Title: MURINE ANTIBODY DISPLAY LIBRARY

Abstract: Murine antibody display libraries, "PHILOtop", for selection of antibodies to desired antigens, including antibodies that display species cross-reactivity e.g. cross-reactivity between human and mouse. Synthetic antibody library of variable domains comprising variably mutated residues within murine framework, including frameworks based on murine germline 186 and/or Vkappa kv4-72.

Declarations under Rule 4.17:
- of inventorship (Rule 4.17(iv))
- with international search report (Art. 21.3)
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2)(ii)
MURINE ANTIBODY DISPLAY LIBRARY

Field of the Invention

This invention relates to antibody display libraries and methods of using such libraries to select an antibody molecule with a desired antigen-binding property. In particular, this invention relates to antibody libraries for selection of mouse antibodies.

Introduction

Monoclonal antibodies and their derivatives represent the fastest growing class of biopharmaceuticals (Walsh 2006). The isolation of good quality human antibodies against a variety of different antigens from single-pot phage display libraries has been performed using human antibody libraries (Marks, Hoogenboom et al. 1991; Griffiths, Williams et al. 1994; Nissim, Hoogenboom et al. 1994; Vaughan, Williams et al. 1996; Marks and Bradbury 2004; Andreasson and Carlsson 2005; Hoogenboom 2005; Silacci, Brack et al. 2005; Donzeau and Knappik 2007; Dubel 2007), also in consideration of the fact that human antibodies are likely to be less immunogenic for biomedical applications in patients.

However, the development of antibody-based therapeutics may require the development and in vivo testing of antibody-based products in syngeneic preclinical settings e.g., murine antibodies in mouse models of pathology.

Current preclinical therapy studies involving human antibodies and antibody derivative therapeutics in mouse models are limited by the mouse immune response and the mouse anti-human antibody (MAHA) generation. To overcome this problem, such studies are limited to use of immuno-deficient mouse strains or to short-time therapies. There is therefore a need for mouse antibodies for use in such studies.

One method of generating mouse antibodies against an antigen of interest is to immunise mice with the antigen. Antibody libraries from immunised mice have been described (Clackson, Hoogenboom et al. 1991). Monoclonal antibodies are routinely generated from mice or rats using hybridoma technology. This approach has clear limits when the antigen is highly conserved among species (resulting in low immunogenicity), or in case of highly toxic or deadly pathogenic antigens. Certain conserved antigens are not immunogenic in rodents (Camemolla, Leprini et al. 1992; Melkko and Neri 2003). The generation of mouse antibodies
against human antigens, such as for mouse models of pathology in which the human antigen is expressed, e.g. tumour xenografts, is therefore a particular difficulty with conserved antigens.

Constructions of non-immune scFv phage display libraries from spleen and bone marrow of unimmunised mice have been described. There are a few reports of naïve single pot mouse antibody libraries in the literature (Gao, Huang et al. 1999; Okamoto, Mukai et al. 2004; Imai, Mukai et al. 2006). These have been used for the isolation of a small number of monoclonal antibodies. However, cloning of antibody gene from non-immune donors is inefficient: this due to the fact that their RT-PCR primers sets, PCR conditions and efficiency of subcloning for construction of a antibody gene library cannot encompass all the antibody diversity (Okamoto, Mukai et al. 2004).

We have now designed and constructed a large mouse antibody phage display library, which capitalises on the previous experience of our laboratory in the construction of antibody libraries based on a single antibody scaffold with combinatorial mutations at residues of the CDR3 loops of heavy and light chains (Pini, Viti et al. 1998; Viti, Nilsson et al. 2000, Silacci, Brack et al. 2005; Ettorre, Rosli et al. 2006; Villa, Trachsel et al. 2008). We describe herein a library of antibody molecules, in which each antibody molecule comprises a variably mutated amino acid sequence within the same murine antibody framework.

Such a library allows selection of antibody molecules that bind mouse antigens, without being subject to the limitations of libraries derived from immunised mice, since the antibodies have not undergone negative selection against "self" antigens in the mouse.

Further, the provision of a synthetic antibody library, not requiring immunisation of a donor mouse with antigen, avoids much laborious laboratory work, and the use of phage display and/or E.coli production gives the possibility of further genetic manipulation.

The antibody library disclosed herein is of exceptional value for use in the selection of antibody molecules against a variety of diverse antigens, including several antigens of interest for therapeutic and/or diagnostic applications, and allows screening for antibodies to conserved antigens, toxic and/or pathogenic antigens. A single-pot antibody library according to the invention may represent a useful source of binding specificities, facilitating preclinical studies in immunocompetent syngeneic mouse models of pathology.
We describe libraries of antibody molecules comprising framework regions derived from murine germline gene segments in antibody VH and/or VL domains, e.g. mouse germline heavy chain \( v \) gene segment 186 (Bothwell, Paskind et al. 1981; Williams, Martinez et al. 2001; Chang and Mohan 2005) and/or mouse germline Vk light chain (kappa) gene segment kv4-72 (Kirschbaum, Roschenthaler et al. 1999). We found that an antibody library, the "PHILOtop library" based on these germline regions as a scaffold for the variably mutated residues was particularly suitable for selecting high quality antibodies in biopanning experiments using a wide variety of antigens.

In fact, although we were able to generate other mouse libraries that largely fulfilled the quality requisites as percentage of clones carrying the full-length insert, bacterial expression of the soluble scFv fragments and display of the scFv-plll fusion protein on the phage particle, only the PHILOtop library was shown to reliably yield good-quality antibodies towards protein antigens used in selection experiments.

Antibody libraries according to the invention library represent a useful complement to human antibody phage display libraries. The new antibody libraries may be used for preclinical research activities, when fully murine antibodies are needed for the \textit{in vivo} implementation of biomedical strategies in mouse models of pathology. As an example, the PHILOtop library has already solved an old problem of our lab, namely the isolation of mouse monoclonal antibodies to extra-domains of fibronectin and of tenascin-C which can be used for the cloning and \textit{in vivo} testing of fully murine immunocytokines for therapeutic applications in rodents (Carnemolla, Borsì et al. 2002; HaNs, Rondini et al. 2002; Gafner, Trachsel et al. 2006; Schliemann and Neri 2007; Schliemann, Palumbo et al. 2009).

\textbf{Brief Description of the Tables and Drawings}

Table I shows the primers used for the construction of scFv libraries MuLibi, MuLib2 and PHILOtop described in the Examples. In the nucleotide sequences shown in Table 1, M indicates A or C, and N indicates A, C, G or T.

Supplementary Table I shows the amino acid sequences of scFv libraries MuLibi, MuLib2 and PHILOtop described in the Examples. The scFv is composed of a heavy chain, a linker and a variable chain. The randomised CDR3 regions are underlined. CDR residues that are optionally present or not present are shown in brackets.
Table II shows the results of biopanning experiments with MuLJbI, MuLib2 and PHILOtop libraries described in the Examples.

Figure 1 illustrates the design and construction of antibody libraries as described in the Examples.

Figure 2 (A, B, C) shows PCR-screening, dot blot and western blot for the MuLibi.

Figure 3 (A, B, C) shows PCR-screening, dot blot and western blot for the MuLib2.

Figure 4 (A, B, C) shows PCR-screening, dot blot and western blot for the PhiloTOP.

Figure 5 shows tissue sections from immunohistochemistry.

Figure 6 shows (A) an alignment between the Philotop VH 186.2-derived region and the germline 186.2 gene segment, and (B) an alignment between the Philotop VL Vk4-72 region and the germline vk4-72 gene segment. Numbering is according to Tomlinson 1995.

**Detailed Description**

This invention relates to a library of antibody molecules, in which each antibody molecule comprises a variably mutated amino acid sequence within a framework, where the framework is generally the same between different antibody molecules in the library.

Unlike antibodies derived from human libraries, antibody molecules derived from the libraries according to the invention are generally non-immunogenic in mice. Thus, preferably, antibody molecules from libraries according to the invention do not significantly elicit the HAMA reaction on administration to a mouse.

Preferably, the library is a library of antibody molecules in which the framework is derived from a murine antibody framework.

