentheses.

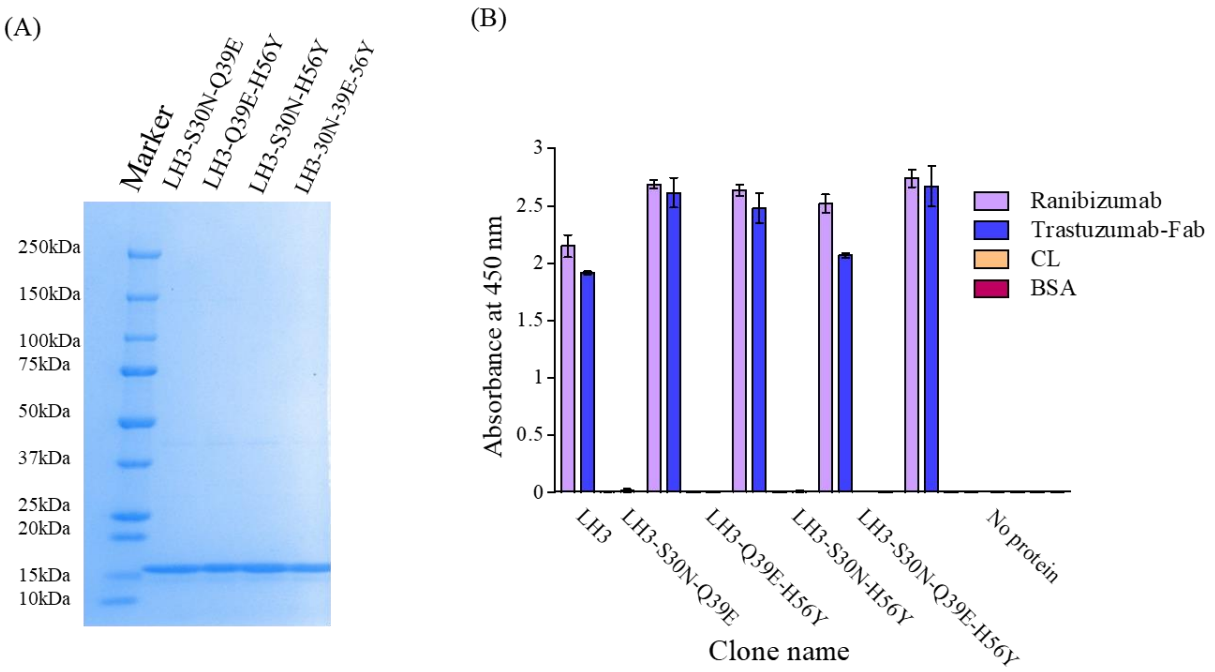

Figure 26. Binding properties of double and triple mutant VHH antibodies. (A) SDS-PAGE of purified VHH clones of combined single-point mutations. (B) Binding specificity of three double mutants and one triple mutant VHH to Ranibizumab and Trastuzumab-Fab by

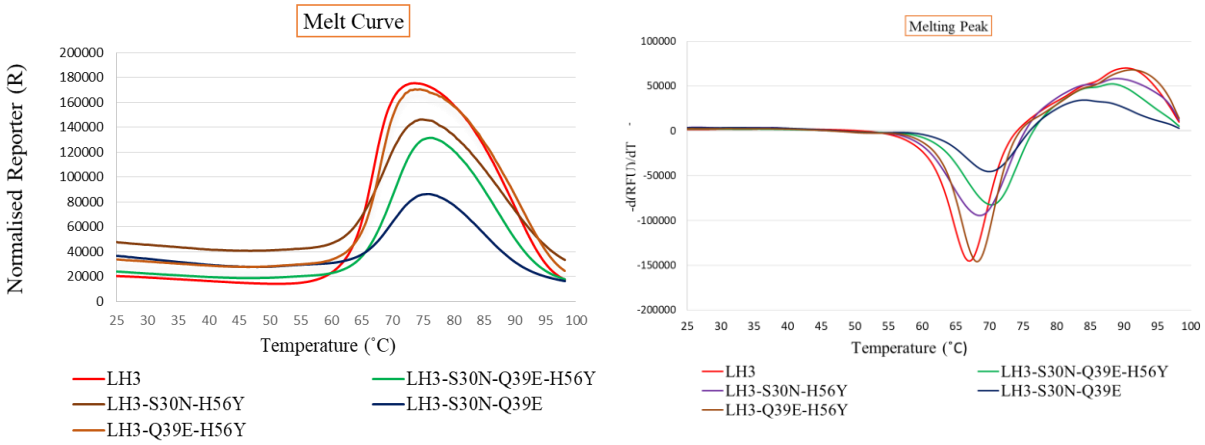

Figure 27. Melting curves and peak for double and triple mutant VHH antibodies.

