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(54) **Title**: VECTOR FOR SCREENING ANTIBODY

(57) **Abstract**: It is an object of the present invention to provide a simple and efficient method for evaluating the VH/VL interaction without expressing/purifying VH and VL, with a purpose of selecting VH and VL, the VH/VL interaction of which is weak in the absence of an antigen but the association constant of which is greatly changed in the presence of the antigen. The prsent invention provides a vector wherein, when the vector is introduced into a host cell, it is capable of secreting: a protein comprising either one of VH fragment or VL fragment of the antibody variable domain from the host cell; and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain from the host cell.

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DESCRIPTION

VECTOR FOR SCREENING ANTIBODY

Technical Field

The present invention relates to a vector for screening antibodies suitable for open sandwich immunoassay, a method for producing the vector, and a method for screening antibodies suitable for open sandwich immunoassay using the vector.

Background Art

Open sandwich assay is a method in which: a VH region polypeptide and a VL region polypeptide of an antigen-specific antibody are prepared; either one of these polypeptides is labeled with a reporter molecule to make a labeled polypeptide, and the other polypeptide is immobilized onto a solid phase to make an immobilized polypeptide; and an antigen-containing sample and the labeled polypeptide are contacted with the solid phase, followed by quantification of the reporter molecule of the labeled polypeptide that has been bound to the immobilized polypeptide. The open sandwich assay is an immunoassay based on a phenomenon that the association constant between VH and VL is increased under the presence of an antigen. Therefore, the indispensable condition is that the VH/VL interaction is weak without an antigen but the association constant is greatly changed in the presence of the antigen.

Hitherto, reported methods for evaluating the VH/VL interaction are as follows: a method comprising causing VH expression and VL expression in a separate manner, purifying each protein, and analyzing the VH/VL interaction by ELISA or gel filtration (Non-Patent Document 1); a method comprising presuming the VH/VL interaction based on CD spectra of scFv (Non-Patent Document 2); and methods comprising causing scFv expression followed by purification and presuming the VH/VL interaction based on thermostability, denaturant resistance, antigen binding kinetics, and the expression level of scFv (Non-Patent Documents 3-6). However, in such methods, proteins must be separately expressed and purified. Therefore, these methods are labor- and time-consuming.

A method called "split-Fv system" has been reported as a method to evaluate the VH/VL interaction without expressing/purifying VH and VL (Patent Document 1). In this method, separate use of *E. coli* between those with and without an amber suppressor function against an amber (stop) codon in an expression vector, makes it possible to separately employ: a method in which VH and VL are respectively expressed as fusion proteins respectively tethered to phage coat proteins pVII and pIX; and a method in which either one of VH and VL is expressed as a fusion protein tethered to a phage coat protein pVII or pIX, and the other one of VH and VL is subjected to secretive expression. This method is capable of evaluating the affinity of the VH/VL complex for an antigen and the VH/VL interaction without the antigen, by changing the type of *E. coli* with a same vector. However, this method has problems in that the use of two phage coat proteins leads to instability of the phage and consequent failure in the stable expression of VH/VL on the phage, and that the distance between VH/VL expressed as fusion proteins respectively tethered to coat proteins pVII and pIX is not enough for their interaction so that the affinity for the antigen is lowered.

Non-Patent Document 1: Y. Chen et al, The Journal of Immunology, vol. 163, 4663-4670 (1999)

Non-Patent Document 2: C. Horne et al, The Journal of Immunology, vol. 129, 660-664 (1982)

Non-Patent Document 3: A. Wörn et al, Biochemistry, vol. 37, 13120-13127 (1998)

Non-Patent Document 4: P. H. Tan et al, Biophysical Journal, vol. 75, 1473-1482 (1998)

Non-Patent Document 5: M. B. Khalifa et al, Journal of Molecular Recognition, vol. 13, 127-139 (2000)

Non-Patent Document 6: J. Chatellier et al, Journal of Molecular Biology, vol. 264, 1-6 (1996)

Patent Document 1: International Publication WO2004 / 016782

Disclosure of the Invention

It is an object of the present invention to provide a simple and efficient method for evaluating the VH/VL interaction without expressing/purifying VH and VL, with a purpose of selecting VH and VL, the VH/VL interaction of which is weak in the absence of an antigen but

the association constant of which is greatly changed in the presence of the antigen.

The inventors of the present invention have conducted intensive studies to solve the above problems. As a result, they have found that the VH/VL interaction can be simply and efficiently evaluated without expressing/purifying VH and VL by establishing a vector that is characterized in that, when it is introduced into a host cell, it is capable of secreting: a protein comprising either one of VH fragment or VL fragment of the antibody variable domain from the host cell; and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain from the host cell. This has led to the completion of the present invention.

The present invention provides a vector which comprises:

a DNA region comprising: (1): a DNA sequence that causes extracellular secretion of peptides encoded by the following DNA sequences (2) and (3); (2): a DNA sequence encoding either one of VH fragment or VL fragment of antibody variable domain; and (3): a DNA sequence encoding a tagged protein; and

a DNA region comprising: (4): a DNA sequence encoding a protein for displaying a peptide encoded by the following DNA sequence (5) on a phage; (5): a DNA sequence encoding the other one of the VH fragment or VL fragment of the antibody variable domain; and (6): a DNA sequence encoding a phage coat protein,

wherein, when the vector is introduced into a host cell, it is capable of secreting: a protein comprising either one of VH fragment or VL fragment of the antibody variable domain from the host cell; and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain from the host cell.

Preferably, the vector is an *E. coli* phage vector or phagemid vector.

Preferably, the DNA sequence in (1) is a DNA sequence encoding a ribosomal binding site and a gIII signal sequence.

Preferably, the DNA sequence in (2) is a DNA sequence encoding a VL fragment of the antibody variable domain and the DNA sequence in (5) is a DNA sequence encoding a VH fragment of the antibody variable domain.

Preferably, the DNA sequence in (3) is a DNA sequence encoding a maltose binding protein.

Preferably, the DNA sequence in (4) is a DNA sequence encoding a ribosomal binding site and an OmpA signal sequence.

Preferably, the DNA sequence in (6) is a DNA sequence encoding a gIII protein.

The present invention further provides a method for producing a vector whererein, when the vector is introduced into a host cell, it is capable of secreting a protein comprising either one of VH fragment or VL fragment of the antibody variable domain from the host cell; and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain from the host cell, the method comprising allowing a recombinase to act on a first vector (A) and a second vector (B) described below so as to cause gene recombination between the first vector and the second vector: (A): a first vector comprising a DNA sequence capable of causing expression of a polypeptide comprising a VH fragment and a VL fragment of the antibody variable domain, by means of extracellular secretion or in a form of a fusion protein tethered to a phage coat protein, and a pair of recombinase recognition sequences that are inserted between a DNA sequence encoding a VH fragment and a DNA sequence encoding a VL fragment; and (B): a second vector comprising a pair of recombinase recognition sequences, and a stop codon inserted between the pair of recombinase recognition sequences.

Preferably, the first vector contains a sequence that can cause secretion expression of a single-chain variable region (scFv) polypeptide.

Preferably, the first vector contains a sequence that can express a single-chain variable region (scFv) in a form of a fusion protein tethered to a phage coat protein.

Preferably, the first vector contains a sequence that can cause secretion expression of a Fab polypeptide.

Preferably, the first vector contains a sequence that can express Fab in a form of a fusion protein tethered to a phage coat protein.

Preferably, the first vector contains a sequence that can cause secretion expression of an F(ab')2 polypeptide.

Preferably, the first vector contains a sequence that can cause secretion expression of an IgG polypeptide.

Preferably, the first vector contains the following (1) to (4) in the order of (1)-(2)-(2)-(3)-(4) or (3)-(2)-(2)-(1)-(4): (1): a VL polypeptide sequence; (2): a recombination site sequence; (3): a VH polypeptide sequence; and (4): a phage coat protein sequence.

Preferably, the second vector contains a DNA sequence encoding a tagged protein.

Preferably, the recombinase is Cre recombinase.

Preferably, recombination takes place between loxP sites.

The present invention further provides a method for evaluating the interaction between VH polypeptide and VL polypeptide, comprising the steps of:

(i): introducing the vector according to any one of claims 1 to 7 or the vector produced by the method according to any one of claims 8 to 18 into a host cell;

(ii): collecting a protein comprising either one of VH fragment or VL fragment of the antibody variable domain which has been secreted from the host cell, and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain; and

(iii) detecting a complex of the VH fragment, the VL fragment, and an antigen by allowing the antigen to contact with the protein comprising either one of VH fragment or VL fragment of antibody variable domain collected in (ii), and phage displaying the other one of the VH fragment or VL fragment of antibody variable domain collected in (ii).

Preferably, the antibody variable domain in which the interaction between the VH fragment and the VL fragment is changed under the presence of the antigen, is selected.

Preferably, a VH polypeptide and a VL polypeptide having a weak interaction are selected from an scFv mixture.

Preferably, an scFv mixture having a high affinity to a target antigen is selected from among scFv mixtures, and then a VH polypeptide and a VL polypeptide having a weak interaction are selected from a mixture of scFv.

Preferably, the complex of the VH fragment, the VL fragment, and an antigen is detected by immunoassay with the use of a labeled anti-phage antibody.

Open sandwich assay is a type of immunoassay based on a phenomenon that the

association constant between VH and VL is increased under the presence of an antigen. For instance, enzyme-labeled VH and an antigen are added to a plate on which a VL chain is immobilized such that a three-element complex comprising VL/VH/antigen is formed in an antigen concentration-dependent manner, which enables ELISA. In order to establish an assay system for open sandwich assay, it is necessary to use an antibody in which the VH/VL interaction varies significantly depending on the presence or absence of an antigen. Hitherto, evaluation of the interaction between VH and VL fragments in the antibody variable domain requires isolation of the VH and VL genes from an antibody-producing cell, incorporation of the genes into separate expression vectors, induction of the expression of VH and VL proteins, and purification. This has been very labor- and time-consuming (requiring several months). In addition, a method called the split Fv method has been suggested. However, in this method, the use of two phage coat proteins causes a phage to be in an unstable state. Accordingly, stable expression of VH/VL is not induced on such a phage. In addition, the VH/VL distance in proteins expressed as a fusion protein of coat proteins VII and pIX is not sufficient for VH/VL interaction, resulting in a decrease in affinity to an antigen, which is problematic. Meanwhile, according to the present invention, it has become possible to simply convert an scFv display phage into a coexpression system of VL-MBP and a VH display phage while avoiding the problem of decrease in expression level on a phage. Accordingly, rapid evaluation of VH/VL interaction is realized. According to the method of the present invention, antibodies further suitable for use in open sandwich ELISA can be selected according to the purpose.

Best Mode for Carrying Out the Invention

Hereafter, the present invention will be more specifically described.

The vector of the present invention comprises:

a DNA region comprising: (1): a DNA sequence that causes extracellular secretion of peptides encoded by the following DNA sequences (2) and (3); (2): a DNA sequence encoding either one of VH fragment or VL fragment of the antibody variable domain; and (3): a DNA sequence encoding a tagged protein; and

a DNA region comprising: (4): a DNA sequence encoding a protein for displaying a peptide encoded by the following DNA sequence (5) on a phage; (5): a DNA sequence encoding the other one of the VH fragment or VL fragment of the antibody variable domain; and (6): a DNA sequence encoding a phage coat protein. The vector is characterized in that, when it is introduced into a host cell, it is capable of secreting: a protein comprising either one of VH fragment or VL fragment of the antibody variable domain from the host cell; and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain from the host cell.

The above DNA sequence (1) is not particularly limited as long as it is a DNA sequence that causes extracellular secretion of a peptide. However, examples thereof include DNA sequences encoding "a ribosomal binding site and a gIII signal sequence", "a ribosomal binding site and an OmpA signal sequence", "a ribosomal binding site and a pelB signal sequence", or the like.

The tagged protein in (3) above is preferably a protein that can be secreted and produced in *E. coli* and contributes to protein stabilization and that has an affinity to a certain substance. Examples thereof include a maltose-binding protein, calmodulin, and an antibody light chain constant region (CL).

The DNA sequence in (4) above is not particularly limited as long as it is a DNA sequence encoding a protein for displaying a peptide on a phage. Examples thereof include DNA sequences encoding "a ribosomal binding site and an OmpA signal sequence", "a ribosomal binding site and a pelB signal sequence", "a ribosomal binding site and a gIII signal sequence", and the like.

The phage coat protein in (6) above is not particularly limited. However, examples thereof include a gIII protein, a gIII protein C-terminal domain (D3), and a gIX protein.

Specifically, the aforementioned vector of the present invention can be constructed by allowing a recombinase to act on a first vector (A) and a second vector (B) described below so as to cause gene recombination between the first vector and the second vector: (A): a first vector comprising a DNA sequence capable of causing expression of a polypeptide comprising a VH fragment and a VL fragment of the antibody variable domain, by means of extracellular

secretion, or in a form of a fusion protein tethered to a phage coat protein, and a pair of recombinase recognition sequences that are inserted between a DNA sequence encoding a VH fragment and a DNA sequence encoding a VL fragment; and (B): a second vector comprising a pair of recombinase recognition sequences and a stop codon inserted between the pair of recombinase recognition sequences.

Examples of a sequence that can be contained in a first vector include, but are not limited to, a sequence that can cause secretion expression of a single-chain variable region (scFv) peptide, a sequence that can express a single-chain variable region (scFv) in a form of a fusion protein tethered to a phage coat protein, a sequence that can cause secretion expression of a Fab peptide, a sequence that can express Fab in a form of a fusion protein tethered to a phage coat protein, a sequence that can cause secretion expression of an F(ab')2 peptide, and a sequence that can cause secretion expression of an IgG peptide. In terms of the configuration, the first vector may contain the following (1) to (4) in the order of (1)-(2)-(2)-(3)-(4) or (3)-(2)-(2)-(1)-(4): (1): a VL peptide sequence; (2): a recombination site sequence; (3): a VH peptide sequence; and (4): a phage coat protein sequence.

In the present invention, a phagemid vector is preferably used. Since a phagemid vector is a plasmid produced to include a part of filamentous phage genome, the phagemid vector has to be transformed into *E. coli*, and further infected with a helper phage. By so doing, coat proteins for particle formation are supplied, by which phages are provided in a form of a mixture of helper phage particles and phagemid particles. In addition, as a simpler method, a phage vector including necessary DNA sequences can also be used. In the case of a phage vector, phages can be directly provided through infection of the phage vector into *E. coli*, and there is no need of using a helper phage.