One aspect of the invention is a library of antibody molecules, wherein each antibody molecule comprises:
a VH domain comprising an amino acid sequence at least 80 %, 85 %, 90 %, 95 % or 99 % identical to the Philotop VH domain SEQ ID NO: 1 in which a CDR, e.g. CDR3, is variably mutated,
and/or
5 a VL domain comprising an amino acid sequence at least 80 %, 85 %, 90 %, 95 % or 99 % identical to the Philotop VL domain SEQ ID NO: 4 in which a CDR, e.g. CDR3, is variably mutated.
The VH domain may comprise SEQ ID NO: 1 in which CDR3 is variably mutated. The VL domain may comprise SEQ ID NO: 4 in which CDR3 is variably mutated.
An aspect of the invention is a library of antibody molecules, wherein each antibody molecule comprises:
a VH domain comprising
15 an amino acid sequence at least 80 %, 85 %, 90 %, 95 % or 99 % identical to the Philotop VH 186.2-derived region SEQ ID NO: 2, and
a variably mutated amino acid sequence
and/or
a VL domain comprising
20 an amino acid sequence at least 80 %, 85 %, 90 %, 95 % or 99 % identical to the Philotop VL kv4-72-derived region SEQ ID NO: 5, and
a variably mutated amino acid sequence.
The VH domain may comprise the Philotop VH 186.2-derived region SEQ ID NO: 2 and a variably mutated amino acid sequence. The VL domain may comprise the Philotop VL kv4-72-derived region SEQ ID NO: 5 and a variably mutated amino acid sequence.

Mouse germline v gene segment 186.2 is described in Bothwell, Paskind et al. 1981; Williams, Martinez et al. 2001; Chang and Mohan 2005. Mouse germline Vk light chain (kappa) gene segment kv4-72 is described in Kirschbaum, Roschenthaler et al. 1999. As shown in the sequence listing and described in the Examples, the Philotop library exemplified herein contains VH and VL domains derived from these germline gene segments.

As exemplified by the Philotop library, particular residues of VH and VL may be different from the germline residues and/or may contribute to the good stability and/or high diversity of antibody binding in antibody molecules in the library.
A VH domain may comprise one or more, or all, of the following residues at the stated positions:
M at residue 20
N at residue 33
T at residue 40
A at residue 50
Y at residue 52
G at residue 53
N at residue 54
Y at residue 56
S at residue 58
Q at residue 61
G at residue 65
A at residue 71

A VL domain may comprise Met at residue 32, e.g. in a sequence of residues SYMHWFQQ.

Figure 6 shows an alignment between the Philotop VH 186.2-derived region (SEQ ID NO: 2) and the germline 186.2, and an alignment between the Philotop VL Vk4-72-derived region (SEQ ID NO: 5) and the germline Vk 4-72.

Diversity within the antibody library stems from inclusion of variably mutated residues within the antibody molecules.

By variably mutated it is meant that different antibody molecules in the library have different mutations at those residues. A residue position in the VH or VL domain is variably mutated if different residues are present at that position when the library is considered as a whole. For example there may be at least 2, at least 4, at least 10, at least 15 or at least 20 different residues at a variably mutated position in the library. The different residues at variably mutated positions may comprise non-germline residues, or in some cases may consist only of non-germline residues.

The use of combinatorial mutation provides a high diversity of binding sequences within the library.
Variability may be achieved by random mutation of residues. Techniques such as site directed mutagenesis and error prone PCR may be used to produce variable mutation. A method described in the examples herein is the generation of variable mutations by PCR using degenerate primers that anneal in the CDR and contain variable oligonucleotide sequences corresponding to certain positions of the CDR.

In a library according to the invention, each antibody molecule preferably comprises a VH domain and a VL domain, forming a VH-VL domain pair.

A VH or VL domain framework comprises four framework regions, FR1, FR2, FR3 and FR4, interspersed with CDRs in the following structure:

\[
\text{FR1} - \text{CDR1} - \text{FR2} - \text{CDR2} - \text{FR3} - \text{CDR3} - \text{FR4}
\]

In germline VH and VL domains, FR1, FR2 and FR3 correspond to the mouse germline v segment FR region, e.g. mouse germline v gene segment 186 for a VH domain, mouse germline Vk gene segment kv4-72 for a VL domain, and FR4 corresponds to a mouse germline j segment.

PHILOtop sequences are exemplified herein and shown in the sequence listing. CDRs and framework regions within the VH and VL domains can be identified by the skilled person - see Chang S et al., Molecular Immunology (2005), 42, 1293-1301.

For example, in the Philotop VH domain:

- CDR1 is from residues 31 to 35 (SYNMH)
- CDR2 is from 50 to 59 (AIYPGNYTS)
- CDR3 is the mutatable sequence following CAR.

Each antibody molecule in a library of the invention may comprise a VH domain consisting of VH complementarity determining regions CDR1, CDR2 and CDR3 and framework regions FR1, FR2, FR3 and FR4, wherein

VH FR1 consists of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the FR1 contained within Philotop VH domain SEQ ID NO: 1 or Philotop VH 186.2-derived region SEQ ID NO: 2,
VH FR2 consists of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the FR2 contained within Philotop VH domain SEQ ID NO: 1 or Philotop VH 186.2-derived region SEQ ID NO: 2,

VH FR3 consists of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the FR3 contained within Philotop VH domain SEQ ID NO: 1 or Philotop VH 186.2-derived region SEQ ID NO: 2 and wherein at least one VH complementarity determining region comprises a variably mutated amino acid sequence.

Each antibody molecule in a library may comprise a VL domain consisting of VL complementarity determining regions CDR1, CDR2 and CDR3 and framework regions FR1, FR2, FR3 and FR4, wherein

VL FR1 consists of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the FR1 contained within Philotop VL domain SEQ ID NO: 4 or Philotop VL kv4-72-derived region SEQ ID NO: 5,

VL FR2 consists of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the FR1 contained within Philotop VL domain SEQ ID NO: 4 or Philotop VL kv4-72-derived region SEQ ID NO: 5,

VL FR3 consists of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the FR1 contained within Philotop VL domain SEQ ID NO: 4 or Philotop VL kv4-72-derived region SEQ ID NO: 5, and wherein at least one VL complementarity determining region comprises a variably mutated amino acid sequence.

Preferably, the VH CDR3 and/or VL CDR3 in the antibody library comprises a variably mutated amino acid sequence.

Residues other than the variably mutated residues may be as shown in the Philotop VH and VL domain sequences. This does not, of course, exclude the possibility that the library will contain antibody molecules in which random errors in the sequence have resulted in accidental mutations from the sequences shown - such mutations occur naturally and typically arise by errors in DNA replication or in transcription or translation. Antibody molecules of the invention may therefore contain non-germline residues or other mutations at positions other than those identified herein as being variably mutated, or that some antibody molecules in a library will contain residues that are different from those identified herein. However, it may be that in at least 90 %, 95% or 99% of VH domains a library only the VH CDR3 residues are variably
mutated and/or different from the residues as shown in the Philotop VH or VL domain sequence, and that in at least 90 %, 95% or 99% of VL domains in a library only the VL CDR3 residues are variably mutated and/or different from the residues as shown in the Philotop VH or VL domain sequence.

Optionally, there may be other mutated (e.g. substituted) residues in the VH and/or VL domains. Mutation may for example comprise substitution of a germline residue for a non-germline residue, or substitution of a non-germline residue for a germline residue. For example a VH or VL domain framework may have up to five, e.g. one, two or three mutated residues in addition to the variably mutated residues. The same mutated residue or residues in the VH and/or VL domain may optionally be present in all or most antibody molecules in the library, e.g. at least 90 %, 95% or 99 % of antibody molecules.

CDR1 sequences may be identical in at least 90 %, 95 %, 99 % or in 100 % of antibody molecules in the library, and CDR2 sequences may be identical in at least 90 %, 95 %, 99 % or in 100 % of antibody molecules in the library, such that sequence diversity within the VH domains of the library is substantially restricted to CDR3.

VH domains of a library may comprise

- a VH CDR1 comprising or consisting of an amino acid sequence at least 90%, 95% or 99% identical to the CDR1 sequence contained within Philotop VH domain SEQ ID NO: 1 or Philotop VH 186.2-derived region SEQ ID NO: 2, and
- a VH CDR2 comprising or consisting of an amino acid sequence at least 90%, 95% or 99% identical to the CDR2 sequence contained within Philotop VH domain SEQ ID NO: 1 or Philotop VH 186.2-derived region SEQ ID NO: 2.

VL domains of a library may comprise

- a VL CDR1 comprising or consisting of an amino acid sequence at least 90%, 95% or 99% identical to the CDR1 sequence contained within Philotop VL domain SEQ ID NO: 4 or Philotop VL kv4-72-derived region SEQ ID NO: 5, and
- VL CDR2 comprising or consisting of an amino acid sequence at least 90%, 95% or 99% identical to the CDR2 sequence contained within Philotop VL domain SEQ ID NO: 4 or Philotop VL kv4-72-derived region SEQ ID NO: 5.

The VH domain amino acid sequence of antibody molecules in the library preferably comprises the amino acid sequence of Philotop VH 182.2-derived region SEQ ID NO: 2. The VL domain
amino acid sequence of antibody molecules in the library preferably comprises the amino acid sequence of Philotop VL kv4-72-derived region SEQ ID NO: 5.