Table 9. Summary of thermal shift assay (Tm)

Clones name	Melting temperature (Tm)／ºC
LH3	66.50
LH3-S30N-Q39E	70.00
LH3-Q39E-H56Y	68.00
LH3-S30N-H56Y	68.50
LH3-S30N-Q39E-H56Y	70.40

4.4 Summary

As our NGS identified LH3 clone was not suitable for as an affinity ligand due to low affinity, we tried to enhance the affinity maturation using error-prone PCR library technique. After harboring suitable mutations, we constructed mutant phage library with the diversity of 1.4×10^8. Phage enrichment was carried out by biopanning against coating Ranibizumab and Trastuzumab-Fab. Seven clones were successfully isolated (**Fig.23**) with a higher specificity and 0 to 2.5 folds higher affinity than parent VHH (**Table 8**). Among seven mutated VHH clones, three of them LH3-Q39E, LH3-H56Y and LH3-S30N showed 1.5 to 2.5 fold higher K_D (70 nM, 112 nM and 92 nM for Ranibizumab whereas 63.6 nM, 113 nM and 83.5 nM for Trastuzumab-Fab respectively) relative to parent VHH (**Fig.25**) as well as difference in antigen binding free energies ($\Delta\Delta G$) relative to the parent VHH (**Table 8**)

Furthermore, we prepared double and triple mutant VHH antibodies focused on these three mutant Q39E, H56Y and S30N. Expressed double and triple mutant VHH with high specificity (**Fig.26**)

and subjected to SPR analysis. Double mutant VHHs were 5-6 folds and triple mutant VHH was more than ten folds higher affinity than parent VHH. Therefore, these mutations can contribute independently to affinity, indicating a clear additivity in the change of mutational energy. In this way, without mutations on CDR3, through the accumulation of the small mutational energies, the increase of affinity was attained, indicating the method employed here and/or mutations found can be applicable for engineering of other VHHs.

In addition, the melting temperature (Tm) of triple mutant VHH was 70.4°C, it indicated that about 4°C higher than parent VHH (**Fig.27 and Table 9**).

4.5 References

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Chapter 5

Characterization and evaluation of Fab drugs purification by VHH-conjugated affinity column

5.1 Background

One of the major milestones in the history of antibody research and the development of monoclonal antibody as therapeutics [**1, 2**]. Over the last three decades, monoclonal antibodies have made a dramatic transformation from scientific tools to powerful human therapeutics. At present, approximately 30 therapeutic monoclonal antibodies are marketed in the United States and Europe in a variety of indications, with sales in the US alone reaching approximately $18.5 billion in 2010 [**3**]. Consequently, the production of therapeutic antibodies necessitates the use of very large cultures of mammalian cells followed by extensive purification steps, under Good Manufacturing Practice conditions, leading to extremely high production costs and limiting the wide use of these drugs [**4**]. Though the production cost is high but there are several purification techniques are well established in the antibody therapeutic market.

However, the fragment antibody (Fab) drugs are getting popularity in recent days due to their incredible properties like smaller in size, higher tissue penetration, lower side effects, easy to protein engineering and low production cost [**5, 6, 7, 8**]. Many more antibody fragments (Fab) drugs are under development. [**5, 9, 10**]. The production of antibody fragments pipeline is expanding with three therapeutic Fabs approved by US Food and Drug Administration those are Abciximab (ReoPro, Eli Lilly), ranibizumab (Ranibizumab, Genentech), and certolizumab pegol (Cimzia, UCB) are manufacture in the periplasm of *E.coli* and many in the active clinical pipeline

and preclinical research. [**11, 12, 13, 14**]. Unfortunately, the Fab antibody drug purification is not well established yet.

Despite the nature of the ligand, several requirements must be met such as exhibiting specific and reversible binding to the protein of interest, as well as stability towards components present in the crude extract. The affinity interaction should be sufficiently high in order to form a stable complex. However, the interaction must not be too high in order to be able to effectively elute the protein by simple change of the buffer. For proper interaction the affinity constant, K_A, should at least be in the range 10^3 to 10^6 M or higher (Labrou and Clonis, 1994). This is equal of a dissociation constant, K_D, of mM to μM. Affinity constants substantially exceeding these values, $K_A = 10^{10}$ to 10^{11} M or higher, often require harsh and sometimes denaturing elution conditions (Labrou and Clonis, 1994). However, the disruption of the complex is much depending on the type of interaction. Hence, some very strong interactions may be quite easily broken. Also, the ligand density of the adsorbent may influence the binding capacity as well as the selectivity. Moreover, the ligand should also possess at least one functional group, which can be used for immobilization to the matrix. These functional groups commonly include NH_2, COOH, CHO, SH, or OH.