In the present invention, the interaction between VH polypeptide and VL polypeptide can be evaluated by the steps of : introducing the above vector of the present invention into a host cell; collecting a protein comprising either one of VH fragment or VL fragment of the antibody variable domain, which has been secreted from the host cell, and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain; and detecting a complex of the VH fragment, the VL fragment, and an antigen by allowing the

antigen to contact with the collected protein comprising either one of VH fragment or VL fragment of antibody variable domain, and phage displaying the other one of the VH fragment or VL fragment of antibody variable domain. The detection of a complex of the VH fragment, the VL fragment, and the antigen is performed by open sandwich immunoassay that will be described below.

Protein antigens are generally assayed by a method called sandwich assay with use of two types of antibodies. Sandwich assay has several merits such as higher specificity and sensitivity, although two types of antibodies which can simultaneously bind to an antigen need to be prepared. However, small molecules having a molecular weight of 1000 or less are too small to be sandwiched by two antibodies. That is to say, a small molecule having a molecular weight of 1000 or less is a monovalent antigen having one antigen determinant, and thus is difficult to sandwich by two antibodies. Such small molecules are usually assayed by a method called competitive assay. However, the competitive assay has demerits such as difficulty in the condition setting, lower sensitivity, and requirements for considerable care and attention in the assay manipulations.

As a method which enables noncompetitive assay of small molecules without such demerits, the inventors of the present invention have reported an immunoassay approach called the open sandwich immunoassay. This assay is based on a principle that "an antibody variable domain (antigen binding site) is unstable without an antigen, but is stabilized once an antigen is bound thereto". An antibody is composed of two chains, an H chain and an L chain. Respective antigen binding sites thereof are called VH and VL, which constitute a minimum antigen recognition unit, namely the variable domain Fv. Recently, cloning of gene fragments encoding VH and VL can be easily performed with use of phage display method, and the like. However, the binding between VH and VL is noncovalent and is often unstable. In many cases, VH and VL are linked by a peptide to be used as a single-chain antibody (scFv).

The inventors of the present invention have found that some of such unstable Fv can be stabilized when an antigen is bound thereto, and the use of this phenomenon had realized simple, quick, and highly sensitive quantification of the antigen concentration. That is to say, they have found that quantification of phage or enzyme immobilized on a VL

fragment-immobilized plate, after being contacted with a sample containing a phage- or alkaline phosphatase-conjugated VH fragment and an antigen, and subsequently washed once, showed a high correlation with the amount of the antigen (UEDA, H. *et al.* Nature Biotechnol. 14, 1714-1718 (1996)).

Further, the inventors of the present invention have developed a method for simple examination of available antibodies regarding the suitability for open sandwich assay (Aburatani, T. *et al.*, Anal. Chem. 75; Hiroshi Ueda, "A novel immunoassay capable of noncompetitive detection of small molecules", Bio Medical Quick Review Nets No. 027 (2004); and Hiroshi Ueda, "Noncompetitive immunoassay of small molecules", Seikagaku (Biochemistry), 76(7), 670-674 (2004))". By the use of this method (split-Fv system), which is similar to commercially available phage antibody system, both the antigen binding ability and the strength of VH/VL interaction of the antibody variable domain of available hybridomas can be conveniently examined by changing the phage-producing *E. coli*, and more suitable antibodies can be selected.

In the present invention, the interaction between VH polypeptide and VL polypeptide can be evaluated by detecting a complex of a VH fragment, a VL fragment, and an antigen, after contacting: a protein comprising either one of VH fragment or VL fragment of antibody variable domain; and a phage displaying the other one of the VH fragment or VL fragment of antibody variable domain; with an antigen. Accordingly, a clone in which the VH/VL interaction largely varies in an antigen-dependent manner can be rapidly screened for. In a case of an antibody fragment in which the VH/VL interaction is weak, the VL fragment (or VH fragment) immobilized on the carrier and the VH fragment (or VL fragment) displayed on the phage are rarely bound directly to each other, and therefore the phage is hardly bound to the carrier. However, in some cases, the VH fragment and the VL fragment of an antibody are both bound to the antigen in the presence of an antigen, and the complex is stabilized, so that the phage can be bound to the carrier via the antigen. Accordingly, quantification of the carrier-tethered phage with use of, for example, an anti-phage antibody enables selection of antibody fragments, the phage-binding amount of which largely varies depending on the presence of the antigen. In a case of such antibody fragment, it is thought that the VH/VL

interaction largely varies when the antigen binds to the fragment. Therefore, such fragment is preferably used for open sandwich ELISA. If the interaction between the VH fragment and the VL fragment of the antibody variable domain is changed double or more under the presence of the antigen, such antibody fragments can be used for the purpose of the present invention.

It is possible to produce, for example, an assay kit as follows, with use of the antibody provided by the method of the present invention, the VH/VL interaction of which is weak in the absence of an antigen but the VH/VL interaction of which is strengthened in the absence of the antigen.

(1) The VL fragment is immobilized onto a tube or a microplate through biotin-avidin interaction or physical adsorption.

(2) A fusion protein of the VH fragment and a reporter enzyme (such as alkaline phosphatase) is produced and is contacted with the VL-immobilized solid phase together with a sample, for a fixed period of time.

(3) After washing, the activity of the immobilized enzyme is measured and is used as an indicator of the antigen concentration in the sample.

In addition, it is also possible to produce an assay kit as follows.

(1) The VH fragment and the VL fragment are labeled with two types of fluorescent dyes having mutually overlapping absorption/fluorescent spectrum (such as fluorescein and rhodamine).

(2) These are mixed with a sample, and left still for about 5 minutes, followed by exclusive excitation of the fluorescent dye having the shorter wavelength with exciting light. The measurement of fluorescence intensities derived from these two types of fluorescent dyes enables detection of fluorescence resonance energy transfer caused by the VH/VL association. The ratio between two fluorescence intensities is used as an indicator of the antigen concentration in the sample. This method enables measurement of the antigen concentration in a shorter time without washing operation, as compared to the former method.

Further, it is also possible to produce an assay kit as follows.

(1) The VH fragment and the VL fragment are expressed as fusion proteins with two types of enzyme fragments, each of which is not active per se or shows low activity, but the

closely contacted pair of which shows an increased activity (such as LacZΔα and LacZΔω), in *E. coli*, followed by purification.

(2) These two types of fusion proteins and a sample is mixed, and left still for a fixed period of time. Then, a substrate (such as luminescent substrate Galacton Plus) is mixed therein. The activity of the fusion protein complex is measured and is used as an indicator of the antigen concentration in the sample. This method enables measurement of the antigen concentration with much higher sensitivity without washing operation, as compared to the former two methods (Yokozeki *et al.*, Anal. Chem. 74(11), 2500-2504, 2002).

The target of assay of the above method can include, firstly, specific proteins, peptides, various hormones, narcotic drugs, and therapeutic drugs in serum for clinical examinations. In addition, the target of assay of the present invention can also include dioxin, bisphenol A, nonyl phenol, and other presumably toxic chemical substances and agrochemicals in environmental water.

The present invention will be more specifically described in the following Examples. However, these Examples are not intended to limit the scope of the present invention.

Examples

In each experiment, water purified with milliQ (Millipore) was used. Hereafter, water is referred to as "milliQ water." The general reagents used were those provided by Sigma (St. Louis, MO, USA), nacalai tesque (Kyoto), Wako Pure Chemical Industries, Ltd. (Osaka), and Kanto Chemical Co., Inc. (Tokyo) (unless otherwise specified). Oligo DNA was synthesized by Texas Genomics Japan (Tokyo) or INVITROGEN.

A T3000 thermocycler (Biometra, Goettingen, Germany) was used for polymerase chain reaction (PCR). Also, a CEQ™ 8000 Genetic Analysis System (BECKMAN COULTER, Tokyo) was used for DNA sequencing.

E. coli TG-1 and XL10-Gold were used. The genotypes thereof are as described below.

TG-1: *supE, hsd* Δ5, *thi,* Δ *(lac-proAB)*/F' [*traD*36, *proAB*$^{+}$, *lacI*^{q}, *lacZ* Δ M15]

XL10-Gold: Tet^{r}, Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *rec*A1,

*gyr*A96, *rel*A1, *lac*, The, [F', *pro*AB, *lacI*^{q}ZΔM15, Tn10(Tet^{r}), Tn5(Kan^{r}), Amy]

YT, 2YT, and LB media, each containing an appropriate antibiotic, were used for culture of *E. coli*. The compositions of the media are as described below.

YT medium: bacto trypton (8 g); bacto yeast extract (5 g); and NaCl (5 g) (per 1 L)

2YT medium: bacto trypton (16 g); bacto yeast extract (10 g); and NaCl (5 g) (per 1 L)

LB medium: bacto trypton (10 g); bacto yeast extract (5 g); and NaCl (10 g) (per 1 L)

SOC medium: bacto trypton (20 g); bacto yeast extract (5 g); NaCl (0.5 g); 5N NaOH (0.2 ml); 1 M Glucose (20 ml); 1 M $MgCl_2$ (10 ml); and 1 M $MgSO_4$ (10 ml) (per 1 L)

Example 1: production of scFv(HyHEL10)/pMK

For conventional scFv, a $(G_4S)_3$ linker comprising 15 amino acid residues is often used. Meanwhile, scFv/pMK (fig. 1) used in the present invention has two loxP sites for recombination, and thus it has a long length corresponding to 45 amino acid residues and contains many types of amino acids. Therefore, it is necessary to confirm whether or not such loxP linker causes loss of antigen-binding property. Also, it is necessary to confirm that desired recombination between produced scFv/pMK and OS/pMI takes place and to further confirm that OS-ELISA can be performed with the use of the resulting recombinant product. For such confirmation, scFv(HyHEL10)/pMK encoding the V_H and V_L genes of an anti-lysozyme antibody (HyHEL-10) that is appropriate for OS-ELISA was produced with the use of scFv/pMK in the manner described below (fig. 1). In addition, scFv(D13HyHEL)/pMKQC was produced for a control experiment, in which V_H of scFv(HyHEL10)/pMK had been replaced by the V_H gene of an anti-lysozyme antibody (D1.3) that is not appropriate for OS-ELISA (1.5).

(1.1) Production of an loxP-linker

Synthetic oligo DNAs described below were annealed to produce an loxP linker sequence (fig. 2).

Lox-rev:

5'-CACAGTGCACAGGTCCAAGCGGCCGCGataacttcgtatagtatacattatacgaagttatCCGGTG GAGGCAATTTAAATGGCGGT-3' (SEQ ID NO: 1)

(The underlined portions represent the *Apa*LI site, the *Not*I site, and the *Swa*I site in that order from the 5' end, and the set of lower-case characters represents the loxP 511 site.)

lox-for:

5'-CCATGGCCGGCTGGGCCGataacttcgtataatgtatgctatacgaagttatGCTGCCACCGCCATTT AAATTGCCTCCA-3' (SEQ ID NO: 2)

(The underlined portions represent the *Sfi*I site and the *Swa*I site in that order from the 5' end, and the set of lower-case characters represents the loxP WT site.)

Lox-rev and lox-for (100 pmol each), 0.2 mM dNTPs, Ex-Taq Buffer (Takara Bio Inc.) (10 μl), and Ex-Taq (Takara Bio Inc.) (5 units) were mixed to prepare a reaction solution (100 μl). A reaction at 95°C for 5 minutes, a reaction of 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and then a reaction at 72°C for 5 minutes were carried out. The obtained PCR reaction solution was subjected to electrophoresis in a TAE buffer (40 mM Tris, 40 mM CH_3COOH, and 1 mM EDTA) with the use of 1.5% agarose gel containing 1 μg/ml ethidium bromide (EtBr). A band with a desired size was excised and purified with a QIAquick gel extraction kit (QIAGEN) to produce an loxP-linker.

The loxP-linker (2 μg) was mixed with *Apa*LI (New England Biolbs) (1 μl), NEBuffer 2 (New England Biolbs) (5 μl), and 1 mg/ml BSA (5 μl). MilliQ water was added thereto to a volume of 50 μl, followed by reaction at 37°C for 2 hours. Thereafter, *Sfi*I (1 μl) was added thereto and the resultant was left still overnight at 50°C for restriction enzyme treatment. The resultant was again subjected to electrophoresis with 1.5% agarose gel. A band with a desired size was excised and purified with a QIAGEN gel extraction kit.

(1.2) Incorporation of the loxP-linker into a vector

The loxP-linker was inserted between the *Apa*LI/*Sfi*I sites of a pCANTAB3-derived phagemid vector pCGJ (C. G. Jakobsen *et al.*, Molecular Immunology, 41, 941-953, 2004) into which several restriction enzyme sites and the like had been inserted. pCGJ (4 μl) and the loxP-linker (4 μl) subjected to *Apa*LI/*Sfi*I treatment and purification in the same manner as above were mixed with T4 DNA ligase (1 μl) and a T4 DNA ligase buffer (1 μl), followed by

ligation at 16°C for 30 minutes. DNA was collected from the ligation solution by ethanol precipitation and resuspended in milliQ water (5 μl), followed by transformation of *E. coli* TG-1 by electroporation. The transformant was cultured overnight at 37°C in a YT agar medium containing 100 μg/ml ampicillin (Amp) and 1% glucose (Glu). The generated colony was subjected to colony PCR with two different primers M13RV and M13back-115. Accordingly, insertion of the insert fragment was confirmed. The M13RV and M13back-115 sequences are as described below.

M13RV: 5'-CAGGAAACAGCTATGAC-3' (SEQ ID NO: 3)

M13back-115: 5'-TGAATTTTCTGTATGAGGTTTTG-3' (SEQ ID NO: 4)

M13RV and M13back-115 (20 pmol each), 0.2 mM dNTPs, Ex-Taq Buffer (2 μl), and Ex-Taq (1 unit) were mixed to prepare a reaction solution (20 μl). A reaction at 95°C for 5 minutes, a reaction of 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and then a reaction at 72°C for 5 minutes were carried out. *Not*I (1 μl), NEBuffer 3 (1 μl), 1 mg/ml BSA (1 μl), and milliQ water (1 μl) were added to the PCR reaction solution (5 μl), followed by a reaction at 37°C. Only in the case in which a desired insert was amplified, cleavage with *Not*I was confirmed. Electrophoresis was carried out with 1.5% agarose gel containing 1 μg/ml ethidium bromide (EtBr). Then, a clone exhibiting a desired band pattern was inoculated in a YT medium (YTAG) (4 ml) containing 100 μg/ml Amp and 1% Glu. Plasmid DNA was extracted from bacteria obtained by overnight culture at 37°C with a QIAquick miniprep kit (QIAGEN). The DNA sequence thereof was confirmed and it was designated as loxP-linker/pMK.