Preferably, the number of variably mutated residues in a CDR, e.g. CDR3, e.g. VH CDR3, is four, five or six residues.

A variably mutated CDR may comprise or consist of a randomised sequence of variable length.

Preferably, a library comprises VH domains in which CDR3 comprises a randomised sequence of four residues, VH domains in which CDR3 comprises a randomised sequence of five residues, and VH domains in which CDR3 comprises a randomised sequence of six residues.

A sequence of residues Cys-Ala-Arg (CAR) may immediately precede the VH CDR3. The Cys-Ala-Arg sequence may be present at residue numbers 92 to 94.

Numbering of antibody domains herein is according to Tomlinson, Cox et al. 1995, as illustrated in Figures 1 and 6. See also Chothia and Lesk 1987.

The first residue (R1) of a VH CDR3 may be residue 95. The first residue (R1) of a VL CDR3 may be residue 91.

VH domains of a library may comprise a FR4 sequence comprising an Asp or Asn residue in the first three residues following VH CDR3. The Asp or Asn residue may be present at the central position of the three, or at residue number 101. The FR4 sequence preferably terminates with residues TVSS.

A VL domain may comprise a CDR3 containing a Pro residue, e.g. a single Pro residue. Pro may be present in most (e.g. at least 90 or 95 %) or all VL domain CDR3 sequences in the library. VL domains may comprise a CDR3 comprising or consisting of a sequence of sequence of six amino acid residues containing five randomised amino acid residues and a proline residue.

Preferably, the proline residue is in a conserved position, e.g. at the 5th position in the sequence of six amino acid residues, or at residue 95. For example, at least 90 or 95 % or all VL domain CDR3 sequences in the library may comprise Pro at the conserved position.
In VL domains, a sequence of residues Cys-Gln-Gln (CQQ) may immediately precede the VL CDR3. The Cys-Gln-Gln sequence may be at residues 88-90.

The VH domains and/or VL domains of a library may comprise a FR4 sequence derived from mouse germline. VH FR4 may comprise or consist of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the Philotop VH domain FR4 SEQ ID NO: 3. VL FR4 may comprise or consist of an amino acid sequence at least 90%, 95% or 99% identical to the sequence of the Philotop VL domain FR4 SEQ ID NO: 6.

A VH domain FR4 may comprise Asp as the second residue following the end of VH CDR3, and/or Tyr as the third residue, and/or Gln as the sixth residue and/or Ser as the fourteenth residue, e.g. as shown in SEQ ID NO: 3.

Preferably, VH domains comprise a PHILOtop VH FR4 sequence SEQ ID NO: 3. Preferably, VL domains comprise a PHILOtop VL FR4 sequence SEQ ID NO: 6.

VH domains of a library may comprise the following sequence:

SEQ ID NO: 2 - R1 - R2 - R3 - R4 - R5 - R6 - SEQ ID NO: 3

wherein

R1, R2, R3 and R4 are amino acid residues;
R5 is an amino acid residue or is not present; and
R6 is an amino acid residue or is not present.

VL domains of a library may comprise the following sequence:

SEQ ID NO: 5 - R1 - R2 - R3 - R4 - Pro - R6 - SEQ ID NO: 6

wherein

R1, R2, R3, R4 and R6 are amino acid residues.

The antibody molecules of the library according to the invention may be scFv antibody molecules. An scFv comprises a VH domain and a VL domain joined by a peptide linker. Any
suitable peptide linker may be used. An example is the PHILOtop 14 residue linker SEQ ID NO: 7, as illustrated in the sequence listing.

A specific example of an scFv of the Philotop library is shown in Supplementary Table 1, using X to represent the R residues.

Antibody molecules in a library may comprise other sequences in addition to antibody sequences, for example antibody molecules may be conjugated as fusion proteins to linker peptides and/or peptide tags for purification, isolation or detection, and/or to polypeptides for display of the antibody molecules such as coat protein of filamentous phage as discussed in detail below. For example, a myc tag may be present, and may conveniently be fused to the end of the VL domain. The myc tag sequence AAEQKLISEEDLNGAA is shown in Figure 1A. One or more spacer amino acids may be included e.g. to facilitate cloning and/or expression. For example, a Gly residue may be included at the C terminus of a VL domain if desired.

A library according to the invention may comprise in the order of 10^4 antibody molecules upwards, for example at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9 or at least 10^10 antibody molecules.

A variety of different antigen-binding antibody molecules are known. Any suitable antibody molecule format may be used in a library of the invention. Antibody molecules may be whole antibodies, which have four polypeptide chains - two identical heavy chains and two identical light chains. The heavy and light chains form pairs, each having a VH-VL domain pair that contains an antigen binding site. The heavy and light chains also comprise constant regions.

As noted, the present invention is concerned with mouse antibody libraries. Thus, where constant domains are present these may be mouse antibody constant domains.

Conveniently, antibody fragments and smaller antibody molecule formats, such as single chain antibody molecules, may be used in libraries according to the invention. For example, the antibody molecules may be scFv molecules, consisting of a VH domain and a VL domain joined by a linker peptide. In the scFv molecule, the VH and VL domains form a VH-VL pair in which the complementarity determining regions of the VH and VL come together to form an antigen binding site. A peptide linker, e.g. the PHILOtop 14 residue linker may be used to link the VH and VL domain.
Other antibody fragments that comprise an antibody antigen-binding site include, but are not limited to, (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment [1, 2, 3], which consists of a VH or a VL domain; (v) isolated CDR regions; (vi) F(ab')2 fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site [4, 5]; (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; [6]). Fv, scFv or diabody molecules may be stabilized by the incorporation of disulphide bridges linking the VH and VL domains [7].

Various other antibody molecules including one or more antibody antigen-binding sites have been engineered, including for example Fab2, Fab3, diabodies, triabodies, tetrabodies and minibodies (small immune proteins). Antibody molecules and methods for their construction and use are described in [8].

Minibodies or small immune proteins (SIP) comprise scFv joined to a heavy chain constant domain CH3 and/or CH4 [9, 10]. For example, an antibody molecule may be an SIP comprising an scFv molecule fused to the CH4 domain of IgE.

Other examples of binding fragments are Fab', which differs from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain, including one or more cysteines from the antibody hinge region, and Fab'-SH, which is a Fab' fragment in which the cysteine residue(s) of the constant domains bear a free thiol group.

A dAb (domain antibody) is a small monomeric antigen-binding fragment of an antibody, namely the variable region of an antibody heavy or light chain [3]. VH dAbs occur naturally in camelids (e.g. camel, llama) and may be produced by immunizing a camelid with a target antigen, isolating antigen-specific B cells and directly cloning dAb genes from individual B cells. dAbs are also producible in cell culture. Their small size, good solubility and temperature stability makes them particularly physiologically useful and suitable for selection and affinity maturation. Camelid VH dAbs are being developed for therapeutic use under the name "nanobodies™".
Synthetic antibody molecules may be created by expression from genes generated by means of oligonucleotides synthesized and assembled within suitable expression vectors, for example as described by Knappik et al. [11] or Krebs et al. [12].

Bispecific or bifunctional antibodies form a second generation of monoclonal antibodies in which two different variable regions are combined in the same molecule [13]. Their use has been demonstrated both in the diagnostic field and in the therapy field from their capacity to recruit new effector functions or to target several molecules on the surface of tumour cells. Where bispecific antibodies are to be used, these may be conventional bispecific antibodies, which can be manufactured in a variety of ways [14], e.g. prepared chemically or from hybrid hybridomas, or may be any of the bispecific antibody fragments mentioned above. These antibodies can be obtained by chemical methods [15, 16] or somatic methods [17, 18] but likewise and preferentially by genetic engineering techniques which allow the heterodimerization to be forced and thus facilitate the process of purification of the antibody sought [19]. Examples of bispecific antibodies include those of the BiTE™ technology in which the binding domains of two antibodies with different specificity can be used and directly linked via short flexible peptides. This combines two antibodies on a short single polypeptide chain. Diabodies and scFv can be constructed without an Fc region, using only variable domains, potentially reducing the effects of anti-idiotypic reaction.

Bispecific antibodies can be constructed as entire IgG, as bispecific Fab'2, as Fab'PEG, as diabodies or else as bispecific scFv. Further, two bispecific antibodies can be linked using routine methods known in the art to form tetravalent antibodies.

Bispecific diabodies, as opposed to bispecific whole antibodies, may also be particularly useful because they can be readily constructed and expressed in E.coli. Diabodies (and many other polypeptides, such as antibody fragments) of appropriate binding specificities can be readily selected e.g. using phage display. If one arm of the diabody is to be kept constant, for instance, with a specificity directed against an antigen of interest, then a library can be made where the other arm is varied and an antibody of appropriate specificity selected. Bispecific whole antibodies may be made by alternative engineering methods as described in Ridgeway et al., 1996 [20].