In this work, we were trying to develop a human Fab antibody purification system employing VHH affinity ligand from Alpaca. Therefore, we isolated a VHH affinity ligand 10 folds higher affinity than parent clones using high throughput sequencing (HTS). Using this affinity ligand, we evaluated the Fab antibody purification system.

5. 2 Methods and materials

5.2.1 Preparation of VHH-conjugated affinity column

VHHs were produced in *Escherichia coli* HB2151 cells infected with cloned phages and purified on HisTrap excel columns (GE Healthcare) from the periplasmic fraction of the bacteria. Purified VHH (5 mg) was immobilized on a HiTrap^{TM} NHS-activated HP column (1mL, GE Healthcare).

Protocols:

1. HiTrap NHS activated column (GE Healthcare, 1 ml) was washed with 5 mL of 1mM HCl
2. 5 mg of VHH ligand was dissolved in 1 mL of 0.2 M $NaHCO_3$ (pH 8.3)
3. 1mL of VHH ligand solution was slowly injected to the column.
4. Incubation at RT for 3 hours
5. Wash the column with 10 mL of 0.2 M $NaHCO_3$ buffer
6. Block the column with 6 mL of blocking buffer (0.5M Monoethanolamine + 0.5M NaCl, pH-7.5)
7. Wash the column with 6 mL of washing buffer (0.1M Sodium acetate + 0.5M NaCl, pH-4.0)
8. Block the column with 6 mL of blocking buffer (0.5M Monoethanolamine + 0.5M NaCl, pH-7.5)
9. Incubation at RT for 30 minutes
10. Wash the column with 6 mL of washing buffer (0.1M Sodium acetate + 0.5M NaCl, pH-4.0)
11. Block the column with 6 mL of blocking buffer (0.5M Monoethanolamine + 0.5M NaCl, pH-7.5)
12. Wash the column with 6 mL of washing buffer (0.1M Sodium acetate + 0.5M NaCl, pH-4.0)

5.2.2 Loading and elution buffer preparation

Before initiating the purification of antibodies, it is important to assess the properties of the sample. According to the nature of column ligand and loading Fab antibody, we prepared sodium phosphate buffer as a loading buffer and glycine-HCl buffer as an elution buffer.

Sodium Phosphate Buffer 1 M, pH 7.0) preparation

1. Solution A:

   Dissolve 138.0 g $NaH_2PO_4–H_2O$ in 1 liter dH_2O (pH 7.0).

2. Solution B:

   Dissolve 142.0 g Na_2HPO_4 in 1 liter dH_2O (pH 7.0).

3. Mix 423 ml Solution A with 577 ml Solution B.
4. Adjust the desired pH 7.0 using HCl or NaOH.
5. Micro filter and store at room temperature.

Glycine-HCl Buffer (0.1 M, pH 3.0) preparation

1. Prepare 800 mL of distilled water in a suitable container.
2. Add 7.5 g of **Glycine** to the solution.
3. Add 832 mg of **Hydrochloric acid** to the solution.
4. Adjust solution to final desired pH using **HCl** or NaOH.
5. Add distilled water until volume is 1 L.

5.2.3 Sample load, column wash and elution

Washing is an essential step to ensure the removal of all unbound and non-specific proteins from the column. Before sample loading, column was washed with 10 mL of 20 mM sodium phosphate buffer (pH 7.0) to remove the unwanted substances and equilibrated the column. Ranibizumab/Trastuzumab-Fab (1 mg) with 50 mL binding buffer (20mM phosphate, pH 7.0) was loaded into the column at a flow rate of 1 mL/min using Profinia Purification System (Bio-Rad). After completion of sample loading through the column, wash was done by 2 × 10 mL of 20 mM phosphate buffer (pH 7.0) for elution of specific antibodies. Non-specific interactions may be due to the interaction of the proteins in a crude sample with the matrix or ligand. After washing the column, binding Fab antibodies were eluted with 100 mM Glycine-HCl, (pH 3.0). The eluate was immediately neutralized with one-tenth volume of neutralizing buffer (1M Tris-HCl, pH 8.0) and the absorbance at 280 nm was measured to determine the yield of recovered antibody.