(1.3) Incorporation of the HyHEL10 gene into loxP-linker/pMK

The V_H and V_L fragments of HyHEL10 were amplified with the use of the following primers.

MVK-BACK12: 5'-CTCCTGTGCACTTGACATTGWGCTSACYCARTCT-3' (SEQ ID NO: 5)

(The underlined portion represents the *Apa*LI site.)

MVL-FOR2: 5'-GATGTGCGGCCGCMCSTWBNABHKYCAVYYTDG-3' (SEQ ID NO: 6)

(The underlined portion represents the *Not*I site.)

VH36-60back1: 5'-GAGGTGCAGGAGTCAGGACCTAGCCTC-3' (SEQ ID NO: 7)

VH36backSfi:

5'-CGCAACTGCGGCCCAGCCGGCCATGGCCGAGGTGCAGGAGTC-3' (SEQ ID NO: 8)

(The underlined portion represents the *Sfi*I site.)

JH-3SgrA1:

5'-ATGACACCGGTGGCCGCTCTCGCTCGAGACAGTGACCAGAGTCCC-3' (SEQ ID NO: 9)

(The underlined portion represents the *Sgr*AI site.)

First, the V_H fragment of HyHEL10 was amplified. VH36-60back1 and JH-3SgrA1 (50 pmol each), HyHEL10/pCANTAB (100 ng) serving as a template, 0.2 mM dNTPs, Ex-Taq Buffer (10 μl), and Ex-Taq (5 units) were mixed to prepare a reaction solution (100 μl). A reaction at 95°C for 5 minutes, a reaction of 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and a reaction at 72°C for 5 minutes were carried out. In order to add the *Sfi*I site to the 5' end of the V_H fragment, PCR reaction was carried out in a similar manner with the use of the obtained PCR reaction solution (1 μl) as a template, VH38backSfi, and JH-3SgrA1. Electrophoresis was carried out with 1.5% agarose gel. A band with a desired size was excised and purified with a QIAquick gel extraction kit to produce V_H (HyHEL10). The obtained DNA fragment (approximately 1 μg) was mixed with *Sgr*AI (New England Biolbs) (1 μl), NEBuffer 4 (New England Biolbs) (5 μl), and 1 mg/ml BSA (5 μl). MmilliQ water was added thereto to a volume of 50 μl, followed by a reaction at 37°C for 2 hours. Then, *Sfi*I (1 μl) was added thereto and the resultant was left still overnight at 50°C for restriction enzyme treatment. The resultant was again subjected to electrophoresis with 1.5% agarose gel. A band with a desired size was excised and purified with a QIAquick gel extraction kit. The obtained insert solution (5 μl) and loxP-linker/pMK (5 μl)

that had been subjected to *Sgr*AI/ *Sfi*I treatment in a similar manner were mixed with ligation high ver2 (Toyobo) (10 μl), followed by a ligation reaction at 16°C for 30 minutes. Ethanol precipitation was carried out for demineralization, followed by transformation of TG-1 by electroporation. The transformant was cultured overnight at 37°C in a YT agar medium containing 100 μg/ml Amp and 1% Glu. The generated colony was subjected to colony PCR with two different primers, M13RV and pHENseq, so as to confirm insertion of an insert fragment. The sequence of pHENseq was as described below.

pHENseq: 5'-CTATGCGGCCCCATTCA-3' (SEQ ID NO: 10)

M13RV and pHENseq (10 pmol each), 0.2 mM dNTPs, Ex-Taq Buffer (1 μl), and Ex-Taq (0.5 unit) were mixed to prepare a reaction solution (10 μl). A reaction at 95°C for 5 minutes, a reaction of 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, and then a reaction at 72°C for 5 minutes were carried out. The obtained PCR reaction solution was subjected to electrophoresis with 1.5% agarose gel containing 1 μg/ml EtBr. A clone exhibiting a desired band pattern was inoculated in a YTAG (4 ml). Plasmid DNA was extracted from bacteria obtained by overnight culture at 37°C with a QIAquick miniprep kit so as to obtain V_H (HyHEL10)/pMK.

The V_L fragment was produced in the same manner as above. V_L (HyHEL10) was amplified with MVK-BACK12 and MVL-FOR2 primers under the above conditions except for the primers, followed by purification. V_L (HyHEL10) and V_H (HyHEL10)/pMK were subjected to restriction enzyme treatment with *Apa*LI/*Not*I. Ligation and transformation of TG-1 were carried out in the same manner as above. Then, a clone into which the V_L (HyHEL10) fragment had been inserted was selected by colony PCR. The selected clone was cultured and then the DNA sequence of the extracted plasmid was confirmed so as to obtain scFv(HyHEL10)/pMK.

(1.4) Modification of the sequence in the vicinity of the *Sgr*AI site of scFv(HyHEL10)/pMK

An amino acid derived from the *Sgr*AI site of scFv(HyHEL10)/pMK includes a Cys residue that might inhibit antibody folding. Therefore, amino acid mutation was carried out by a

quick change method.

Two different primers (15 pmol each), scFv(HyHEL10)/pMK (1 ng), 0.2 mM dNTPs, PfuUltra High-Fidelity DNA Polymerase (Stratagene) (2.5 units), and 10 x PfuUltra reaction Buffer (Stratagene) (5 μl) were mixed to prepare a reaction solution (50 μl). A reaction at 95℃ for 1 minute and then a reaction of 18 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 7 minutes were carried out. The sequences of the primers used are as described below.

SgrA1(TGA)rev: 5'-TCGAGCGAGAGCGGCGCCACCGGTGCCCATCATCATCACCAT-3' (SEQ ID NO: 11)

SgrA1(TGA)for: 5'-ATGGTGATGATGATGGGCACCGGTGGCGCCGCTCTCGCTCGA-3' (SEQ ID NO: 12)

*Dpn*I (1 μl) was added to the reaction solution, followed by treatment at 37°C for 1 hour for disintegration of the methylated template DNA. Then, the resultant (5 μl) was used to transform XL-10 Gold (100 μl), followed by overnight culture at 37°C on a YTAG agar medium. On the following day, the generated colony was collected by pricking with a bamboo skewer and was inoculated in 2YTAG (4 ml), followed by overnight culture at 37°C. After plasmid extraction from the culture solution, the DNA sequence was confirmed so as to obtain scFv(HyHEL10)/pMKQC.

(1.5) Production of scFv(D13HyHEL)/pMKQC

For a control experiment, scFv(D13HyHEL)/pMKQC having a V_H/V_L pair that is characterized by being inappropriate for OS-ELISA was produced as a control for scFv(HyHEL10)/pMKQC having a V_H/V_L pair that is characterized by being appropriate for OS-ELISA.

The V_H fragment of D1.3 and the V_L fragment of HyHEL10 were amplified with the use of the primers described below.

VH3: CTTTCTATGCGGCCCAGCCGGCCATGGCCCAGGTRCAGCTGAAGGAGTC (SEQ ID NO: 13)

(The underlined portion represents the *Sfi*I site.)

JH2: ACTGCTCGAGACTGTGAGAGTGGTGCC (SEQ ID NO: 14)

(The underlined portion represents the *Xho*I site.)

MVK-BACK12: 5'-CTCCTGTGCACTTGACATTGWGCTSACYCARTCT-3' (SEQ ID NO: 15)

(The underlined portion represents the *Apa*LI site.)

JK1/2: 5'-TTTCTCGTGCGGCCGCACGTTTKATTTCCAGCTTGG-3' (SEQ ID NO: 16)

(The underlined portion represents the *Not*I site.)

VH3 and JH2 (25 pmol each), pKST2/D1.3 (100 ng) serving as a template, 0.2 mM dNTPs, Ex-Taq Buffer (10 μl), and Ex-Taq (5 units) were mixed to prepare a reaction solution (50 μl). A reaction at 95°C for 1 minute, a reaction of 25 cycles at 95°C 30 seconds, 55°C 30 seconds, and 72°C 30 seconds, and then a reaction at 72°C for 2 minutes were carried out. The obtained PCR reaction solution was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) so as to obtain the D1.3 V_H gene.

The D1.3 V_H gene was inserted between the *Sfi*I/*Xho*I sites of pMKQC (dummy) lacking a portion of V_H of HyHEL10 and the full-length V_L of HyHEL10. pMKQC subjected to *Sfi*I/*Xho*I treatment and purification in the same manner as above and the D1.3 V_H gene were subjected to a ligation reaction with the use of a Rapid DNA Dephos & Ligation kit (Roche). The ligation solution (1 μl) was used to transform an XL10-Gold chemical competent cell. The transformant was cultured overnight at 37°C in a YT agar medium containing 100 μg/ml ampicillin (Amp) and 1% glucose (Glu). The generated colony was subjected to colony PCR with two different primers M13RV and pHENseq so as to confirm insertion of the insert fragment. The sequences of M13RV and pHENseq are as described below.

M13RV: 5'-CAGGAAACAGCTATGAC-3' (SEQ ID NO: 17)

pHENseq: 5'-CTATGCGGCCCCATTCA-3' (SEQ ID NO: 18)

M13RV and pHENseq (7.5 pmol each) and Premix Taq (*Ex Taq*™ Version) (Takara Bio Inc.) (7.5 μl) were mixed to prepare a reaction solution (15 μl). A reaction at 95°C for 1

minute, a reaction of 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, a reaction at 72°C for 1 minute were carried out. The obtained PCR reaction solution was analyzed by electrophoresis with the use of 1.5% agarose gel containing 1 μg/ml ethidium bromide (EtBr). A YT medium (YTAG) (4 ml) containing 100 μg/ml Amp and 1% Glu was inoculated with the clone exhibiting a desired band pattern. After plasmid DNA extraction from bacteria obtained by overnight culture at 37°C with a Wizard® *Plus* Minipreps DNA Purification kit (Promega), the DNA sequence was confirmed so as to obtain D13VH/pMK.

D13VH/pMK (approximately 2 μg) was mixed with *Apa*LI (New England Biolbs) (1 μl), NEBuffer 2 (New England Biolbs) (5 μl), and 1 mg/ml BSA (5 μl). MilliQ water was added thereto to a volume of 50 μl, followed by reaction at 37°C for 2 hour. Thereafter, *Sfi*I (1 μl) was added thereto and the resultant was left still overnight at 50°C for restriction enzyme treatment. The resultant was again subjected to electrophoresis with 1.5% agarose gel. Then, a band with a desired size was excised and purified with a Wizard® *Plus* Minipreps DNA Purification kit.

Subsequently, the V_L gene of HyHEL10 to be inserted into D13VH/pMK was produced as described below. MVK-BACK12 and JK1/2 (25 pmol each), HyHEL10/pCANTAB (100 ng) serving as a template, 0.2 mM dNTPs, Ex-Taq Buffer (10 μl), and Ex-Taq (5 units) were mixed to prepare a reaction solution (50 μl). A reaction at 95°C for 1 minute, a reaction of 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and then a reaction at 72°C for 2 minutes were carried out. The obtained PCR reaction solution was purified with a Wizard® SV Gel and PCR Clean-Up System so as to obtain the V_L gene of HyHEL10.

The V_L gene of HyHEL10 was inserted between the *Sfi*I/*Xho*I sites of D13VH/pMK. D13VH/pMK subjected to *Sfi*I/*Xho*I treatment and purification in the same manner as above and the V_L gene of HyHEL10 were subjected to ligation reaction with a Rapid DNA Dephos & Ligation kit (Roche). The obtained ligation solution (1 μl) was used to transform an XL10-Gold chemical competent cell. The transformant was cultured overnight at 37°C in a YT agar medium containing 100 μg/ml ampicillin (Amp) and 1% glucose (Glu). The

generated colony was subjected to colony PCR with two different primers M13RV and pHENseq so as to confirm insertion of an insert fragment in the same manner as above. The clone exhibiting a desired band pattern was inoculated in a YT medium (YTAG) (4 ml) containing 100 μg/ml Amp and 1% Glu. After plasmid DNA extraction from bacteria obtained by overnight culture at 37°C with a Wizard® *Plus* Minipreps DNA Purification kit, the DNA sequence was confirmed so as to obtain scFv(D13HyHEL)/pMKQC.

Example 2: Production of OS/pMI

An OS/pMI donor vector having MBP, RBS, a start codon, and an OmpA signal between the 2 loxP sites was produced based on a pSTV28 vector having the pACYC replication origin, antibiotic resistance (chloramphenicol (Cm) resistance) which differs from that of scFv/pMK, and the ability to coexist with a vector such as pUC or pBR in an identical bacterium (Takara Bio Inc.). Fig 3 is a flowchart of the production method.

First, *Sfi*I and *Not*I sites were introduced upstream of the lacZ α gene by PCR. The MBP gene amplified with a reverse primer containing *Sfi*I, *Swa*I, and the loxP 511 sequence and a forward primer containing *Not*I was incorporated into pSTV28 with the use of the *Sfi*I and *Not*I sites. Then, the full-length vector was amplified using 2 primers that are hybridized downstream of the lacZ α gene and are separately elongated in the upstream and downstream directions. The upstream-directed primer contains RBS, a start codon, and an OmpA signal sequence. The downstream-directed primer contains an loxP WT sequence. The amplified linear vector was subjected to self-ligation so as to obtain OS/pMI.