A library according to the invention may be used to select an antibody molecule that binds one or more antigens of interest. Selection from libraries is described in detail below. Following
selection, the antibody molecule may then be engineered into a different format and/or to contain additional features. For example, the selected antibody molecule may be converted to a different format, such as one of the antibody formats described above. The selected antibody molecules, and antibody molecules comprising the VH and/or VL CDRs of the selected antibody molecules, are an aspect of the present invention. Antibody molecules and their encoding nucleic acid may be provided in isolated form.

Antibody fragments can be obtained starting from an antibody molecule by methods such as digestion by enzymes e.g. pepsin or papain and/or by cleavage of the disulphide bridges by chemical reduction. In another manner, the antibody fragments can be obtained by techniques of genetic recombination well known to the person skilled in the art or else by peptide synthesis by means of, for example, automatic peptide synthesizers, or by nucleic acid synthesis and expression.

It is possible to take monoclonal and other antibodies and use techniques of recombinant DNA technology to produce other antibodies or chimeric molecules that bind the target antigen. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the CDRs, of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP-A-184187, GB 2188638A or EP-A-239400, and a large body of subsequent literature.

Antibody molecules may be selected from a library and then modified, for example the in vivo half life of an antibody molecule can be increased by chemical modification, for example PEGylation, or by incorporation in a liposome.

An antibody molecule selected from the library may be fused to a cytokine, e.g. murine cytokine, allowing the use of syngeneic mouse models of disease. The use of fully murine immunocytokines would enable more significant therapy studies.

A library according to the invention may be screened for antibody molecules that bind one or more antigens of interest, and/or that bind a particular region or epitope of an antigen of interest.

A library of antibody molecules according to the invention may be used for screening for an antibody molecule that binds one or more antigens, e.g. using biopanning techniques or iterative colony filter screening.
A library of antibody molecules according to the invention may optionally be combined with one or more additional libraries. However, one advantage of the invention is that it already provides a "single pot" antibody library that may be used alone for screening and selection of antibodies to a given target molecule. Thus, we envisage that the antibody library would comprise only the antibody molecules as described herein.

The present invention provides a method of obtaining one or more antibody molecules able to bind an antigen, the method including bringing into contact a library of antibody molecules according to the invention and said antigen, and selecting one or more antibody molecules of the library able to bind said antigen.

A method may comprise:

- providing a library according to the invention; and
- contacting the library with the antigen, so that the antigen binds to one or more antibody molecules in the library; and
- selecting nucleic acid encoding an antibody molecule that binds to the antigen.

The selecting step may comprise isolating the antibody molecule that is bound to the antigen, for example the antigen may be attached to magnetic beads or other molecules that may be recovered, thereby also recovering the antibody. The antibody molecule may be linked to its encoding nucleic acid, e.g. it may be part of a particle or replicable genetic package that contains the nucleic acid. Alternatively the selecting step may comprise isolating bacteria that express the antibody molecule, such as in the technique of iterative colony filter screening as described below. Nucleic acid encoding the antibody molecule that binds the antigen may then be isolated, if desired.

As discussed earlier, a variety of library formats and suitable screening methods are known.

A library of antibody molecules may be a bacterial library, e.g. *E. coli*. Thus, the antibody molecules may be expressed in bacteria. This may be achieved by providing bacteria containing nucleic acid molecules encoding the antibody molecules of the library, and culturing the bacteria so that they express the antibody molecules. Nucleic acid molecules encoding the antibody molecule library are an aspect of the invention, as are bacteria containing such nucleic acid. The bacteria may conveniently be stored as glycerol stocks.
Antibody molecules may be secreted from bacteria. This allows use of the technique of iterative colony filter screening (ICFS), a two-filter sandwich assay in which hundreds of millions of antibody-expressing bacterial colonies can be screened (Giovannoni 2001). In ICFS, bacterial cells (typically E. coli) expressing the library are grown on a porous master filter in contact with a second filter coated with the antigen of interest. Antibody molecules are secreted by the bacteria and diffuse on to the second filter and thus are brought into contact with the antigen. Detection of antigen binding on the second filter allows the recovery of a number of bacterial cells, including those expressing the binding specificity of interest. In turn, those bacteria may be submitted to a second round of screening for the isolation of specific antibody molecules. Iteration of the steps refines the population of selected antibody molecules. Using this methodology, a number of specifically binding antibodies of different amino acid sequences may be recovered.

Alternatively, antibody molecules of a library may be displayed on particles or molecular complexes, rather than secreted. Suitable replicable genetic packages include yeast, bacterial or bacteriophage (e.g. T7) particles, viruses, cells or covalent, ribosomal or other in vitro display systems, each particle or molecular complex containing nucleic acid encoding the antibody VH variable domain displayed on it, and optionally also a displayed VL domain if present.

Selections using cells or protein mixtures have also been documented in the literature [21, 22, 23] and these techniques may be applied in the present invention.

Phage display is an established technique for selection of antibody molecules of desired specificity, in which the library of antibody molecules is displayed on filamentous bacteriophage (Winter, Griffiths et al. 1994). See also WO92/01047, 24, 25. Filamentous bacteriophage are viruses that infect bacteria, and thus the phage library may be maintained in a bacterial library. The antibody molecules may be fused to an inner coat protein pill or to the major coat protein pVIII of the phage by inserting synthetic DNA encoding the peptide into phage gene III or gene VIII respectively. Three (or possibly five) copies of pill are thought to be located at the tip of the phage particle and about 500 copies of pVIII are thought to be present per phage. pill is responsible for attachment of the phage to the bacterial F-pilus and for infection, and pVIII is responsible for coating the single stranded phage DNA. The pill protein has two domains. Fusions can be made to the N terminus of pill or the N-terminal domain can be removed and fusions made to the second domain; however, phage lacking the N-terminal domain are not infective. A gene encoding scFv or other single chain antibody
molecule can be inserted into gene III, resulting in expression of the antibody molecule fused to the N terminus of pill and incorporated into the phage, allowing the phage to bind antigen. Dimeric antibody fragments, e.g. heterodimeric Fab, can be displayed by linking the heavy or light chain to a coat protein and secreting the other chain into the bacterial periplasm, where the two chains associate.

Nucleic acid molecules according to the invention may comprise a nucleotide sequence encoding an antibody molecule fused to a coat protein of filamentous bacteriophage, e.g. pill or pVIII. Such nucleic acid molecules may be used either to express the library of antibody molecules displayed on phage that infect bacteria or to obtain soluble antibody secreted from the bacteria. By inserting an amber stop codon between the antibody molecule gene and the coat protein gene, when phage is grown in an amber suppressor strain of E. coli the amber codon is read as an amino acid and the antibody fused to the coat protein is displayed on the surface of the phage. When the phage is grown in a non-suppressor strain, the amber codon is read as a stop codon, and soluble protein is secreted from the bacteria. As illustrated in Figure 1, the amber codon may conveniently be included between sequences encoding a purification tag and the coat protein sequence, respectively.

Following selection of antibody molecules able to bind the antigen and displayed on bacteriophage or other library particles or molecular complexes, nucleic acid may be taken from the phage or other particle or molecular complex displaying a said selected antibody molecule. Such nucleic acid may be used in subsequent production of an antibody molecule or an antibody VH or VL variable domain by expression from nucleic acid with the sequence of nucleic acid taken from a bacteriophage or other particle or molecular complex displaying a said selected antibody molecule.

Thus, following selection, nucleic acid encoding the antibody molecule that binds the antigen may be expressed to produce the antibody molecule. Optionally the antibody molecule and/or its encoding nucleic acid may be subjected to further modifications, such as antibody reformatting as discussed elsewhere herein. The antibody molecule and/or its encoding nucleic acid may be formulated into compositions for therapeutic or diagnostic use. Thus, methods of the invention may comprise formulating an antibody molecule and/or its encoding nucleic acid into a composition comprising a pharmaceutically acceptable excipient, following isolation from the library and optional subsequent modifications.
As described elsewhere herein, the antibody libraries of the invention are particularly suitable for use in providing antibodies for treatment and/or diagnostic use in mice, especially in mouse models of human disease.

Once one or more antibody molecules have been selected from the library, the antibody molecules may be further characterised to determine their properties in a variety of assays according to the purpose for which the antibody molecule is intended. Assays may include determining affinity of the antibody molecule for binding the antigen or antigens of interest, cross-reactivity with other antigens, epitope mapping to determine which region of an antigen is bound by the antibody molecule, immunohistochemistry, and other in vitro or in vivo tests. Certain steps in the antibody isolation procedure and in the downstream characterisation of binding specificities may be robotised, as described for the ETH2-Gold library [26].