5.2.4 Column regeneration

When scaling up chromatography systems the possibility of repeated use of the resins must be considered, thereby reducing the cost. Also, if the product is aimed for therapeutic purposes as injected pharmaceuticals the avoidance of contamination is of extreme importance. Attention regarding germ inactivation, pyrogen removal, and virus clearance must be taken. To achieve this, a decontamination step more commonly known as a cleaning-in-place (CIP) step is included in the chromatography protocol.

0.1 M of NaOH was used for cleaning the column resins for 15 minutes and 30 minutes to removal of tightly bound, precipitated, or denatured proteins, as well as lipids. Consequently, column was washed with 10 mL of 20 mM sodium phosphate buffer and load the Fab antibody drugs to determine the binding abilities after alkaline pH regeneration of affinity column.

5. 3 Results and discussion

To check the usability of VHHs as affinity ligand for the column chromatography, we prepared VHH-conjugated column using 5 mg of VHH affinity ligand according to methods and materials.

We first tested the group B and C VHH acidic elution profile on Profinia Purification System (BioRad). 1 mg of Ranibizumab/ or Trastuzumab-Fab with 50 mL loading buffer was loaded through VHH-conjugated column. Subsequently, the column was washed with loading buffer and eluted the Fabs from the column with 100 mM glycine-HCl at pH 3.0. The system generated chromatograph indicated that the acidic elution of group C VHH-conjugated column was broaden (**Fig. 28B**) and group B VHH-conjugated column was suitable for the acidic elution indicating easy elution with a sharp peak (**Fig. 28A**). However, this group B VHH column could not be used as affinity ligand due to the lack of alkaline pH resistance (**Fig. 31**).

Furthermore, we checked the column binding ability of NGS identified original LH3-VHH to Fabs. Similarly, 1 mg of whole bodies (Bevacizumab and Trastuzumab) harboring Fabs (Ranibizumab, and Trastuzumab-Fab) Ranibizumab or Trastuzumab-Fab dissolved in 50 mL loading buffer was injected into the LH3 VHH-conjugated affinity column and the elution was step-wisely done with 100 mM glycine-HCl at pH 3.0. After elution of Fabs, we estimated the amount of eluted Fabs by optical density at 280 nm. The recovered amount indicated the column absorption ability was 80% for Ranibizumab and 75% for Trastuzumab. Also, we analyzed SDS-PAGE with the excluded and eluted fractions. The results indicated that the flow through fractions contained Fabs in SDS-PAGE gel (**Fig. 29 and Table 10**). So, we confirmed that NGS-identified LH3 VHH cannot be as an affinity ligand for weak absorption into column due to low affinity.

Finally, we checked triple mutant VHH-conjugated column to purify Fabs by acidic elution at pH 3.0 in a similar way as mentioned above. In this case, the absorption of whole body harboring

Fab was almost complete (100%) without no exclusions, indicating the suitable profile as an affinity ligand (**Fig. 30 and Table 11**).

Subsequently, the resistance of this triple mutant against alkaline pH was checked. The column was exposed to 0.1 M sodium hydroxide solution for 15 and 30 min at room temperature. After this regeneration process, the binding capacities of the alkaline-treated column were examined. The changes of the capacity to absorb Fabs are indicated (**Fig. 31 and Table 12**). The engineered triple mutant VHH maintained its capacity up to 87% even after 30 min treatment, but group B VHH (containing Cys residue on CDR3) rapidly lost its binding capacity to around 50%, demonstrating the advantages of triple mutant VHH against alkaline pH.

At the end of the work, we checked the immunoreactivity of Fabs after acidic elution at pH 3.0 by protein ELISA (**Fig. 32**), it retained more than 96% activity.