(2.1) Introduction of *Sfi*I and *Not*I sites into pSTV28

The full-length vector was amplified by PCR using primers separately containing *Sfi*I and *Not*I sites at one end, so that the *Sfi*I and *Not*I sites were introduced upstream of the lacZ gene. The primer sequences used are described below.

pSTV-Not1: 5'-AAAAAAAGCGGCCGCTTACACAGGAAACAGCTATGACC-3' (SEQ ID NO: 19)

(The underlined portion represents the *Not*I site.)

pSTV-Sfi1: 5'-AAAAAAAAGGCCCACACGGCCGCCTGGGGTGCCTAATGAGTG-3' (SEQ ID NO: 20)

(The underlined portion represents the *Sfi*I site.)

pSTV-Not1 and pSTV-Sfi1 (15 pmol each), pSTV28 (50 ng) serving as an template, 0.2 mM dNTPs, 10 x Buffer for Pfu (Stratagene) (5 μl), and Pfu turbo (Stratagene) (1 unit) were mixed to prepare a reaction solution (50 μl). A reaction at 94°C for 30 seconds and then a reaction of 20 cycles at 98°C for 10 seconds, 68°C for 30 seconds, and 72°C for 10 minutes were carried out. *Dpn*I (Promega) (1 μl) was added to the obtained PCR product, followed by reaction at 37°C for 1 hour. The resultant was purified with a Wizard® SV Gel and PCR Clean-Up System. The obtained DNA fragment (approximately 1 μg) was mixed with *Not*I (1 μl), NEBuffer 3 (5 μl), and 1 mg/ml BSA (5 μl). MilliQ water was added thereto to a volume of 50 μl, followed by reaction at 37°C for 2 hours and purification. Thereafter, the resultant was further mixed with *Sfi*I (1 μl), NEBuffer 3 (5 μl), and 1 mg/ml BSA (5 μl). MilliQ water was added thereto to obtain a system in a volume of 50 μl. The system was subjected to overnight reaction at 50°C, followed by agarose gel electrophoresis and purification of a band with a desired size.

(2.2) Production of an MBP insert and incorporation into a vector

The maltose binding protein (MBP) gene was produced and incorporated into a vector. The MBP gene was produced using pMAL-p2 (New England Biolbs) as a template and two different primers MBP-N and MBP-C. At such time, *Swa*I and *Sfi*I sites and loxP 511 were added to the 5'-end of MBP-N. In addition, a *Not*I site, a stop codon, and an Avi tag were added to the 5'-end of MBP-C. Accordingly, a gene was produced in which these sequences had been separately inserted into both ends of MBP.

MBP-N:

5'-AAAAAAAAGGCCGTGTGGGCCTTTATTTAAATTTTataacttcgtatagtatacattatacgaagttatCCAAAATCGAAGAAGGTAAACTG-3' (SEQ ID NO: 21)

(The underlined portions represent the *Sfi*I and *Swa*I sites in that order from the 5'-end, and the set of lower-case characters represents the loxP 511 site.)

MBP-C:

5'-AAAAAAAGCGGCCGCAAAttattcatgccattcaatcttctgagcttcaaaaatatcattaagaccAGTCTGCGC GTCTTTCAGGGC-3' (SEQ ID NO: 22)

(The underlined portion represents the *Not*I site, and the set of lower-case characters represents a complementary strand corresponding to the Avi tag and the stop codon.)

MBP-N and MBP-C (50 pmol each), pMAL-p2 (100 ng) serving as a template, 0.2 mM dNTPs, Ex-Taq Buffer (10 μl), and Ex-Taq (5 units) were mixed to prepare a reaction solution (100 μl). A reaction at 95°C for 30 seconds, a reaction of 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 60 seconds, and then a reaction at 72°C for 2 minutes were carried out. The resultant was purified in the same manner as described above (2.1), followed by *Not*I/*Sfi*I treatment and another purification.

The MBP gene and the vector DNA produced above (2.1) were ligated to each other by Ligation high ver2 for transformation of an XL10-Gold chemical competent cell. The transformant was cultured overnight at 37°C in a YT agar medium containing 34 μg/ml Cm. A single colony was further cultured overnight in a YT medium (YTC) (4 ml) containing 34 μg/ml Cm. Plasmid DNA was extracted from the resulting bacteria with the use of a Wizard® *Plus* Minipreps DNA Purification kit so as to obtain MBP/pSTV28.

(2.3) Incorporation of loxP WT and the OmpA signal sequence

In the last step, linear DNA obtained by amplifying MBP/pSTV28 with the use of pSTV-loxWT, pSTV-Signal 1, and pSTV-Signal 2 was subjected to self-ligation. Thus, OS/pMI in which the OmpA signal and loxP WT had been inserted downstream of the lacZ α gene of MBP/pSTV28 was produced. The OmpA signal sequence to be therein inserted has a long length. Thus, PCR was carried out 2 times with the use of pSTV-Signal 1 and pSTV-Signal 2 so as to produce OS/pMI.

pSTV-loxWT:

5'-p-ATAACTTCGTATAGCATACATTATACGAAGTTATGCTGTCAAACATGAGAATTAC AAC-3'

(SEQ ID NO: 23)

(The underlined portion represents loxP WT and "p" refers to 5' phosphorylation.)

pSTV-Signal 1

5'-CACTGCAATCGCGATAGCTGTCTTTTTCATATGATAtctcctGTGTGAAATTATCATCG ATAAGCTCATTCGCC-3' (SEQ ID NO: 24)

(The underlined portion represents a complementary strand corresponding to the first half of the OmpA signal, and the set of lower-case characters represents a complementary strand of RBS.)

pSTV-Signal 2

5'-p-TTTGTCATCGTCGTCCTTGTAGTCAGCTTGCGCAACGGTAGCGAAACCAGCCA GTGCCACTGCAATCGCGATAGCTGT-3' (SEQ ID NO: 25)

(The full-length strand represents a complementary strand of the OmpA signal sequence and "p" refers to 5' phosphorylation.)

pSTV-loxWT and pSTV-Signal 1 (15 pmol each), MBP/pSTV28 (50 ng) serving as a template, 0.2 mM dNTPs, 10 x Buffer for Pfu (5 μl), and Pfu turbo (1 unit) were mixed to prepare a reaction solution (50 μl). A reaction at 94°C for 30 seconds and then a reaction of 20 cycles at 98°C for 10 seconds, 68°C for 30 seconds, and 72°C for 10 minutes were carried out. The obtained PCR reaction solution (1 μl) was used as a template and another PCR was carried out under the above conditions with the use of pSTV-loxWT and pSTV-Signal 2. Thus, a linear vector to which the full-length OmpA Signal sequence had been added was obtained. *Dpn*I (Promega) (1 μl) was added to the PCR reaction solution, followed by incubation at 37°C for 1 hour and then purification with the use of a Wizard® SV Gel and PCR Clean-Up System. Further, T4 DNA polymerase (New England Biolbs) (1 μl) was allowed to act on the linear vector (approximately 1 μg), followed by ligation at 16°C for 30 minutes and then transformation of an XL10-Gold chemical competent cell. The transformant was cultured overnight at 37°C in a YT agar medium containing 34 μg/ml Cm. A single colony was further cultured overnight in YTC (4 ml). Plasmid DNA was extracted

from the resulting bacteria with the use of a Wizard® *Plus* Minipreps DNA Purification kit. Then, the DNA sequence was confirmed to obtain OS/pMI.

Example 3: ELISA with scFv(HyHEL10)/pMK and scFv(D13HyHEL10)/pMKQC

An scFv display phage having an loxP linker with a length longer than that of an ordinary loxP linker was prepared using scFv(HyHEL10)/pMK and scFv(D13HEL)/pMKQC so as to confirm the HEL-binding property.

(3.1) Preparation of a phage with the use of scFv(HyHEL10)/pMK

TG-1 (100 μl) was transformed by electroporation with the use of scFv(HyHEL10)/pMK (10 ng), followed by overnight culture at 37°C on a YT agar medium (YTAG) plate containing 1% glucose and 100 μg/ml ampicillin. Thus, an scFv(HyHEL10) display pIII-expressing cell line (scFv(HyHEL10)/pMK/TG-1) was produced.

The colony generated on the YTAG agar medium plate subjected to overnight culture was pricked with a bamboo skewer, and was inoculated in a 2YT liquid medium (2YTAG) (4 ml) containing 1% glucose and 100 μg/ml ampicillin, followed by overnight culture at 37°C until $O.D._{600}$ reached 0.5. 3×10^{10} cfu of a helper phage KM13 was added to the culture solution. The mixture was left still at 30°C for 30 minutes for infection. Then, centrifugation at 3300 g for 10 minutes was carried out and the supernatant was discarded. The resultant was resuspended in a 2YT liquid medium (2YTAK) (4 ml) containing 0.1% glucose, 100 μg/ml ampicillin, and 50 μg/ml kanamycin and cultured overnight at 30°C.

The culture solution was centrifuged at 3,300 g for 30 minutes. The recovered supernatant was added with PEG/NaCl (20% Polyethylene glycol 6000, 2.5 M NaCl) (800 μl), and left still on ice for 1 hour, followed by centrifugation at 3,300 g for 30 minutes. The supernatant was discarded. The resultant was suspended in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) (200 μl) and further centrifuged at 11,600 g for 10 minutes so as to recover the supernatant. For comparison, a phage was prepared from scFv(D13HyHEL)/pMKQC and scFv(HyHEL10)/pCANTAB encoding scFv having a $(G_4S)_3$ linker in the same manner as above. In addition, the prepared phage solution was diluted so as to be infected with TG-1 in logarithmic

growth phase. Then, the phage titer (colony-forming ability per 1 ml of phage: cfu/ml) was determined.

(3.2) ELISA with a prepared phage

A 50 mM $NaHCO_3$ solution (pH 9.6) containing 10 μg/ml HEL or a PBS solution containing 10 μg/ml bovine serum albumin (BSA) (NaCl (5.84 g), Na_2HPO_4 (4.72 g), and $NaH_2PO_4 \cdot 2H_2O$ (2.64 g) per 1 L (pH 7.2)) was dispensed in a Falcon 3912 microplate at 100 μl each. The microplate was left still at 4°C for 16 hours. After discarding solutions from the microplate, the microplate was blocked with PBS containing 2% skim milk (200 μl) at room temperature for 2 hours. Next, the microplate was washed with PBS containing 0.1% Tween 20 (PBS-T), added with PBS containing 2% skim milk (100 μl) and 10^8 cfu phage obtained above, and left still at room temperature for 90 minutes. In order to detect the scFv display phage immobilized in the above operations, the microplate was washed with PBS-T. Then, the microplate was added with 5000-fold diluted HRP/anti-M13 monoclonal conjugate (Amersham) in PBS containing 2% skim milk and left still at room temperature for 1 hour. The microplate was then washed with PBS-T three times. Thereafter, a previously prepared enzyme reaction solution (100 mM sodium acetate (50 ml; pH 6.0), 10 mg/ml TMBZ (in DMSO) (500 μl), and H_2O_2 (10 μl)) was added to respective wells at 100 μl each to initiate the reaction. After incubation in dark for about 5 minutes, the reaction was stopped with 3.2 N H_2SO_4 (50 μl), and the absorbance was read at 450 nm (with reference at 655 nm) using a plate reader.

As shown in fig. 4, an scFv display phage prepared from scFv(HyHEL10)/pMK exhibited a more significant antigen (HEL)-binding property than that of BSA serving as a blank sample, as in the case of a phage derived from scFv(HyHEL10)/pCANTAB. In addition, scFv(D13HyHEL)/pMKQC was also subjected to a similar experiment, and significant HEL binding was confirmed.

Example 4: Model panning

A model library was produced by mixing an scFv(HyHEL10)/pMKQC-derived phage with an scFv(9-3)/pMKQC-derived phage having an antibody gene lacking an

HEL-binding property at a ratio of 1:5000. Model panning for selecting scFv(HyHEL10)/pMKQC was carried out by panning with HEL.

(4.1) The HEL-binding property and the antibody display rate of a scFv(9-3)/pMKQC phage

The 9-3 gene was amplified by the same technique described above (1.3) with the use of HEL-immunized mouse spleen cell-derived mRNA as a template. After incorporation into loxP-linker/pMK, mutation at the *Sgr*AI site was carried out in the same manner as described above (1.4) to obtain scFv(9-3)/pMKQC. The resultant was used to prepare a phage. ELISA was performed on an HEL-immobilized plate (under the same antigen immobilization conditions described above (3.2)) to evaluate the antigen-binding property. In this Example, 20-fold scale phage preparation was conducted for mass phage preparation. Further, in order to increase the antibody display rate, culture was carried out with the addition of 1 mM IPTG after infection with KM13.

(4.2) Panning of Model Libraries

2.5×10^{12} cfu of scFv(9-3)/pMKQC-derived phage and 5.0×10^{8} cfu of scFv(HEL10)/pMKQC-derived phage were mixed together to obtain a model library.

A 50 mM $NaHCO_3$ solution (3.6 ml, pH 9.6) containing 50 μg/ml HEL was put into a Nunc Maxisorp immuno test tube (Nunc) and left still overnight at 4°C for 16 hours to immobilize antigens. After washing with PBS three times, blocking was performed with PBS containing 2% skim milk (MPBS) (3.6 ml) at room temperature for 2 hours. After washing with PBS three times, the tube was poured with MPBS (3.6 ml) containing 1.0×10^{12} cfu of model library phage, and was rotated for 1 hour and left still for 1 hour at room temperature to immobilize these phages. After discarding the phage solution, the tube was washed with PBS-T twenty times, added with Trypsin-PBS (10 mg/ml trypsin stock (50 μl) and PBS (450 μl)) (500 μl), and repeatedly inverted at room temperature for 10 minutes to effect elusion. The eluted phage solution (250 μl) was added to TG-1 (1.75 ml) in logarithmic growth phase, and left still at 37°C for 30 minutes to infect these phages. 100-fold and 10,000-fold dilutions of this solution were spotted on a YTAG agar medium and incubated at 37°C overnight. The

titer of the eluted phages was measured. The remaining solution was subcultured in a 2YTAG liquid medium (10 ml) at 37°C until the OD_{600} reached 0.4. Then, the solution was added with 5 x 10^{10} cfu of KM13, and left still at 37°C for 30 minutes to effect infection of the helper phage. After centrifugation at 3,000 g for 10 minutes, the supernatant was discarded, and the pellet was resuspended with 2YTAK containing IPTG (50 ml), followed by incubation at 30°C overnight. The culture solution was centrifuged at 3,300 g for 15 minutes. The recovered supernatant (40 ml) was added with PEG/NaCl (10 ml), and left still on ice for 1 hour, followed by centrifugation at 3,300 g for 30 minutes. PEG/NaCl was discarded. The pellet was suspended with 2 ml of TE, followed by centrifugation at 11,600 g for 10 minutes. *E. coli* debris was removed, and the supernatant was recovered.