In particular, antibody libraries according to the invention provide a suitable platform for the isolation of antibodies that display species cross-reactivity, especially cross-reactivity between human and mouse i.e. the antibody binds the mouse antigen and the corresponding human antigen. Antibody libraries of the invention are especially suited for screening with antigens that are conserved between human and mouse, or which have conserved domains, such as the extra domains of fibronectin (e.g. ED-B) and of tenascin C.

The design of the antibody libraries of the invention is compatible with affinity maturation strategies, based on library construction by combinatorial mutagenesis of residues in CDR1 and/or CDR2 loops. Such strategies have extensively been used by our group in the past (Pini, Viti et al. 1998; Brack, Silacci et al. 2006; Silacci, Brack et al. 2006; Villa, Trachsel et al. 2008), yielding the L19, F16 and F8 antibodies, whose derivatives are currently being investigated in multiple clinical trials (Santimaria, Moscatelli et al. 2003; Sauer, Erba et al. 2009).

**Experimental Examples**

We describe herein the design, construction and functional characterisation of three large synthetic mouse antibody libraries, containing more than one billion antibody clones each. The libraries (termed "MuLib1", "MuLib2" and "PHILoTop") feature the use of single chain Fv antibody fragments (Huston, Levinson et al. 1988) fused to the minor coat protein pill of filamentous phage (Hoogenboom, Raus et al. 1990; McCafferty, Griffiths et al. 1990; Hoogenboom, Griffiths et al. 1991). The large majority of the antibody clones tested before
selection of antigen exhibited a good expression in the bacterial supernatant and an efficient
display on filamentous phage. However, among the three libraries, only the PHILOtop library
has consistently yielded specific binders against a variety of different antigens, including
monoclonal antibodies to "difficult" tumour-associated antigens such as the alternatively-
spliced EDA and EDB domains of fibronectin (Zardi, Carnemolla et al. 1987; Peters, Chen et al.

The three libraries were considered to be equally promising from the design, the cloning and
the performed quality controls. Only biopanning experiments revealed that MuLJb1 and MuLib2
were unable to yield antibody to the majority of the tested antigens. Furthermore, an analysis a
posteriori revealed that only the positive antibody clones raised from the MuLJb1 and MuLib2
were able to bind to protein L, while unselected clones from the same libraries did not display
such binding properties (data not shown). This suggests that the MuLJb1 and MuLib2 library
antibody clones do not fold properly, resulting in a very poor selection outcome.

In contrast, the PHILOtop library was shown to reliably yield good-quality antibodies towards
all protein antigens used so far in selection experiments.

As an illustrative example of the practical performance of the PHILOtop library, we describe
the isolation of a specific monoclonal antibody to the ED-B domain of fibronectin, a marker of
angiogenesis (Carnemolla, Neri et al. 1996), showing the ability of the antibody to recognise
the native antigen in ELISA, real-time interaction analysis in a BIAcore 3000 instrument, as
well as in sections of F9 teratocarcinoma.

Materials and Methods

Growth media, helper phage, and general procedures used for library selections and screening
procedures were essentially as described by Viti et al. (Viti, Nilsson et al. 2000). Unless stated
otherwise, chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland).

Library construction and cloning

Synthetic genes (GenScript Corporation; NJ, USA) were used as templates for the PCR
amplification of the heavy variable chain (VH) genes 98-3G (Williams, Martinez et al. 2001)
and 186 (Bothwell, Paskind et al. 1981; Williams, Martinez et al. 2001 ; Chang and Mohan
2005), and for the amplification of the light variable chains (Vk) genes dv-36 (Schable, Thiebe
et al. 1999), V1C (Corbet, Milili et al. 1987; Ng, Lavigneur et al. 1989) and kv4-72 (Kirschbaum,
Roschenthaler et al. 1999). The amplified fragments were used for the construction in the scFv
format of the MuLib1 library (98-3G/dv-36), the MuLib2 library (98-3G/V1C) and the PHILOtop
library (186/kv4-72); the linker Gly4SerGly4SerGly4 was introduced between the heavy and
the light variable chain amplified genes (Figure 1). The resulting scFv segments were used for
library construction as follows.

Antibody residues are numbered according to (Chothia and Lesk 1987; Tomlinson, Cox et al.
1995), and are indicated in Figure 1A. Sequence variability in the variable heavy chains
component of the libraries was introduced by PCR using partially degenerated primers (Figure
1B, Table 1), in a process that generate random mutations at position 95-99 of the VH CDR3.
The variable light chain components of the libraries were generated in a similar fashion,
introducing random mutations at position 91, 92, 93, 94 and 96 in the Vk CDR3 (Figure 1B,
Table 1).

VH/Vk combinations were assembled in scFv format by PCR assembly (Figure 1B), using gel
purified VH and Vk segments as templates. The assembled VH/Vk fragments were doubly-
digested with Ncol/NotI (New England Biolabs; MA, USA) and cloned (T4 DNA ligase, New
England Biolabs; MA, USA) into Ncol/NotI-digested pHEN1 phagemid vector (Hoogenboom,
Griffiths et al. 1991). The resulting ligation product was electroporated into electrocompetent
Escherichia coli TG1 cells according to Viti et al. (Viti, Nilsson et al. 2000). The libraries were
electroporated each on three different days, thereby obtaining three different sub-libraries for
each library, named MuLib1 H4K, H5K and H6K, Mulib2 H5K, H6K and H7K, and PHILOtop
H4K, H5K and H6K. The libraries were stored as glycerol stocks, rescued and used for phage
production according to standard protocols (Viti, Nilsson et al. 2000).

Library characterization

A total of 36 clones for each library were tested by PCR screening using the primers LMB3long
and fdseqlong (Table 1) and the REDTaq ReadyMix to verify the correct size of the insert. For
all the libraries fifteen clones (five for each sub-library) were selected at random and
sequenced (Big Dye Terminator v1.1 Cycle Sequencing kit; ABI PRISM 310 Genetic Analyzer;
Applied Biosystems, Foster City, CA, USA) to check for the absence of frameshifts and
pervasive contamination. The percentage of clones expressing soluble antibody fragments
was determined by dot blot analysis of bacterial supernatants (ELIFA system; Perbio, Lausanne, Switzerland) using anti-myc mouse mAb 9E10 (Marks, Hoogenboom et al. 1991) and anti-mouse horseradish peroxidase immunoglobulins (Sigma-Aldrich) as detecting reagents or Protein L-horseradish peroxidase (Pierce; Rockford, IL, USA). Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences). The display of the scFv-plll fusion protein on the phage surface was evaluated by Western blot. Different amounts of purified phage were loaded on a SDS gel and then transferred to NC membrane (Protran BA 85; Schleicher & Schuell, Dassel Germany). As detecting reagents anti-pill mouse mAb (MoBiTec; Göttingen, Germany) and anti-mouse horseradish peroxidase immunoglobulins (Sigma-Aldrich) were used. Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences).

Antigens

Human α-2-Macroglobulin was purchased by BIODESIGN International (MILAN ANALYTICA AG, La Roche, Switzerland). Glutathione-s-transferase (GST) was expressed and purified from the GST Gene Fusion Vector pGEX-4-T2 (Amersham Biosciences) according to the manufacturer's instructions. Human apo-transferrin as well as bovine Hemoglobin were purchased by Sigma-Aldrich. The recombinant EDA containing human fibronectin fragment (termed 11A12) (Borsi, Castellani et al. 1998); the recombinant EDB containing human fibronectin fragment (termed 7B89) (Carnemolla, Neri et al. 1996); and the recombinant domain C of human tenascin-C (termed BCD) (Silacci, Brack et al. 2006) were expressed and purified as previously described.