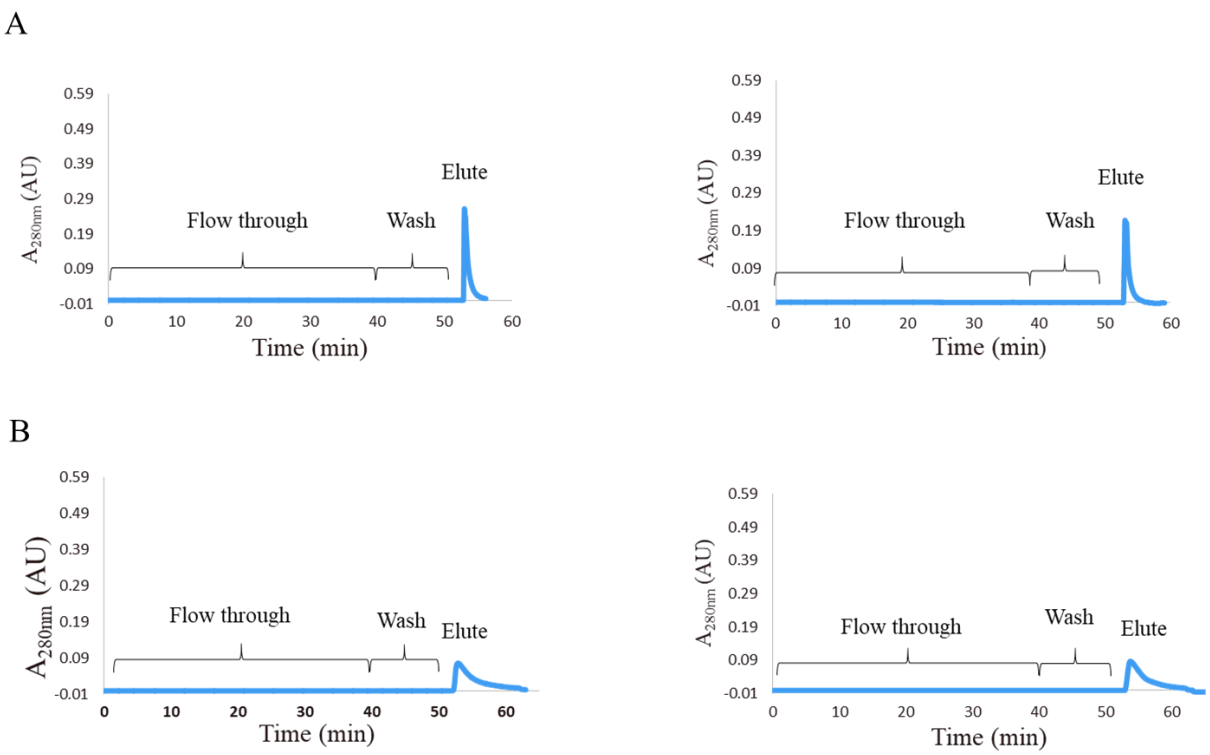
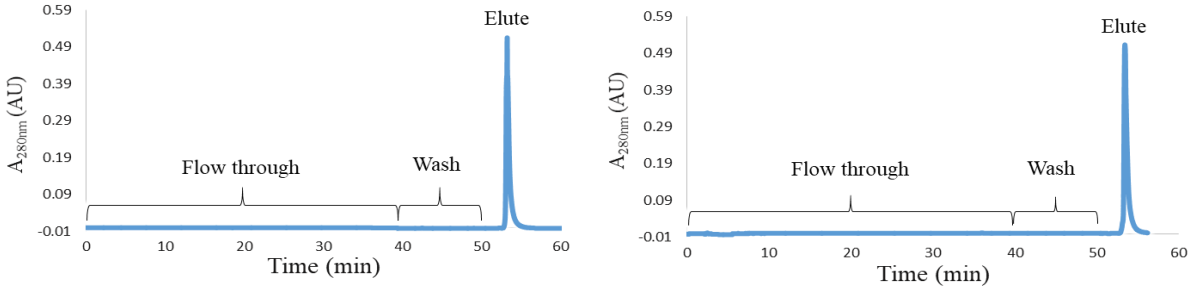


Figure 28. Elution profiles of Fabs (Bevacizumab: left panels, and Trastuzumab: right panels) purification on affinity columns conjugated with group B VHH- (A) and group C VHH- (B).

Table 10. Recovery of Ranibizumab and Trastuzumab-Fab using LH3 VHH-conjugated column