(4.3) Polyclonal phage ELISA

A 50 mM $NaHCO_3$ solution (pH 9.6) containing 10 μg/ml HEL, a PBS solution containing 10 μg/ml BSA, 1000-fold diluted anti-Myc antibody in PBS, and PBS were respectively dispensed in a Falcon 3912 microplate at 100 μl per well. The microplate was left still at 4°C for 16 hours. Then, this plate was reacted with respective phages before and after panning at 5 x 10^9 cfu per well, followed by ELISA in the same conditions described above (3.2). As a result, as shown in Fig. 5, the signal to HEL was observed to remarkably increase after panning. The results revealed that an scFv(9-3)/pMKQC-derived phage having a high level of HEL-binding property was concentrated.

(4.4) Monoclonal phage ELISA

A monoclonal phage antibody was prepared from each phage obtained via panning, followed by determination of the antigen-binding property.

30 colonies produced upon titer determination were subcultured in 2YTAG (100 μl) that had been dispensed on a 96-well plate (Corning) and cultured overnight at 250 rpm and 37°C. The obtained preculture solution (approximately 2 μl) was subcultured in a fresh 2YTAG (200 μl), followed by culture at 250 rpm and 37°C for 2 hours. Then, 2YTAG (25 μl) containing 10^9 cfu KM13 was added thereto and reaction was carried out at 250 rpm and 37°C

for 1 hour to effect infection with a helper phage. The supernatant was discarded via centrifugation at 1,800 g for 10 minutes. Then, the resultant was resuspended in 2YTAK (200 μl) containing 1 mM IPTG and cultured overnight at 30°C. On the following day, monoclonal phage ELISA was performed with the use of the supernatant recovered via centrifugation at 1,800 g for 10 minutes.

A 50 mM $NaHCO_3$ solution (pH 9.6) containing 10 μg/ml HEL, a PBS solution containing 10 μg/ml BSA, 1000-fold diluted anti-Myc antibody in PBS, and PBS were respectively dispensed in a Falcon 3912 microplate at 100 μl per well and left still at 4°C for 16 hours. After blocking in the same manner as described above (3.2), PBS containing 4% skim milk (50 μl) and the above prepared monoclonal phage solution (50 μl) were mixed together and added to each well, followed by reaction at room temperature for 1.5 hours. Thereafter, an HEL-binding phage was detected under the same conditions described above (3.2). Consequently, as shown in Fig. 6, it has been revealed that many clones have a specific HEL-binding property. Signals derived from the anti-Myc antibody-immobilized plate are thought to be propotional to the scFv display rate of a phage. Therefore, it would be possible to accurately estimate the binding property of scFv of an identical clone by dividing signals on the HEL-immobilized plate by signals on the anti-Myc antibody-immobilized plate. As shown in Fig. 7, as a result of correction with the signal intensity (display rate) to the anti-Myc antibody, many clones (21 out of 30 clones) were found to exhibit significant signals to HEL.

(4.5) Analysis based on *Xho*I cleavage pattern

In view of the presence of the *Xho*I site in the V_H fragment of scFv(HyHEL10)/pMK, the proportion of scFv(HyHEl10)/pMKQC was estimated based on the *Xho*I cleavage pattern of a DNA fragment amplified by colony PCR using M13RV and pHENseq.

The colony used for monoclonal phage ELISA was pricked with a bamboo skewer and immersed in a mixed solution containing M13RV and pHENseq (5 pmol each), GoTaq mix (Promega) (5 μl), and milliQ water (5 μl). The solution was subjected to a reaction at 95°C for 5 minutes, a reaction of 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, and then a reaction at 72°C for 5 minutes for DNA fragment amplification.

10 x NEBuffer 2 (1 μl), 1 mg/ml BSA (1 μl), and *Xho*I (2 units) were added thereto, followed by cleavage at 37°C for 4 hours. Electrophoresis was performed with 1.5% agarose gel so as to confirm a band pattern. As shown in fig. 8, many clones exhibited a pattern specific to scFv(HyHEL10)/pMK. Further, the clones were perfectly identical to clones that exhibited significant signals to HEL in Fig. 7 (table 1). In addition, no clone lacking the scFv gene was observed. Accordingly, it was concluded that scFv/pMK is a practical phage display system that can be used for panning.

Table 1: Samples thought to be scFv(HyHEL10)/pMKQC in the case of monoclonal ELISA correspond to those thought to be scFv(HyHEL10)/pMKQC in the case of analysis of PCR patterns of *Xho*I-cleaved fragments.

	1	2	3	4	5	6	7	8	9	10
ELISA				○	○	○		○	○	○
PCR				○	○	○		○	○	○

	11	12	13	14	15	16	17	18	19	20
ELISA	△	△		○	○	○	○	○	○	○
PCR	△	△		○	○	○	○	○	○	○

	21	22	23	24	25	26	27	28	29	30
ELISA		○	○	○		○	○	○		
PCR		○	○	○		○	○	○		

Example 5: Homologous recombination with Cre recombinase

As shown in fig. 9, recombination was carried out using Cre recombinase (Novagen), *Swa*I-treated scFv(HyHEL10)/pMK and scFv(D13HEL)/pMK linear vectors serving as acceptors, a circular plasmid OS/pMI serving as a donor, and a DNA fragment, namely, OS-fragment, containing a sequence sandwiched between 2 loxP sites of OS/pMI. The occurrence or nonoccurrence of desired recombination was confirmed by PCR using a recombination reaction

solution as a template and sequencing.

(5.1) Recombination of scFv(HyHEL10)/pMK and OS/pMI with the use of Cre recombinase

In order to increase recombination efficiency, *Swa*I-treated linear-scFv(HyHEL10)/pMK was used as an accepter. In a preparation method, scFv(HyHEL10)/pMK (approximately 2 μg) was mixed with NEBuffer 3 (5 μl), *Swa*I (10 units), and 1 mg/ml BSA (5 μl). MilliQ water was added thereto to a volume of 50 μl. The resultant was treated at 25°C for 2 hours, followed by agarose gel electrophoresis and purification.

Two different donors were used, which were OS/pMI, and an OS-fragment containing loxP 511, MBP, a start codon, an ompA sequence, loxP WT, and the like of OS/pMI. Such an OS-fragment was produced by PCR using two different primers, which were pSTV-Pro to be annealed upstream of loxP 511 and pSTV-p15A to be annealed downstream of loxP WT (Fig. 10).
pSTV-Pro: 5'-AGGTTTCCCGACTGGAAAGCG-3' (SEQ ID NO: 26)
pSTV-p15A: 5'-TACGCGCAGACCAAAACG-3' (SEQ ID NO: 27)

pSTV-Pro and pSTV-p15A (50 pmol each), OS/pMI (100 ng) serving as a template, 0.2 mM dNTPs, Ex-Taq Buffer (10 μl), and Ex-Taq (5 units) were mixed to prepare a reaction solution (100 μl). A reaction at 95°C for 5 minutes, a reaction of 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, and then a reaction at 72°C for 5 minutes were carried out. After agarose gel electrophoresis, a band with a desired size was excised and purified so as to produce an OS-fragment.

Recombination reaction was carried out using the accepter and the donor produced above.

Cre recombinase (Novagen) (1 unit) was added to linear-scFv(HyHEL10)/pMK (0.25 μg) and OS/pMI or OS-fragment (0.25 μg) so as to prepare a reaction solution (50 mM Tris-HCl (pH 7.5), 33 mM NaCl, 10 mM $MgCl_2$) in a total volume of 30 μl, followed by reaction at 37°C for 1 hour. Then, the resultant was deactivated at 70°C for 5 minutes and left at room temperature for 10 minutes for cooling.

Also, a recombination experiment was conducted for scFv(D13HyHEL)/pMKQC in a

similar manner.

(5.2) Confirmation of the recombinant product

The progress of the recombination reaction of scFv(HyHEL10)/pMK was confirmed by PCR of the reaction solution and sequencing. First, PCR reaction was carried out using the reaction solution as a template, an OS3rev primer to be annealed to the MBP sequence, and pHENseq to be annealed to gIII (Fig. 11).

OS3rev: 5'-GCTGTTGAAGCGTTATCG-3' (SEQ ID NO: 28)

OS3rev and pHENseq (5 pmol each), a recombination reaction solution (1 μl), and GoTaq mix (5 μl) were mixed, and milliQ water was added thereto to prepare a reaction solution (10 μl). A reaction at 95°C for 5 minutes, a reaction of 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes, and then a reaction at 72°C for 5 minutes were carried out. Amplification of a DNA fragment with a desired size was confirmed by 1.5% agarose gel electrophoresis.

The obtained recombination reaction solution (1 μl) was used for transformation of TG-1, followed by overnight culture at 37°C in a YTAG agar medium. The obtained clones were subjected to colony PCR under the aforementioned conditions. Then, a clone thought to have OS(HyHEL10)/pMK was selected, and was inoculated in a 2YTAG (4 ml). Plasmids were extracted from bacteria obtained by overnight culture at 37°C. The DNA sequence designed as predetermined was confirmed to be obtained.

OS(D13HEL)/pMK, which is a recombinant product of scFv(D13HyHEL)/pMKQC, was obtained in a similar manner.

Example 6: OS-ELISA with the use of OS(HyHEL10)/pMK and OS(D13HEL)/pMK

A V_L display MBP and a V_H display phage were produced with the use of OS/pMK produced in a Cre/lox system. It was confirmed whether or not they would be able to be applied to OS-ELISA in practice.

TG-1 (100 μl) was transformed using OS(HyHEL10)/pMK (10 ng) by electroporation

and cultured overnight at 37°C in a YTAG agar medium plate. Thus, a V_L–MBP/V_H-pIII-expressing cell line (OS(HyHEL10)/pMK/TG-1) was produced. The colony generated on the YTAG agar medium plate subjected to overnight culture was pricked with a bamboo skewer, and was inoculated in a 2YTAG liquid medium (4 ml), followed by culture at 37°C until $O.D._{600}$ reached 0.5. The culture solution was added with 3×10^{10} cfu of a helper phage KM13, and left still at 30°C for 30 minutes to effect infection. After centrifugation at 3,300 g for 10 minutes, the supernatant was discarded, and the resultant was resuspended with a 2YTAK liquid medium (4 ml) and cultured overnight at 30°C. The culture solution was centrifuged at 3,300 g for 30 minutes. The recovered supernatant was added with PEG/NaCl (800 μl), and left still on ice for 1 hour, followed by centrifugation at 3,300 g for 30 minutes. The supernatant was discarded. The resultant was suspended in TE (200 μl) and further centrifuged at 11,600 g for 10 minutes so as to recover the supernatant containing V_L–MBP and V_H-phage.

The supernatant containing V_L–MBP and V_H-phage was recovered using OS(D13HEL)/pMK in a similar manner.

A PBS solution containing 0.9 μg/ml Monoclonal Anti-maltose binding protein (Sigma) was dispensed in a Falcon 3912 microplate at 100 μl each. The microplate was left still at 4°C for 16 hours. After discarding solutions from the microplate, the microplate was blocked with PBS containing 2% skim milk (200 μl) at room temperature for 2 hours. Next, the microplate was washed PBS-T, added with a mixture of a V_L-MBP/V_H-phage solution (50 μl) and PBS containing 2% skim milk and 0-200 μg/ml antigen (HEL) (50 μl) obtained from the above procedure, and left still at room temperature for 90 minutes. In order to detect V_H display phage that had been immobilized in the above operations, the microplate was washed with PBS-T. The microplate was added with 5000-diluted HRP/anti-M13 Monoclonal Conjugate (Amersham) in PBS containing 2% skim milk and left still at room temperature for 1 hour. The microplate was then washed with PBS-T three times. Thereafter, a previously prepared enzyme reaction solution (100 mM sodium acetate (pH 6.0) (50 ml), 10 mg/ml TMBZ (in DMSO) (500 μl), and H_2O_2 (10 μl)) was added to respective wells at 100 μl each to initiate the reaction. After incubation in dark for about 5 minutes, the reaction was stopped with the 3.2 N H_2SO_4 (50 μl)

and the absorbance was read at 450 nm (with reference at 655 nm) using a plate reader.

As shown in Fig. 12, an increase in ELISA signal was confirmed along with an increase in antigen (HEL) concentration by OS-ELISA with the use of OS(HyHEL10)/pMK-derived V_H display phage and MBP-V_L, indicating that they can be applied to OS assay. However, in the case of OS-ELISA with the use of OS(D13HyHEL)/pMK-derived V_H display phage and MBP-V_L, strong signals were observed even in the absence of an antigen, so that substantially no signal increase was confirmed along with an increase in HEL concentration. Thus, as a result of conversion into OS/pMK, it has become possible to simply recognize a single-strand antibody appropriate for OS assay.

Brief Description of the Drawings

Fig. 1 shows a production scheme of scFv/pMK.

Fig. 2 shows the loxP-linker sequence.

Fig. 3 shows a production scheme of OS/pMI.

Fig. 4 shows the antigen-binding property of the scFv (anti-HEL)-phage.

Fig. 5 shows results for polyclonal ELISA.

Fig. 6 shows results for monoclonal ELISA 1.

Fig. 7 shows ELISA signals corrected with signals to an anti-c-myc antibody.

Fig. 8 shows results for analysis of the genes of clones obtained by panning.

Fig. 9 shows a recombination reaction of scFv/pMK and OS/pMI with the use of Cre recombinase.

Fig. 10 shows annealing sites of pSTV-Pro and pSTV-p15A.

Fig. 11 shows annealing sites of OS3rev and pHENseq.

Fig. 12 shows results of OS-ELISA using an OS/pMK/TG-1 culture supernatant. Numerical reference 1 denote a culture supernatant of TG-1 transformed with OS(HyHEL10)/pMK and KM13. Numerical reference 2 denotes a control experiment (culture supernatant of non-transformed TG-1). Numerical reference 3 denotes a culture supernatant of TG-1 transformed with OS(D13HyHEL)/pMK and KM13. Numerical reference 4 denotes a control experiment (culture supernatant of non-transformed TG-1),

respectively.

Fig. 13 schematically shows conversion of an scFv into a coexpression system of VL-MBP and a VH display phage according to the method of the present invention.