Library selections on immunotubes

All selections were performed using recombinant or commercially available antigens with purity >90%. Immunotubes (Nunc; Wiesbaden, Germany) were coated with antigen at a concentration of 50 mg/ml in PBS, over-night at room temperature. Immunotubes were then rinsed with PBS and blocked for 2 hours at room temperature with 2% MPBS. After rinsing with PBS, >10^{12} phage particles in 2% MPBS were added to the immunotubes. The immunotubes were first incubated on a shaker for 30 minutes and then for 90 minutes standing upright at room temperature. Unbound phages were washed away by rinsing the immunotubes ten times with PBS 0.1% Tween 20 and ten times with PBS. The bound phages were eluted in 1 ml of 100 mM triethylamine and inverting the tube for 7 minutes. Triethylamine was neutralized by
adding 0.5 ml 1 M Tris-HCl pH 7.4. The eluted phages were used for the infection of exponentially growing *E. coli* TG1, for 40 min at 37°C. Dilution series of bacteria were then plated on small 2xTY agar plates, 100 µg/ml ampicillin (Applichem; Darmstadt, Germany), 0.1% glucose (2xTY-Amp-Glu), and incubated at 30°C overnight to determine the titer of the eluted phage. The remaining phage-infected bacteria were centrifuged for 10 min at 3300 g and 4°C, the pellet resuspended in 0.5 ml 2xTY and spread on a large 2xTY-Amp-Glu agar plate and incubated at 30°C overnight. The following day the bacteria were rescued from the large plate using 5 ml 2xTY, 10% glycerol and a sterile glass loop. The rescued bacteria were stored at -80°C. The rescued bacteria were used to inoculate 50 ml 2xTY-Amp-Glu (initial OD<sub>600 nm</sub> between 0.05 and 0.1) and the culture was grown at 37°C and 200 rpm until OD<sub>600 nm</sub> 0.4 - 0.5. Of this culture, 10 ml (4 - 5x10<sup>8</sup> bacteria/ml) were infected with 100 µl helper phage VCS-M13 (1x1 0<sup>12</sup> phage) at 37°C for 40 min. The bacteria were then centrifuged for 10 min at 3300 g and 4°C, the pellet resuspended in 100 ml 2xTY-Amp-Glu including kanamycin (2xTY-Amp-Kan-Glu) and the culture incubated at 30°C overnight. The overnight culture was centrifuged for 30 min at 3300 g and 4°C. The supernatant was transferred to new bottles and 10 ml 20% PEG/2.5 M NaCl was added for each 40 ml of supernatant. The mixture was incubated on ice for 40 min and then centrifuged for 30 min at 3300 g and 4°C. The pellet was resuspended in 40 ml sterile H<sub>2</sub>O and 10 ml PEG/NaCl was added. The mixture was again incubated on ice for 40 min and then centrifuged for 30 min at 3300 g and 4°C. The pellet containing the precipitated phage was resuspended in 2 ml sterile PBS containing 15% glycerol. In order to remove cell debris, the solution was then centrifuged for 3 min at 15000 g and the pellet was discarded. Phage used on the same day for a further round of panning were kept on ice; otherwise they were stored at -20°C. More details about selection protocols can be found in Viti et al (Viti, Nilsson et al. 2000).

**ELISA screening**

Bacterial supernatants containing scFv fragments were screened for binding to antigen by ELISA essentially as described (Viti, Nilsson et al. 2000). Individual colonies were inoculated in 180 µl 2xYT-Amp-Glu in 96-well plates (Nunclon Surface, Nunc). The plates were incubated for 3 hours at 37°C in a shaker incubator. The cells were then induced with isopropyl-thiogalactopyranoside (IPTG; Applichem) at a final concentration of 1 mM, and grown overnight at 30°C. The bacterial supernatants assayed were tested in ELISA experiment as described in (Marks, Hoogenboom et al. 1991) using the anti-myc tag 9E10 mAb and anti-mouse horseradish peroxidase immunoglobulins.
Immunohistochemistry on frozen tissue sections

Immunohistochemistry with anti-EDB scFv fragment (C12) was performed essentially as described by Brack et al. 2006. Ten µm thick sections were treated with ice-cold acetone, rehydrated in TBS (50mM Tris, 100mM NaCl, pH 7.4), blocked with fetal bovine serum (Invitrogen) and then incubated with supernatant of *E.coli* cultures secreting soluble murine scFv (myc-tagged) together with the biotinylated 9E10 anti-myc antibody (5 µg/ml). Bound antibody was detected using streptavidirrbiotinylated alkaline phosphatase complex (Biospa, Milano, Italy) and subsequent staining reaction with Fast-Red TR (Sigma). Hematoxylin solution (Sigma) was used for counterstaining.

**PROTOCOL:**

**REAGENTS**

- Glycergel mounting Medium Dako 0563
- Gill Hematoxilin Solution n.2 Sigma HHS-16
- FastRED TABLETTES
- Aprotinin Sigma A6279
- Silicon Pen (DAKO)

**TBS:**

- 50mM TRIS
- 100 mM NaCl
- adjust pH 7.4
- 0.01%Aprotinin

**Immunohistochemistry with biotinylated 9E10 for the detection of scFv antibodies:**

- Fix the sections in cold aceton (-20°C). Do not use plastic dishes 10’
- Let the slides dry at RT 30”
- evt. apply silicon with a pen, then: Immerse in TBS 5’
- dry back of the slides with paper without touching the sections
- Block with 100% FCS in TBS, >100 µl/section
  pour off blocking sol. and submerge 5’ in TBS 30’
- Add the primary antibodies (C12 scFv ~20 ng/µl)
  Incubate in a moist chamber 1h
• Wash with TBS 2 x 5'
  
Add biotinylated 9E10 (e.g. OD 0.38 dil 1:15), diluted in TBS/3%BSA, 100 µl/section 1h 
Incubate in a moist chamber 
• Wash with TBS 2 x 5'
  
• Add Streptavidin-Alkaline Phosphatase (Biospa F014-62) 1:150 diluted in TBS/3% BSA 30'
  
Incubate in a moist chamber 
• Wash with TBS 2 x 5'
  
• Dry with paper the back of the slides 
• Add the substrate (dissolve fastRed powder in Sol. Fast Red as already explained in the "Buffer" section). Use 500 µl/slide. 15'
  
Incubate in a moist chamber 
• Transfer the slides in GillIs Hematoxilin solution n.2 for 2'
  
• Quickly transfer the slides in water and rinse with water for 6 times 
• Mount the slides with Glycergel 

For the negative control we omit to incubate the tissues with the anti-EDB antibody.

Results

In order to generate a large, stable and highly diverse library of functional antibody fragments with similar biophysical properties, we chose to clone our synthetic antibody libraries in a phagemid vector of proven quality (Hoogenboom, Griffiths et al. 1991; Silacci, Brack et al. 2005), restricting sequence diversity to the CDR3 of VH and Vκ domains, in which all other positions were kept constant (Pini, Viti et al. 1998). We chose to express antibody fragments in scFv format (Huston, Levinson et al. 1988), as this single polypeptide recombinant antibody format exhibits better expression yields and phage display properties compared to Fab fragments. The complete sequences of the three scFv libraries can be found in Supplementary Table 1 and/or in the sequence listing.

Design, construction and characterization of MuLibi murine antibody library

The scFv antibody scaffold used for the MuLibi library was based on the germline gene 98-3G for the VH domain (Williams, Martinez et al. 2001), and on the gene dv-36 (Schable, Thiebe et al. 1999) for the Vκ domain. The 98-3G and dv-36 genes were chosen as scaffolds for library construction due to their similarity to the VH and Vκ domains DP47 and DPK22, previously
used by our group for antibody library construction (Pini, Viti et al. 1998; Silacci, Brack et al. 2005). Sequence diversity was confined to the CDR3 loops, which are known to largely contribute to antigen recognition (Figure 1B), while the remaining parts of the antibody molecule were kept constant. A completely randomized sequence of four, five or six amino acids residues (followed by the conserved Phe-Asp-Tyr sequence) was appended to the VH germline segment giving rise to the three sub-libraries H4K, H5K and H6K, respectively. A partially randomized sequence of six amino acid residues was appended in the Vk, forming CDR3 loops which contained a fixed proline residue in a conserved position (Figure 1A). The flexible polypeptide Gly4SerGly4SerGly4 (Huston, Levinson et al. 1988) was used to link the variable heavy chain to the variable light chain in the scFv fragments. The resulting scFv antibody fragments were cloned in the phagemid vector pHENM (Hoogenboom, Griffiths et al. 1991), which appends the short peptidic myc-tag at the C-terminal extremity of the recombinant antibody (Figure 1B).

For library construction, CDR3 regions were randomized by PCR using partially degenerate primers (Table I and Figurei). The resulting VH and Vk gene segments were assembled by PCR, subcloned into the phagemid vector pHEN1 and electroporated into freshly prepared electrocompetent E.coli TG1 cells. Three different sub-libraries (H4K, H5K and H6K) contained a total of 1.5 x 10^9 individual clones.

The quality and functionality of the MuLibi library was assessed by PCR colony screening, dot blot, Western blot, DNA sequencing and test selection against standard antigens. PCR screening showed that 36/36 randomly picked individual clones from the library contained an insert on the correct size (Figure 2A). A dot blot experiment based on myc tag detection revealed that >70% of the antibody clones in the library expressed soluble scFv fragments (Figure 2B). The efficiency of the display of svFv-PLL fusion proteins on the surface of filamentous phage was evaluated by Western blot, using an anti-pill antibody as primary detection reagent. From the ratio of intensities of the bands corresponding to pill or scFv-PLL fusions (Figure 2C), and considering 3-5 copies of pill are present at the tip of the phage (Stengele, Brass et al. 1990), we estimated that at least every second phage particle would display a scFv-PLL fusion protein on its surface.