Target	Loading amount (mg)	Recovery (%)
Bevacizumab	1mg	80%
Trastuzumab	1mg	75%

A



B

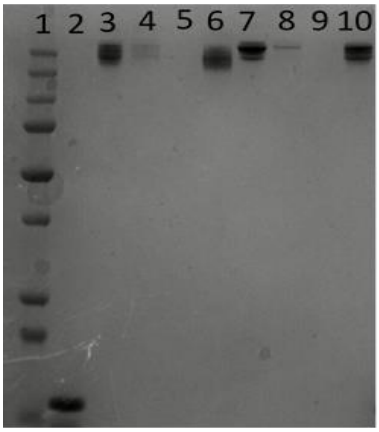
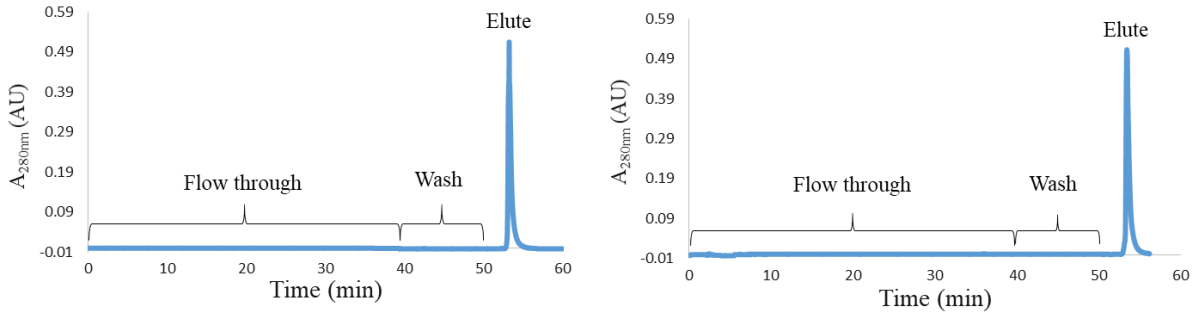


Figure 29. Evaluation of LH3 VHH-conjugated column. (A) Elution profiles of whole bodies (Bevacizumab: left panels and Trastuzumab: right panels) harboring Fabs (Ranibizumab, and Trastuzumab-Fab) purification on affinity columns conjugated with LH3 VHH. (B) SDS-PAGE electrophoresis with excluded and eluted fractions of purification system. Lane 1 is marker with kDa band. Lane 2 LH3 VHH. Lane 3 and 7 before loading Bevacizumab and Trastuzumab respectively. Lane 4 and 8 flow-through of Bevacizumab and Trastuzumab respectively. Lane 5 and 9 washing fraction for both and lane 6 and 10 elution fractions of Bevacizumab and Trastuzumab respectively.

Table 11. Recovery of Ranibizumab and Trastuzumab-Fab using LH3-30N-Q39E-H56Y VHH-conjugated column

Target	Loading amount (mg)	Recovery (%)
Bevacizumab	1mg	98.6
Trastuzumab	1mg	99.6

A



B

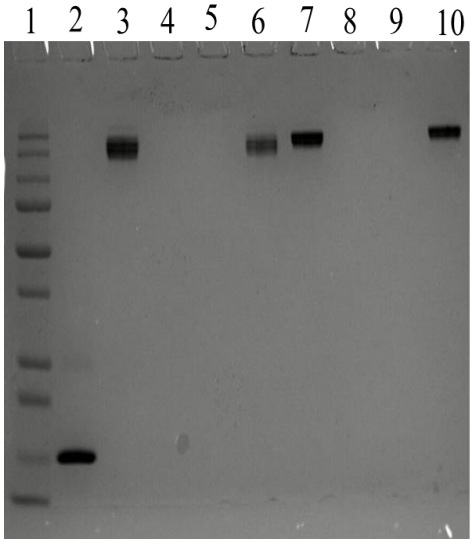


Figure 30. Evaluation of triple mutant VHH-conjugated column. (A) Elution profiles of whole bodies (Bevacizumab: left panels and Trastuzumab: right panels) harboring Fabs (Ranibizumab, and Trastuzumab-Fab) purification on affinity columns conjugated with LH3 VHH. (B) SDS-PAGE electrophoresis with excluded and eluted fractions of triple mutant VHH-conjugated column purification. Lane 1 is marker with kDa. Lane 2 VHH. Lane 3 and 7 before loading Bevacizumab and Trastuzumab, Lane 4 and 8 flow-through of Bevacizumab and Trastuzumab respectively. Lane 5 and 9 washing fraction for both and lane 6 and 10 elution fractions of Bevacizumab and Trastuzumab respectively

Table 12. Summary of alkaline regeneration combined mutation immobilized column

Fab drugs	Recovery (%) before NaOH wash.	Recovery (%) after 15 mins NaOH wash	Recovery (%) after 30 mins NaOH wash
Bevacizumab	98.6	90.2	86.77
Trastuzumab	99.6	93.40	87.20