CLAIMS

1. A vector which comprises:

a DNA region comprising: (1): a DNA sequence that causes extracellular secretion of peptides encoded by the following DNA sequences (2) and (3); (2): a DNA sequence encoding either one of VH fragment or VL fragment of antibody variable domain; and (3): a DNA sequence encoding a tagged protein; and

a DNA region comprising: (4): a DNA sequence encoding a protein for displaying a peptide encoded by the following DNA sequence (5) on a phage; (5): a DNA sequence encoding the other one of the VH fragment or VL fragment of the antibody variable domain; and (6): a DNA sequence encoding a phage coat protein,

wherein, when the vector is introduced into a host cell, it is capable of secreting: a protein comprising either one of VH fragment or VL fragment of the antibody variable domain from the host cell; and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain from the host cell.

2. The vector according to claim 1, wherein the vector is an *E. coli* phage vector or phagemid vector.

3. The vector according to claim 1 or 2, wherein the DNA sequence in (1) is a DNA sequence encoding a ribosomal binding site and a gIII signal sequence.

4. The vector according to any one of claims 1 to 3, wherein the DNA sequence in (2) is a DNA sequence encoding a VL fragment of the antibody variable domain and the DNA sequence in (5) is a DNA sequence encoding a VH fragment of the antibody variable domain.

5. The vector according to any one of claims 1 to 4, wherein the DNA sequence in (3) is a DNA sequence encoding a maltose binding protein.

6. The vector according to any one of claims 1 to 5, wherein the DNA sequence in (4) is a DNA sequence encoding a ribosomal binding site and an OmpA signal sequence.

7. The vector according to any one of claims 1 to 6, wherein the DNA sequence in (6) is a DNA sequence encoding a gIII protein.

8. A method for producing a vector whererein, when the vector is introduced into a host

cell, it is capable of secreting a protein comprising either one of VH fragment or VL fragment of the antibody variable domain from the host cell; and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain from the host cell, the method comprising allowing a recombinase to act on a first vector (A) and a second vector (B) described below so as to cause gene recombination between the first vector and the second vector: (A): a first vector comprising a DNA sequence capable of causing expression of a polypeptide comprising a VH fragment and a VL fragment of the antibody variable domain, by means of extracellular secretion or in a form of a fusion protein tethered to a phage coat protein, and a pair of recombinase recognition sequences that are inserted between a DNA sequence encoding a VH fragment and a DNA sequence encoding a VL fragment; and (B): a second vector comprising a pair of recombinase recognition sequences, and a stop codon inserted between the pair of recombinase recognition sequences.

9. The method according to claim 8, wherein the first vector contains a sequence that can cause secretion expression of a single-chain variable region (scFv) polypeptide.

10. The method according to claim 8, wherein the first vector contains a sequence that can express a single-chain variable region (scFv) in a form of a fusion protein tethered to a phage coat protein.

11. The method according to claim 8, wherein the first vector contains a sequence that can cause secretion expression of a Fab polypeptide.

12. The method according to claim 8, wherein the first vector contains a sequence that can express Fab in a form of a fusion protein tethered to a phage coat protein.

13. The method according to claim 8, wherein the first vector contains a sequence that can cause secretion expression of an F(ab')2 polypeptide.

14. The method according to claim 8, wherein the first vector contains a sequence that can cause secretion expression of an IgG polypeptide.

15. The method according to any one of claims 8 to 14, wherein the first vector contains the following (1) to (4) in the order of (1)-(2)-(2)-(3)-(4) or (3)-(2)-(2)-(1)-(4): (1): a VL polypeptide sequence; (2): a recombination site sequence; (3): a VH polypeptide sequence; and (4): a phage coat protein sequence.

16. The method according to any one of claims 8 to 15, wherein the second vector contains a DNA sequence encoding a tagged protein.

17. The method according to any one of claims 8 to 16, wherein the recombinase is Cre recombinase.

18. The method according to any one of claims 8 to 17, wherein recombination takes place between loxP sites.

19. A method for evaluating the interaction between VH polypeptide and VL polypeptide, comprising the steps of:

(i): introducing the vector according to any one of claims 1 to 7 or the vector produced by the method according to any one of claims 8 to 18 into a host cell;

(ii): collecting a protein comprising either one of VH fragment or VL fragment of the antibody variable domain which has been secreted from the host cell, and a phage displaying the other one of the VH fragment or VL fragment of the antibody variable domain; and

(iii) detecting a complex of the VH fragment, the VL fragment, and an antigen by allowing the antigen to contact with the protein comprising either one of VH fragment or VL fragment of antibody variable domain collected in (ii), and phage displaying the other one of the VH fragment or VL fragment of antibody variable domain collected in (ii).

20. The method according to claim 19, wherein the antibody variable domain in which the interaction between the VH fragment and the VL fragment is changed under the presence of the antigen, is selected.

21. The method according to claim 19, wherein a VH polypeptide and a VL polypeptide having a weak interaction are selected from an scFv mixture.

22. The method according to claim 19, wherein an scFv mixture having a high affinity to a target antigen is selected from among scFv mixtures, and then a VH polypeptide and a VL polypeptide having a weak interaction are selected from a mixture of scFv.

23. The method according to any one of claims 19 to 22, wherein the complex of the VH fragment, the VL fragment, and an antigen is detected by immunoassay with the use of a labeled anti-phage antibody.

Fig. 1

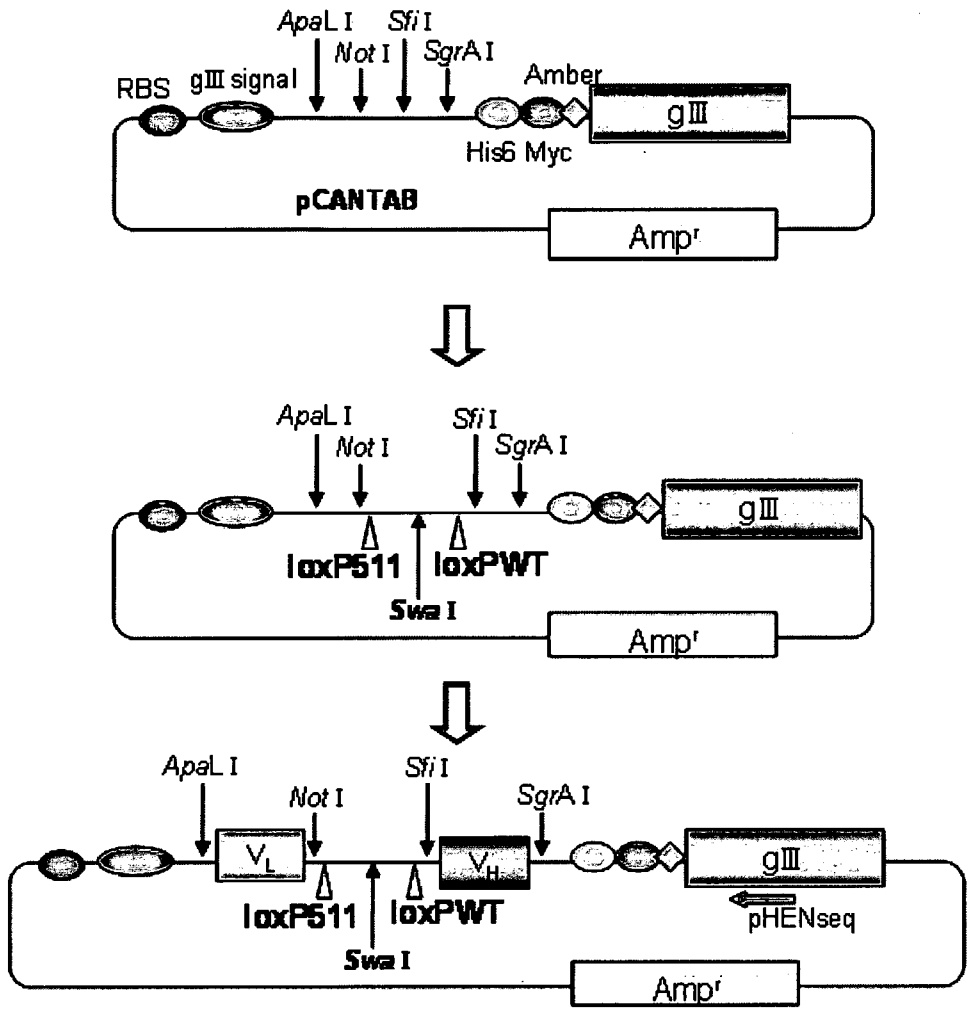
ApaL I
Sfi I
Not I
SgrA I
RBS
gIII signal
Amber
gIII
His6 Myc
pCANTAB
Amp^r
loxP511
loxPWT
Swa I
V_L
V_H
pHENseq

Fig.2

gcggccgcgataacttcgtatagtatacattatacgaagttatccggtggaggc
AlaAlaAlaIleThrSerTyrSerIleHisTyrThrLysLeuSerGlyGlyGly

aATTTAAATggcggtggcagcataacttcgtatagcatacattatacgaagttatcg
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AlaGlnProAla

Fig.3

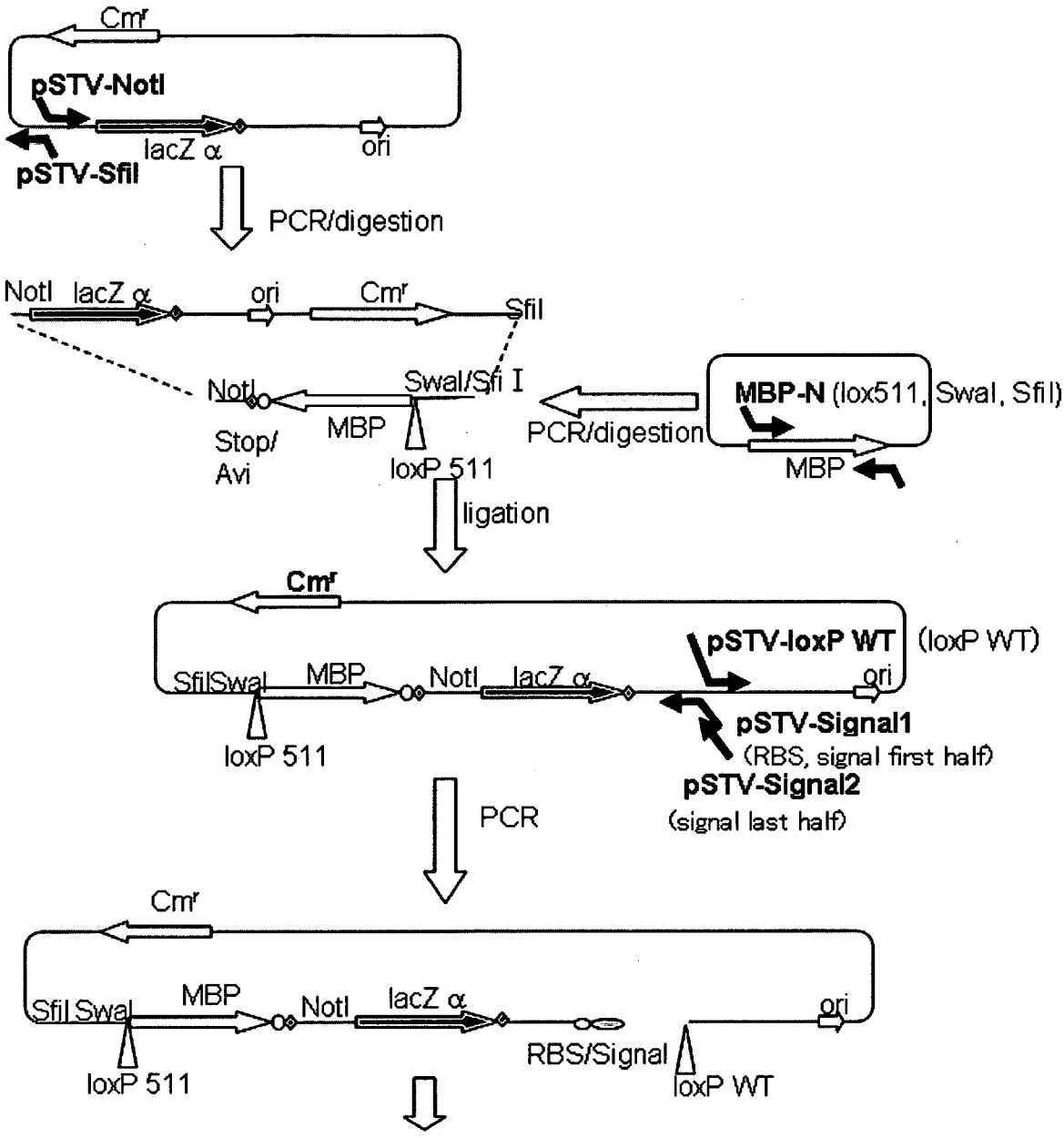
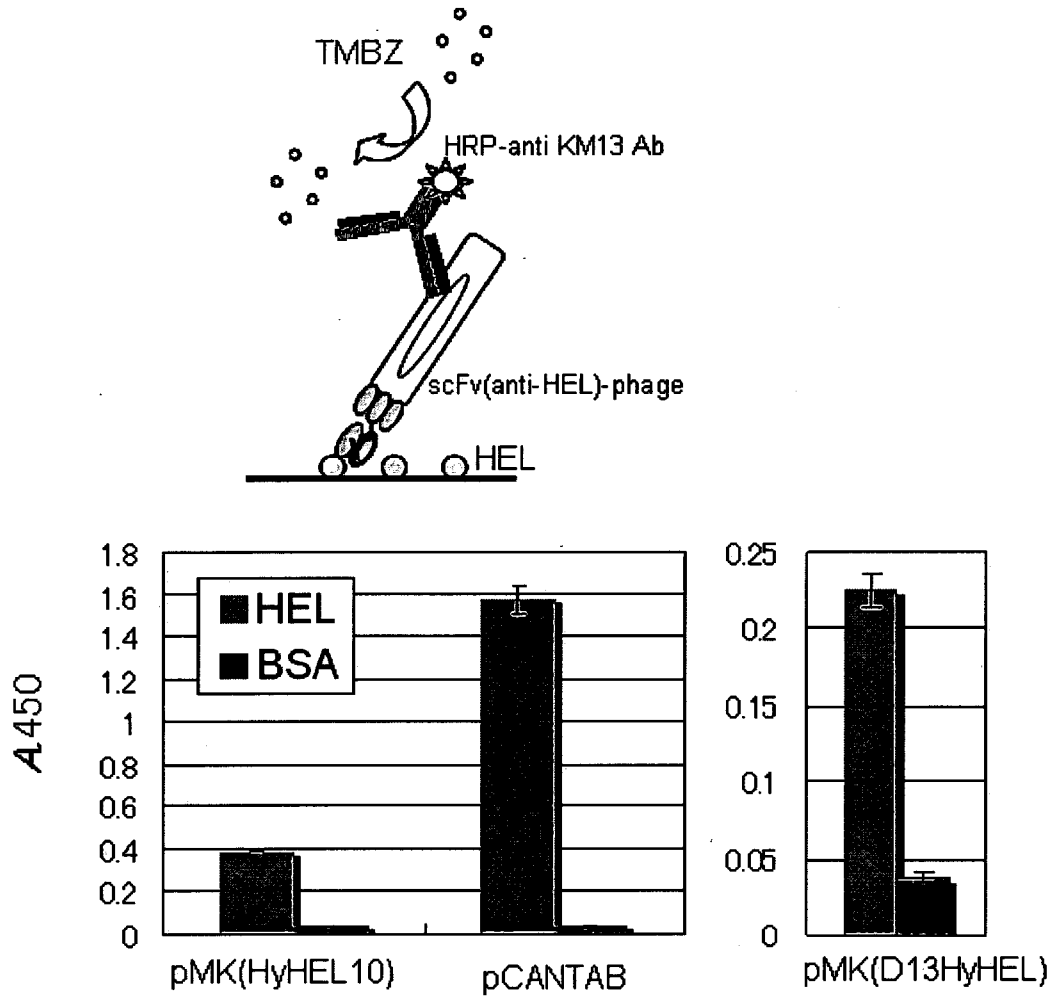