Fifteen randomly picked clones were sequenced, revealing that all amino acid sequences in the CDR3 regions of both heavy and light variable chains were diverse (data not shown). The functionality of the MuLJbI antibody library was tested performing selections against a panel of antigens on immunotubes (Table II).
Only biopanning experiments against the large plasma protein α-2-Macroglobulin resulted in the generation of positive clones, which were found to be specific for the cognate antigen and to carry at least three different amino acid sequences.

**Design, construction and characterization of MuLib2 murine antibody library**

The scFv antibody scaffold used for the MuLib2 library was based on the germline gene 98-3G for the VH (Williams, Martinez et al. 2001) as for the MuLibi library, but we chose the germline Vk gene V1C (Corbet, Milili et al. 1987; Ng, Lavigueur et al. 1989) for library construction. This gene frequently pairs with the 98-3G-derived VH domains in murine antibody sequences, as judged by a BLAST search of the UniProt Knowledgebase (Swiss-Prot + TrEMBL) database. Sequence diversity was confined to the CDR3 loops (Figure 1B), while the remaining parts of the antibody molecule were kept constant. A completely randomized sequence of five, six or seven amino acids residues (followed by the conserved Phe-Asp-Tyr sequence) was appended to the VH germline segment giving rise to the three sub-libraries H5K, H6K and H7K, respectively. A partially randomized sequence of six amino acid residues was appended in the Vk, forming CDR3 loops, while keeping a conserved proline residue fixed (Figure 1A). Library cloning was performed as described in the previous section for the MuLibi library and yielded three sub-libraries (H5K, H6K and H7K), which contained a total of 2.7 x 10^9 individual clones.

PCR screening confirmed that 36/36 individual clones randomly picked from the library contained an insert on the correct size (Figure 3A) and dot blot analysis revealed that ~80% of the analyzed library clones expressed soluble scFv fragments (Figure 3B). The analysis of antibody display on the minor coat protein pill revealed a display rate similar to the one observed for the MuLibi library (Figure 2C and 3C).

Fifteen randomly picked clones were sequenced, revealing that all amino acid sequences in the CDR3 regions of both heavy and light variable chains were diverse. The functionality of the MuLib2 antibody library was tested performing selections against a panel of antigens on immunotubes (Table II). Also for this library, however, functional and selective antibody binders could only be isolated when using α-2-Macroglobulin as an antigen.
Design, construction and characterization of PHILOtop murine antibody library

The PHILOtop library design was based on the germline gene 186 for the VH (Bothwell, Paskind et al. 1981; Williams, Martinez et al. 2001; Chang and Mohan 2005), and on the Vk germline gene kv4-72 (Kirschbaum, Roschenthaler et al. 1999).

The Philotop library uses a similar template based on the mouse 186 and kv4-72 germlines, but with some positions mutated and with variations in the CDR3.

Changes from germline in the Philotop VH region derived from 186.2 were as follows (Figure 6):

- M at residue 20
- N at residue 33
- T at residue 40
- A at residue 50
- Y at residue 52
- G at residue 53
- N at residue 54
- Y at residue 56
- S at residue 58
- Q at residue 61
- G at residue 65
- A at residue 71

A completely randomized sequence of four, five or six amino acids residues (followed by the conserved Phe-Asp-Tyr sequence) was appended to the VH germline segment giving rise to the three sub-libraries H4K, H5K and H6K, respectively. As for the previous libraries, five aminoacid positions were randomized in the CDR3 loop of the Vk domain (Figure 1A). The partially degenerate primers used for library construction can be found in Table I and Figure 1B. Library cloning yielded three sub-libraries (H4K, H5K and H6K) corresponding to the different length of the CDR3 loops of the VH domain, which contained a total of $1.5 \times 10^9$ individual clones.

PCR screening showed that 36/36 randomly picked clones from the library contained an insert on the correct size (Figure 4A). A dot blot experiment revealed that approximately 90% of the analyzed library clones expressed soluble scFv fragments (Figure 4B). Importantly, bacterial
supernatants that scored positive with an anti-myc reagent could also be detected with with Protein L. In line with the good antibody expression data observed in the bacterial supernatants, a Western blot analysis indicated that the band corresponding to pill was approximately four times more intense than the scFv-pill band (Figure 4C), compatible with an average expression of one antibody fragment per phage particle.

Fifteen randomly picked clones were sequenced, revealing that all amino acid sequences in the CDR3 regions of both heavy and light variable chains were diverse. However sequence analysis showed that 2/15 clones contained frameshift mutations. These results were in agreement with the soluble antibody expression data.

The functionality of the PHILOtop library was tested performing selection on immunotubes against a panel of antigens. With this library, it was possible to isolate specific antibody clones against all the tested antigens (Table II), including splice isoforms of fibronectin and of tenascin-C as biomedically-relevant tumor-associated antigens (Neri and Bicknell 2005; Schliemann, Palumbo et al. 2009). For the selections towards fibronectin splice isoforms, larger recombinant proteins containing the EDA or EDB domains were used in analogy to what previously described by our group (Camemolla, Neri et al. 1996; Borsi, Castellani et al. 1998), yielding at least 1 and 10 different sequences which were also capable of specific recognition of the recombinant EDA and EDB domains, respectively. Sequence analysis of individual selected clones revealed substantial amino acid diversity in the CDR3 regions of selected clones, with certain consensus preferences for antibody clones specific to the EDB domain of fibronectin and to transferrin.

In order to demonstrate that the clones isolated from the library performed well not only in ELISA but also for practical immunochemical applications, one anti-EDB antibody was produced in E.coli, purified to homogeneity and analyzed both by BIAcore and by immunohistochemistry on frozen tumor sections, confirming its ability to recognize the cognate antigen with good affinity and in tumor neo-vascular structures.

Immunohistochemistry experiments were performed probing the antibody isolated from the PHILOtop library on sections of F9 murine teratocarcinoma tumor grown in 129Sv Ev mice.

Figure 5 shows the stained tissue sections. Figure 5 A shows tissue treated with the antibody. Figure 5 B shows the negative control. The white parts of the sections (indicated for illustrative
purposes by the white arrow) correspond to the lumen of the blood vessels within the tumor mass.

The black parts of the section in Figure 5A (indicated for illustrative purposes by the black arrow) correspond to the staining of the ED-B domain of fibronectin a marker of angiogenesis which is known to have a peri-vascular pattern of expression.

In the negative control experiment Figure 5B no black staining of the ED-B domain of fibronectin around the vascular structures is visible.
Tables

Table I. The use of primers for the construction of the libraries is depicted in Figure 1

<table>
<thead>
<tr>
<th>Library</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MuLib1</strong> and <strong>PHILOtop</strong> primers</td>
<td></td>
</tr>
<tr>
<td>a LMB3long</td>
<td>5'-CAGGAAACAGCTATGACCATTAC-S'</td>
</tr>
<tr>
<td>b1 98-3G_CDR304ba</td>
<td>5'-TCCCTGACCCCCATGCTAAAAMMNMMMNMMNNACGGTGACAGTAATACATGGCC-3'</td>
</tr>
<tr>
<td>b2 98-3G_CDR305ba</td>
<td>5'-TCCCTGACCCCCATGCTAAAAMMNMMMNMMNNACGGTGACAGTAATACATGGCC-3'</td>
</tr>
<tr>
<td>c 98-3G_CDR306ba</td>
<td>5'-TTTGGCTTGGCCCCATGCTAAAAMMNMMMNMMNNACGGTGACAGTAATACATGGCC-3'</td>
</tr>
<tr>
<td>b 98-3G_CDR307ba</td>
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<tr>
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<td>5'-TCCCTGACCCCCATGCTAAAAMMNMMMNMMNNACGGTGACAGTAATACATGGCC-3'</td>
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M and N are defined according to the IUPAC nomenclature (M=A/C, N=A/G/T).
### Supplementary Table I. Aminoacid sequence of scFv libraries