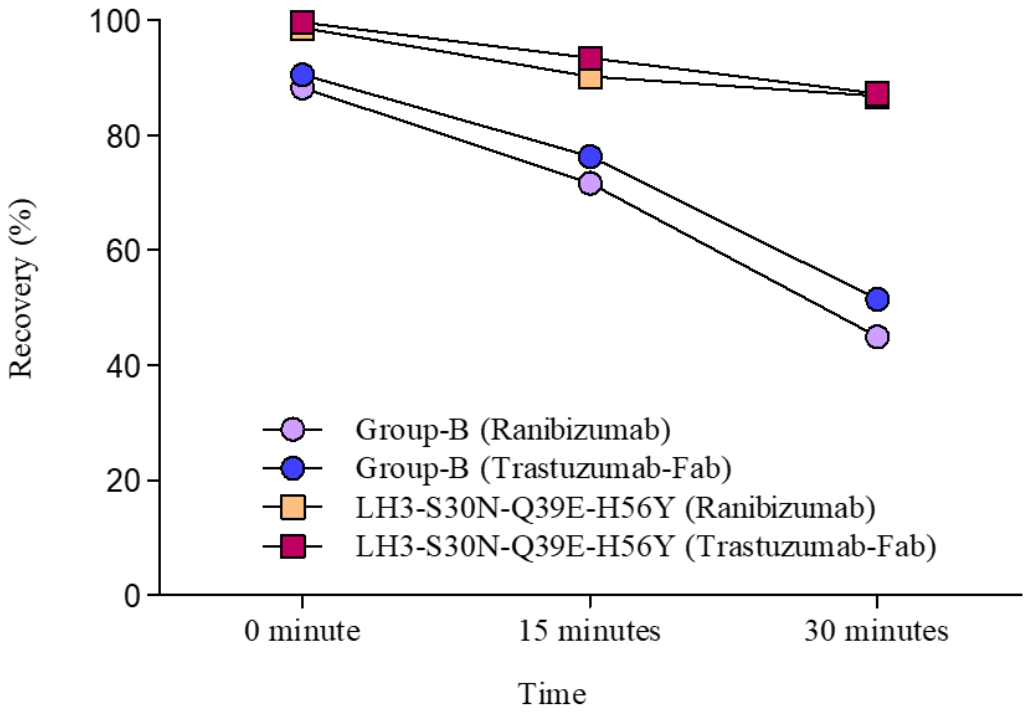


Figure 31. The changes of binding capacity of VHH-conjugated affinity column after repeated alkaline treatment. The VHH-conjugated column was treated with 0.1 M NaOH solution for 15 mins and 30 mins, respectively. Recovery was estimated by the relative amount (%) of yields of the Fab absorbed to the column compared to initial binding yields of Fab.

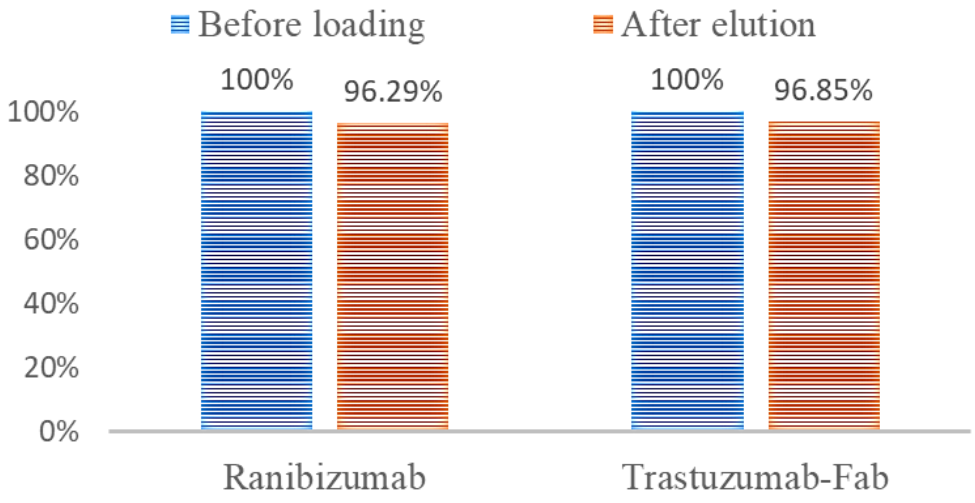


Figure 32. Evaluation of Ranibizumab and Trastuzumab-Fab binding efficiency after elution with pH 3.0 through LH3-S30N-Q39E-H56Y VHH-conjugated column.