Cmr
pSTV-NotI
lacZ α
ori
pSTV-SfiI
PCR/digestion
NotI
lacZ α
ori
Cmr
SfiI
NotI
SwaI/Sfi I
MBP
Stop/
Avi
loxP 511
PCR/digestion
MBP-N (lox511, SwaI, SfiI)
MBP
ligation
Cmr
pSTV-loxP WT
(loxP WT)
SfiISwaI
MBP
NotI
lacZ α
ori
loxP 511
pSTV-Signal1
(RBS, signal first half)
pSTV-Signal2
(signal last half)
PCR
Cmr
SfiI SwaI
MBP
NotI
lacZ α
ori
RBS/Signal
loxP 511
loxP WT

Fig.4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HRP-anti KM13 Ab
scFv(anti-HEL)-phage
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A450
HEL
BSA
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pMK(HyHEL10)
pCANTAB
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0.2
0.15
0.1
0.05
0
pMK(D13HyHEL)

Fig.5

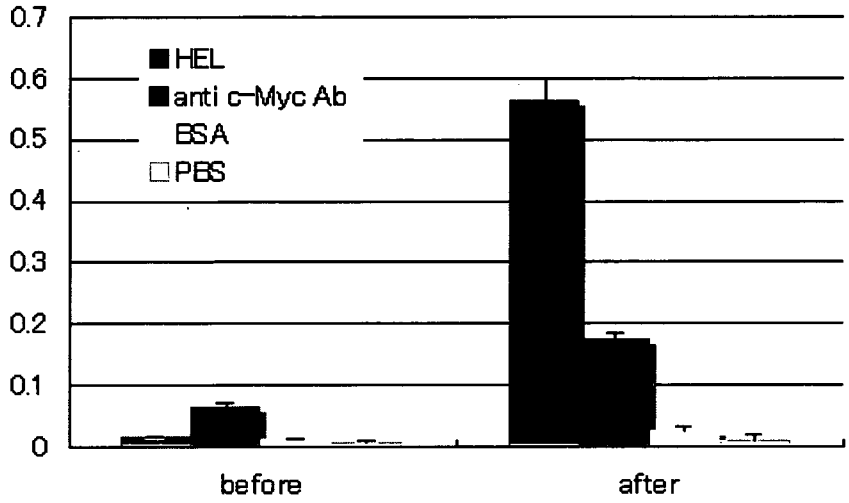
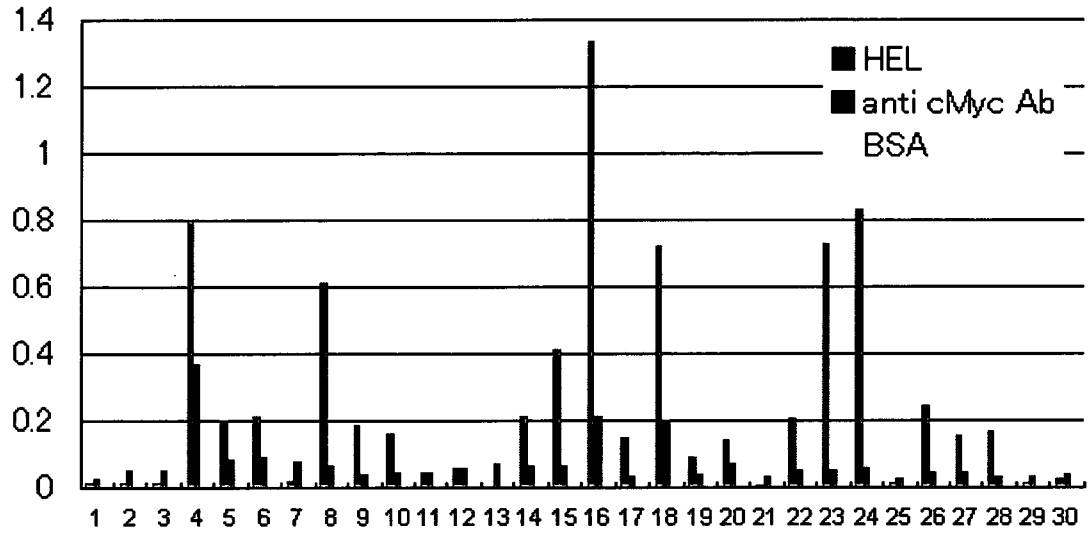


Fig.6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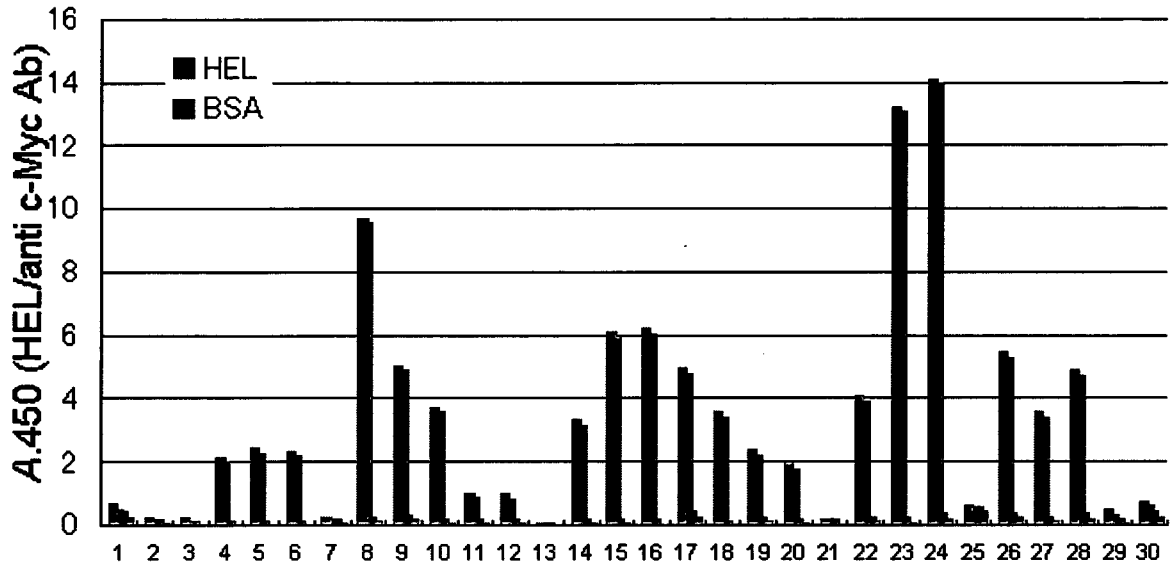
1.2
1
0.8
0.6
0.4
0.2
0
HEL
anti cMyc Ab
BSA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Fig.7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HEL
BSA
0
2
4
6
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10
12
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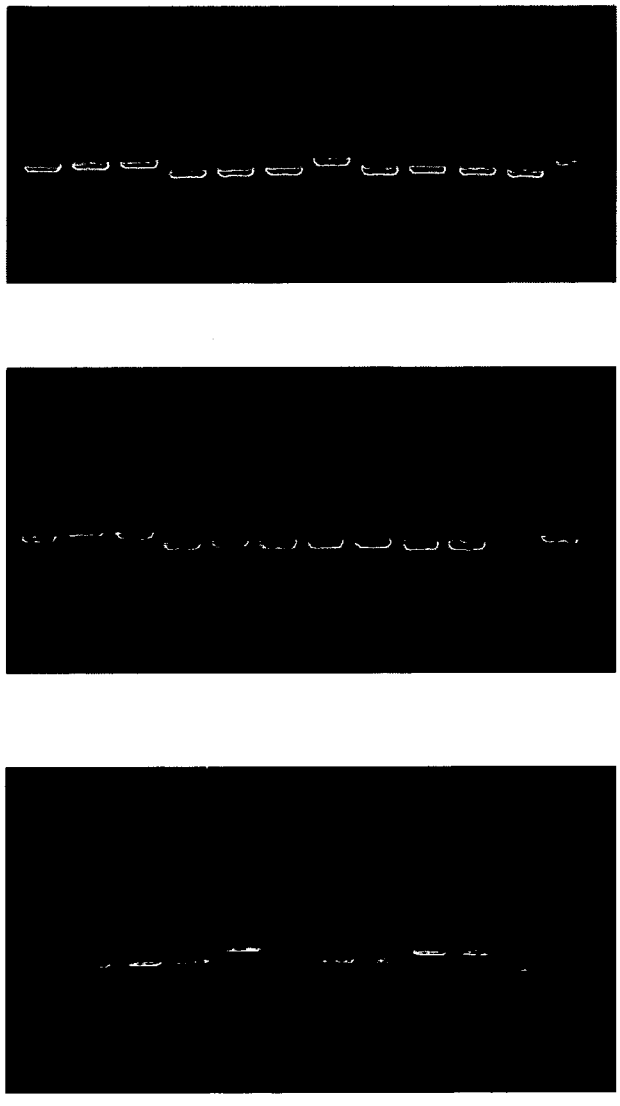
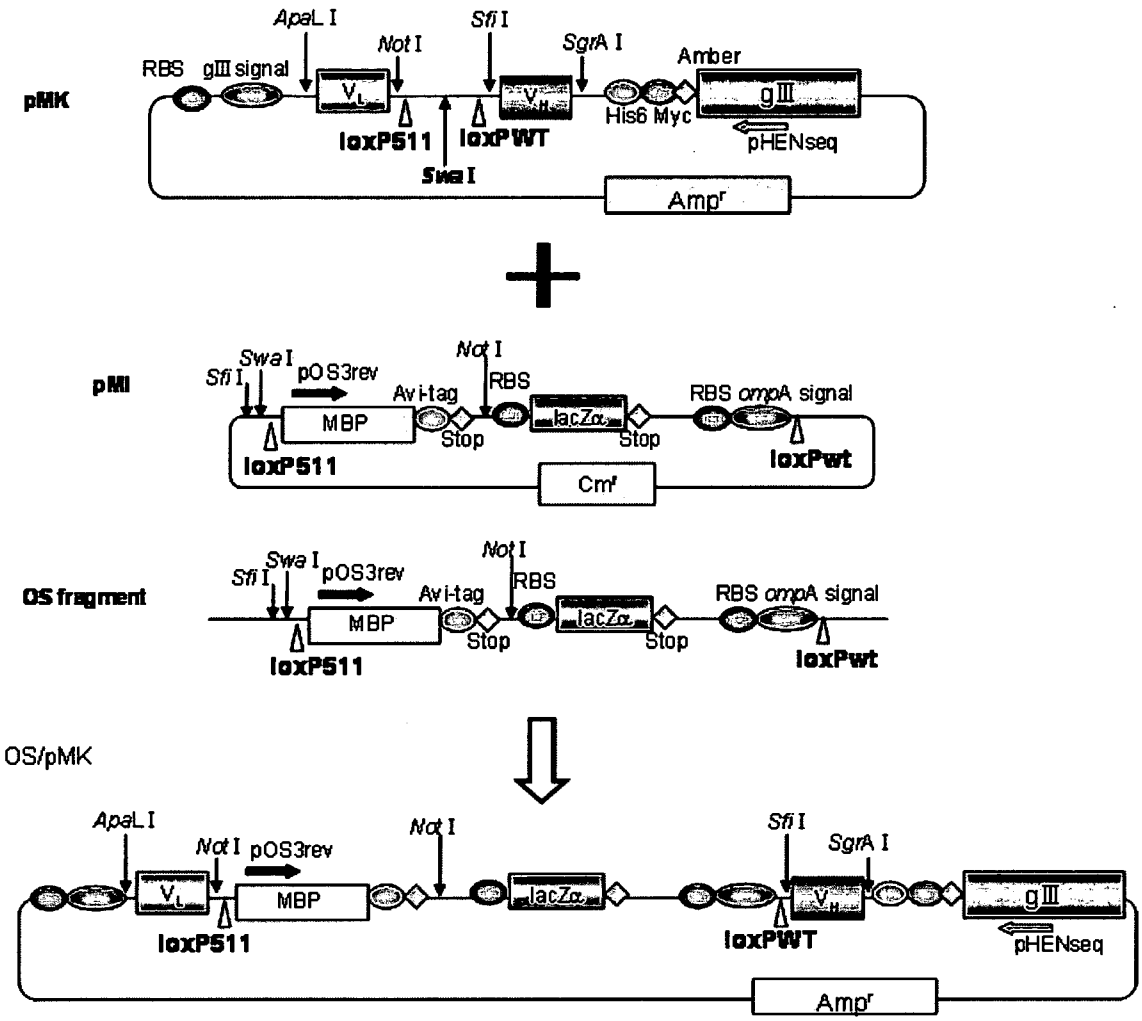
Fig.8



P: scFv(HyHEL10)/pMKQC

N: scFv(9-3)/pMKQC

Fig.9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ApaL I
Not I
Sfi I
SgrA I
Amber
RBS
gIII signal
VL
VH
gIII
His6 Myc
pHENseq
loxP511
loxPWT
Swa I
Ampr
pMI
Swa I
Sfi I
pOS3rev
Not I
Avi-tag
RBS
MBP
lacZα
Stop
Stop
RBS ompA signal
loxP511
loxPwt
Cmr
OS fragment
Swa I
Sfi I
pOS3rev
Not I
Avi-tag
RBS
MBP
lacZα
Stop
Stop
RBS ompA signal
loxP511
loxPwt
OS/pMK
ApaL I
Not I
pOS3rev
Not I
Sfi I
SgrA I
VL
MBP
lacZα
VH
gIII
loxP511
loxPWT
pHENseq
Ampr

Fig.10

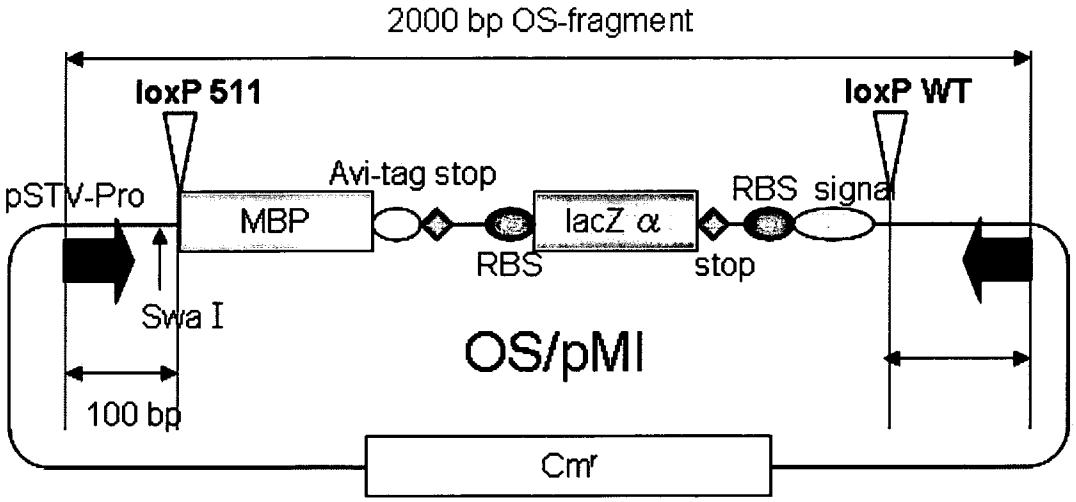

2000 bp OS-fragment
loxP 511
loxP WT
pSTV-Pro
Avi-tag stop
MBP
lacZ α
RBS signal
RBS
stop
Swa I
OS/pMI
100 bp
Cmr

Fig.11

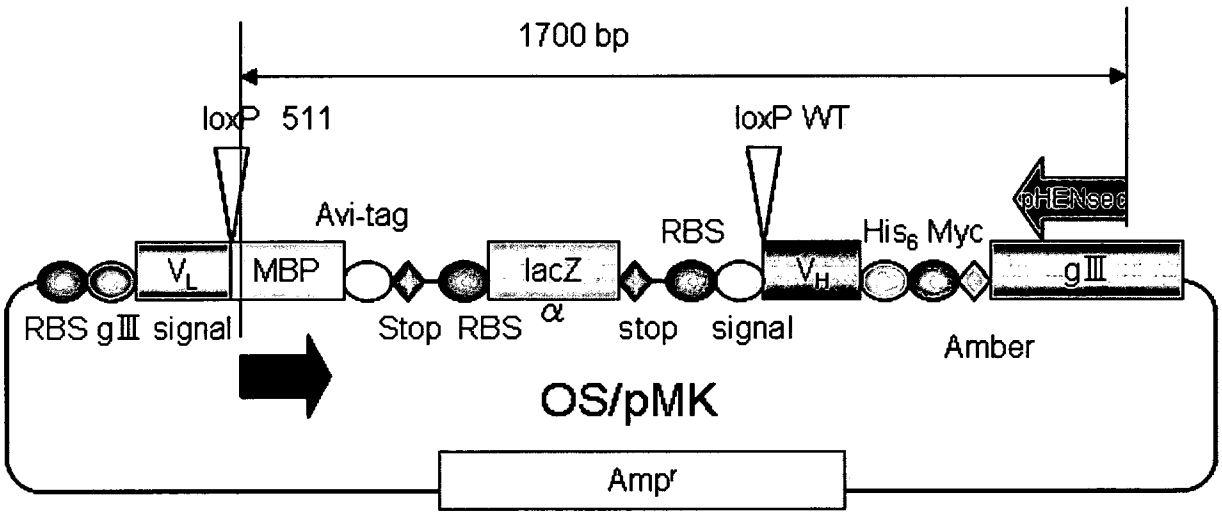

1700 bp
loxP 511
loxP WT
Avi-tag
RBS
His6 Myc
pHENseq
VL
MBP
lacZ
VH
gIII
RBS gIII signal
Stop RBS
α
stop
signal
Amber
OS/pMK
Ampr

Fig.12

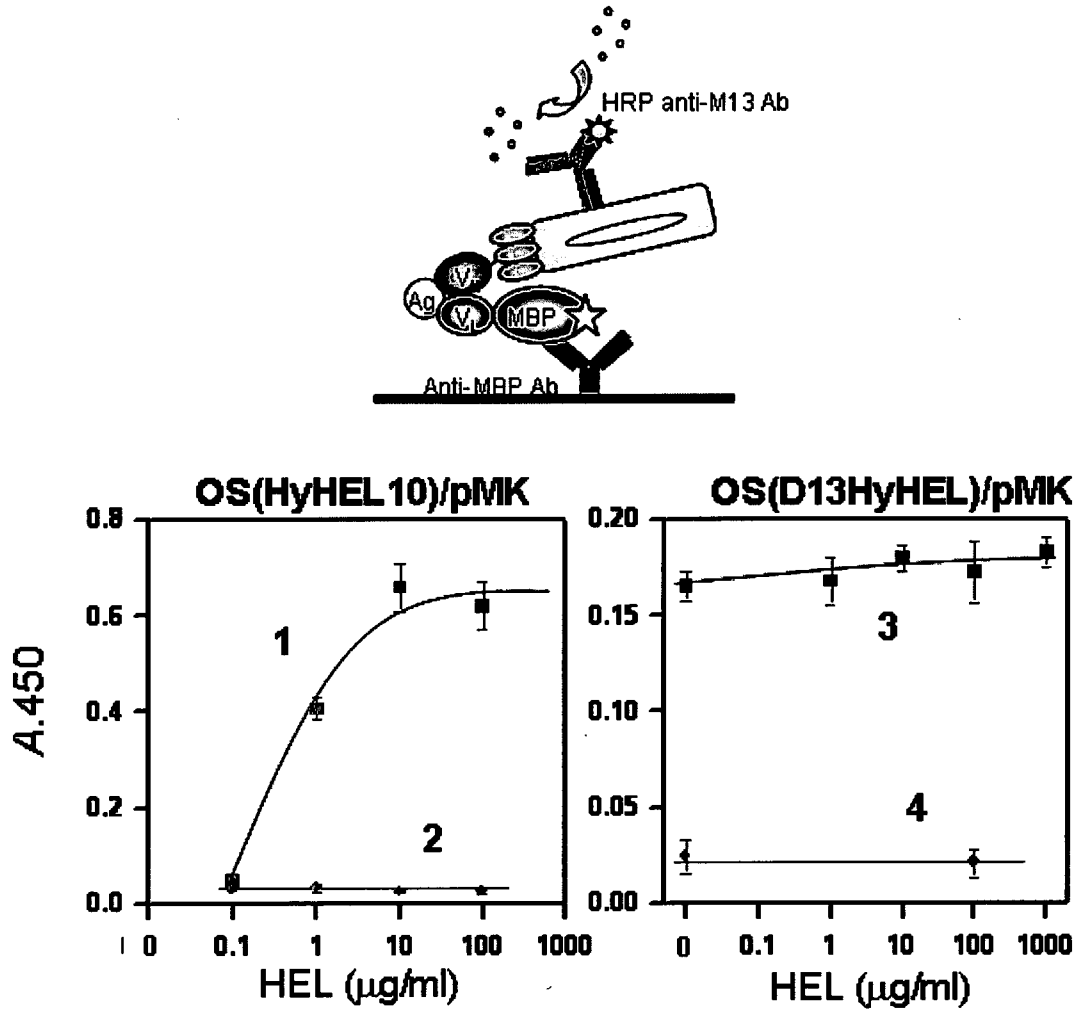
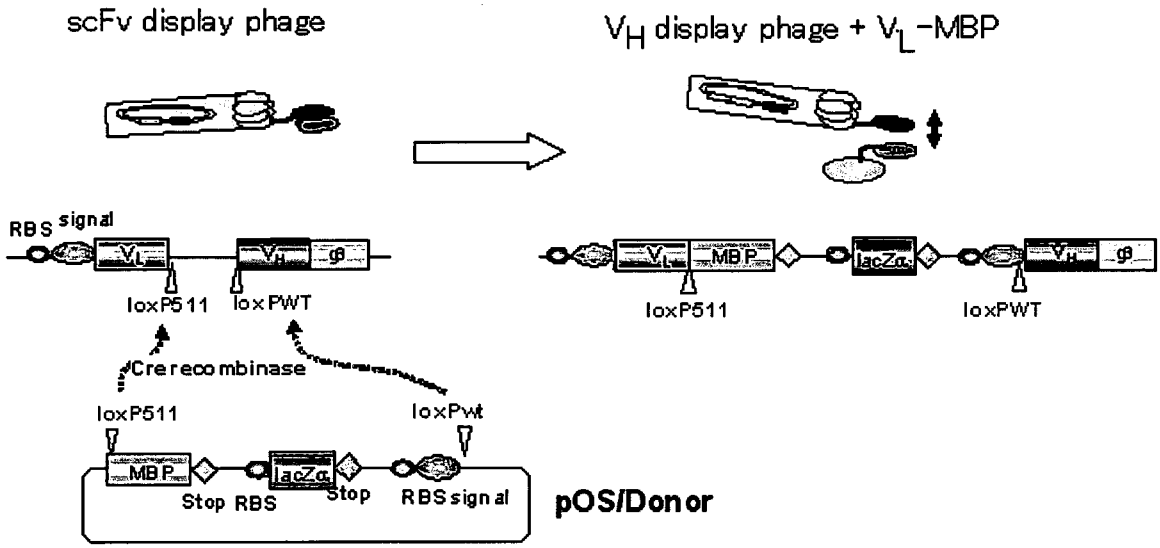

HRP anti-M13 Ab
Ag
MBP
Anti-MBP Ab
OS(HyHEL10)/pMK
OS(D13HyHEL)/pMK
A.450
0.8
0.6
0.4
0.2
0.0
0.20
0.15
0.10
0.05
0.00
1
2
3
4
0
0.1
1
10
100
1000
HEL (μg/ml)

Fig.13


scFv display phage
VH display phage + VL-MBP
RBS
signal
VL
VH
gβ
loxP511
loxPWT
Crerecombinase
loxP511
loxPwt
MBP
lacZα
Stop
RBS
Stop
RBSsignal
pOS/Donor
MBP
lacZα
loxP511
loxPWT

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2009/052944

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/53 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABURATANI T ET AL: "A General Method To Select Antibody Fragments Suitable for Noncompetitive Detection of Monovalent Antigens" ANALYTICAL CHEMISTRY, vol. 75, no. 16, 15 August 2003 (2003-08-15), pages 4057-4064, XP001175789 AMERICAN CHEMICAL SOCIETY. COLUMBUS, US ISSN: 0003-2700 cited in the application the whole document	1,2,4, 19-23
Y		3,5-18
X	EP 1 536 005 A1 (UNIV TOKYO [JP]) 1 June 2005 (2005-06-01) cited in the application	1,2,4, 19-23
Y	the whole document	3,5-18

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 June 2009	16/07/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pérez-Mato, Isabel

INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2009/052944

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THOMASON LYNN ET AL: "Unit1.16: Recombineering: Genetic Engineering in Bacteria Using Homologous Recombination" CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vol. Suppl.78, April 2007 (2007-04), pages 1.16.1-1.16.24, XP002531244 ISSN: 1934-3647 the whole document	8-18
Y	HARTLEY JAMES L ET AL: "DNA cloning using in vitro site-specific recombination" GENOME RESEARCH, vol. 10, no. 11, 1 November 2000 (2000-11-01), pages 1788-1795, XP002187669 COLD SPRING HARBOR LABORATORY PRESS, WOODBURY, NY, US ISSN: 1088-9051 the whole document	8-18
Y	SIEGEL ROBERT W ET AL: "Recombinatorial cloning using heterologous lox sites" GENOME RESEARCH, vol. 14, no. 6, June 2004 (2004-06), pages 1119-1129, XP002531245 ISSN: 1088-9051 the whole document	8-18
Y	TERPE K: "Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 60, no. 5, 1 January 2003 (2003-01-01), pages 523-533, XP002298417 SPRINGER VERLAG, BERLIN, DE ISSN: 0175-7598 page 528, column 2	5
Y	DI GUANA C ET AL: "Vectors that facilitate the expression and purification of foreign peptides in Escherichia coli by fusion to maltose-binding protein" GENE, vol. 67, no. 1, 15 July 1988 (1988-07-15), pages 21-30, XP025705742 ELSEVIER, AMSTERDAM, NL ISSN: 0378-1119 [retrieved on 1988-07-15] the whole document	5

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INTERNATIONAL SEARCH REPORT

International application No
PCT/JP2009/052944

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOLLIGER PHILIPP ET AL: "Engineered antibody fragments and the rise of single domains" NATURE BIOTECHNOLOGY, vol. 23, no. 9, 1 September 2005 (2005-09-01), pages 1126-1136, XP008076746 NATURE PUBLISHING GROUP, NEW YORK, NY, US ISSN: 1087-0156 the whole document	12

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/JP2009/052944

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1536005 A1	01-06-2005	AU 2003266502 A1	03-03-2004
		WO 2004016782 A1	26-02-2004
		US 2006252028 A1	09-11-2006