5.4 Summary

We considered that three properties of VHH were required as an affinity ligand. The first is strong affinity less than several tens nM at least. The second is acidic elution profile, which would be induced from rapid decrease of affinity under low pH as compared with high affinity at neutral pH. Although we did not actually examine the affinities of VHHs under pH3.0, it was considered that the clone of group C (Sb_5) did not drop the affinity at pH 3.0 as compared with LH_4 in B group (**Fig. 28**) or triple mutant LH_3 (**Fig. 30**), which leads to difficulty of Fab elution from the column. The third one is the stability to alkaline pH. The regeneration by alkaline solution is generally used in protein-A column for the IgG purification. Although it cannot be concluded that the stability of LH_3 triple mutant finally designed is sufficient for use, it was demonstrated that the advantage of our clone prior to the clone containing Cys residue on CDR3 (**Fig.31**).

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Chapter 6

Summary

6.1 Discussion and conclusion

In the field of monoclonal antibody therapeutics, the use of Fab antibody drugs is getting popularity in recent days due to several advantages compared with full-length immunoglobulin including smaller in size, higher tissue penetration, lower side effects, easy to protein engineering and overall low production cost. However, the use of Fab antibody drugs is not still available due to that Fab purification system has not well established yet.

In this study, we approached to design a high affinity VHH specific to Fab, using VHH phage display library of immunized Alpaca through biopanning followed by high throughput sequencing analysis on next generation sequencer (NGS). Finally designed VHH was found to function well as an affinity ligand for the purification system for Fab, showing the characters of the high affinity and alkaline resistance.

In chapter 1, described the general introduction about the conventional antibody and the variable domain of heavy chain antibody (VHH), and also their application in research and therapeutics. Furthermore, the advantages in the use of next generation sequencing (NGS) to search desirable VHH antibody beyond the efficiency of the conventional screening were described.

In chapter 2, described the construction of VHH phage library from Fab-immunized Alpaca and the isolation of Fab-specific VHH through biopanning followed by the conventional screening.

In chapter 3, the high throughput sequencing (HTS) technology on NGS was employed to find the desirable VHHs that was proven very powerful in identifying the specific clones from the huge members of VHHs enriched by biopanning. Several useful candidates identified from the analysis of NGS data showed the suitable binding specificity but low affinity.

In chapter 4, the affinity maturation of VHH obtained in Chapter 3 was done using error-prone PCR library technique to enhance the binding affinity of VHH clone. Finally, we succeeded in the design of triple mutant VHH with ten folds higher binding affinity towards Fabs as compared with the parent VHH.

In chapter 5, we prepared VHH-conjugated affinity column and evaluated its usefulness in Fab purification. Finally designed VHH-conjugated column made it possible to bind two kinds of Fab completely and to elute them, and also showed the high resistance against alkaline pH used in regeneration of the column, indicating the VHH affinity ligands suitable for the purification of Fab was successfully designed.

In chapter 6, the results of this study were summarized, and the usefulness of our purification system was discussed from a viewpoint of industrial applications, comparing with other methods.

In conclusion, we successfully fabricated VHH as suitable affinity ligand from the immunized phage library using HTS followed by random mutagenesis. We were able to show that application of HTS on pooled phage libraries before/after biopanning is an effective and suitable identification technique of the target sequence in phage library. The combination of single-point mutation might be a guideline for affinity maturation without using computational predictions. Considering the

center of attention, our refined VHH is expected to be a highly functional affinity ligand to establish a cost-effective, timesaving, and convenient human Fab antibody purification system with high yield and purity.

List of publications

Published papers:

1. Rafique A, Satake K, Kishimoto S, Khan KH, Kato DI and Ito Y: Efficient screening and Design of Variable Domain of Heavy Chain Antibody Ligands Through High Throughput Sequencing for Affinity Chromatography to Purify Fab Fragments. *Monoclonal Antibodies in Immunodiagnosis and Immunotherapy* 2019; 38 (5): 190-200.
2. Khan KH, Himeno A, Kosugi S, Nakashima Y, Rafique A, Imamura A, Hatanaka T, Kato DI and Ito Y: IgY-binding peptide screened from a random peptide library as a ligand for IgY purification. *Journal of Peptide Science* 2017; 23 (10): 790-797.

Poster and oral presentation:

Poster presentation: Isolation and characterization of Fab-specific Alpaca VHH antibodies with enhanced affinity maturation by functional mutations. The 18th Annual Meeting of the Protein science Society of Japan (Niigata, Japan), **2018.06.26-28**

Oral presentation: Efficient screening and design of VHH ligands through high throughput sequencing for affinity chromatography to purify Fab fragments. The 26th Annual Meeting of the Japanese Society for Biotechnology. Kyushu Branch, Nagasaki, **2019.12.07**

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Md. Abdur Rafique