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<th>Heavy chain</th>
<th>Linker</th>
<th>Light chain</th>
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</thead>
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<tr>
<td><strong>MuLib1</strong></td>
<td>EVQLVESGGGLVKPGG SLKLSCAASGFVTSSYA MSWVRQPSPEKRLEWVA EISSGGSYTTYFPDTVTG RFTISRDNKNTLYLEM SSLRSEDTAMYCARXX XX(X)(X)FDYWGWGQGTTL TVSS</td>
<td>GGGGSGGG GSGGGG</td>
<td>DIVLTQSPASLSVSLGE TATLSCRSSESVGSYLA WYQQKAEQVPRLLISH ASTRAGGVPRFSGTG SGTDFTLTISLEPEDAA VYYCQQXXXXPXTFGA GTKLEIKR</td>
</tr>
<tr>
<td><strong>MuLib2</strong></td>
<td>EVQLVESGGGLVKPGG SLKLSCAASGFVTSSYA MSWVRQPSPEKRLEWVA EISSGGSYTTYFPDTVTG RFTISRDNKNTLYLEM SSLRSEDTAMYCARXX XXX(X)(X)FDYWGWGQGTTL TVSS</td>
<td>GGGGSGGG GSGGGG</td>
<td>DIVMTQTPLSPLPGD QASISCRSSQIVHSNG NTYLEWYLQKPGQSPK LLIYKVSNRFSGVPDREF SGSQSGTDFTYLKISRVE AEDLGVYYCFQXXXXXP XTFGGGTKLEIKR</td>
</tr>
<tr>
<td><strong>PHILoTop</strong></td>
<td>QVQLQQPGAEILVKPGQA SVKMSCKASGTYFTSYN MHWVKQTPGRGLEWIG AIYPNGYTSYNQKFKG KATLTADKSSSTAYML SSSLTEDAVYYCARXX XX(X)(X)FDYWGWGQGTTL TVSS</td>
<td>GGGGSGGG GSGGGG</td>
<td>QIVLSQSPAILSSASPGEK VTMTCRASSVSYMHW FQQKPGSPKWYIAT SNLASCVPVRFSGSGS GTSYSLTISREVAEDAA TYYCQQXXXXXPXTFGG GTKLEIKRTVAA</td>
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Table II. Biopanning experiments

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<tr>
<th>Antigen</th>
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<th>Muũb2</th>
<th>PHILOtop</th>
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<tr>
<td></td>
<td>Positive clones/ screened clones</td>
<td>Rounds of panning</td>
<td>Positive clones/ screened clones</td>
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<tr>
<td>α2-Macroglobulin</td>
<td>16/282</td>
<td>4</td>
<td>6/282</td>
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<tr>
<td>Glutathione S-transferase</td>
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<td>Transferrin</td>
<td>0/282</td>
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<tr>
<td>BCD Domain of Tenascin C</td>
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<td>0/188</td>
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<td>Hemoglobin</td>
<td>n.p.</td>
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</tbody>
</table>

The MuLibi, Muũb2 and PHILOtop libraries were biopanned against several different antigens. The ratio between "positive antibody clones/ screened antibody clones" and the number of rounds performed for each antigen are shown in this table.
Sequence Listing

SEQ ID NO: 1 PHILOtop VH domain
QVQLQQPGAEVLKPGASVKMSCKASGYTFTSYNHWVKQTPGRGLEWIGAIYPNGYTSYN
QKFKGKATLTADKSSSTAYMQLSSLTSEDAYVYCARXXX(X)(X)FDYWGVGQTTTVSS

SEQ ID NO: 2 PHILOtop VH 186.2-derived region
QVQLQQPGAEVLKPGASVKMSCKASGYTFTSYNHWVKQTPGRGLEWIGAIYPNGYTSYN
QKFKGKATLTADKSSSTAYMQLSSLTSEDAYVYCAR

SEQ ID NO: 3 PHILOtop VH domain FR4
FDYWGVGQTTTVSS

SEQ ID NO: 4 PHILOtop VL domain
QIVLSQPSAILSASPGEKVTMCRASSVSYSYMHWFQQKPGSSPKPWIYATSNLASSGVPRFS
GSGSGTSTISLTSRVEAEDAATYYCQQXXXXPXTFGGGTKEIKRTVAA

SEQ ID NO: 5 PHILOtop VL kv4-72-derived region
QIVLSQPSAILSASPGEKVTMCRASSVSYSYMHWFQQKPGSSPKPWIYATSNLASSGVPRFS
GSGSGTSTISLTSRVEAEDAATYYCQQ

SEQ ID NO: 6 PHILOtop VL domain FR4
TFGGGTKLEIKRTVAA

SEQ ID NO: 7 PHILOtop Linker
GGGGSGGGGGGGGGG

SEQ ID NO: 8 PHILOtop scFv scaffold
QVQLQQPGAEVLKPGASVKMSCKASGYTFTSYNHWVKQTPGRGLEWIGAIYPNGYTSYN
QKFKGKATLTADKSSSTAYMQLSSLTSEDAYVYCARXXX(X)(X)FDYWGVGQTTTVSSGG

SEQ ID NO: 9 MuLibi VH
EVQLVESGGGLVKPGGLKLSCAASGFTSSYAMSWVRQSPEKRLLEWVAEISSGGSYTYYP
DTVTGRFTISRDNAKNTLYEMSSLRSEDAMAYCARXXX(X)(X)FDYWGVGQTTTVSS


Primer sequences are shown in Table I.

References


Giovannoni et al., Nucleic Acids Research, 29, No. 5 e27 (2001)


Williams, G. S., A. Martinez, et al. (2001). "Unequal VH gene rearrangement frequency within the large VH7183 gene family is not due to recombination signal sequence variation, and mapping of the genes shows a bias of rearrangement based on chromosomal location." J Immunol 167(1): 257-63.


Additional references:

12 Krebs et al. *Journal of Immunological Methods* 254, 67-84 (2001)
17 Staerz U. D. and Bevan M. J. *PNAS* 83 (1986)
Claims

1. A library of antibody molecules, wherein each antibody molecule comprises a VH domain comprising
   an amino acid sequence at least 80%, 85%, 90%, 95% or 99% identical to SEQ ID NO: 2, and
   a variably mutated amino acid sequence.

2. A library of antibody molecules according to claim 1, wherein each antibody molecule comprises a VH domain consisting of VH complementarity determining regions CDR1, CDR2 and CDR3 and framework regions FR1, FR2, FR3 and FR4, wherein
   VH FR1 consists of an amino acid sequence at least 90%, 95% or 99% identical to the FR1 sequence contained within SEQ ID NO: 2,
   VH FR2 consists of an amino acid sequence at least 90%, 95% or 99% identical to the FR2 sequence contained within SEQ ID NO: 2,
   VH FR3 consists of an amino acid sequence at least 90%, 95% or 99% identical to the FR3 sequence contained within SEQ ID NO: 2 and wherein
   at least one VH complementarity determining region comprises a variably mutated amino acid sequence.

3. A library according to claim 1 or claim 2, comprising a variably mutated amino acid sequence in CDR3 of the VH domain.

4. A library according to claim 3, wherein the VH domain comprises
   a VH CDR1 consisting of an amino acid sequence at least 90%, 95% or 99% identical to the CDR1 sequence contained within SEQ ID NO: 2, and
   a VH CDR2 consisting of an amino acid sequence at least 90%, 95% or 99% identical to the CDR2 sequence contained within SEQ ID NO: 2.

5. A library according to claim 4, wherein the VH domain amino acid sequence comprises the amino acid sequence SEQ ID NO: 2.

6. A library according to any of claims 3 to 5, wherein the CDR3 comprises a randomised sequence of four, five or six amino acid residues.
7. A library according to claim 6, wherein the library comprises VH domains in which CDR3 comprises a randomised sequence of four residues, VH domains in which CDR3 comprises a randomised sequence of five residues, and VH domains in which CDR3 comprises a randomised sequence of six residues.

8. A library according to any of the preceding claims, wherein the sequence of residues Cys-Ala-Arg immediately precedes the VH CDR3.

9. A library according to any of the preceding claims, wherein the VH domain comprises a FR4 sequence comprising an Asp or Asn residue in the first three residues following VH CDR3.

10. A library according to claim 9, wherein the VH domain comprises a VH FR4 sequence SEQ ID NO: 3.

11. A library according to claims 1 to 10, wherein the VH domain comprises the sequence:

SEQ ID NO: 2 - R1 - R2 - R3 - R4 - R5 - R6 - SEQ ID NO: 4

wherein

R1, R2, R3 and R4 are amino acid residues;
R5 is an amino acid residue or is not present; and
R6 is an amino acid residue or is not present.

12. A library of antibody molecules, wherein each antibody molecule comprises a VL domain comprising

an amino acid sequence at least 80%, 85%, 90%, 95% or 99% identical to SEQ ID NO: 5, and

a variably mutated amino acid sequence.

13. A library of antibody molecules according to claim 6, wherein each antibody molecule comprises a VL domain consisting of VL complementarity determining regions CDR1, CDR2 and CDR3 and framework regions FR1, FR2, FR3 and FR4, wherein

VL FR1 consists of an amino acid sequence at least 90%, 95% or 99% identical to the FR1 sequence contained within SEQ ID NO: 5,
VL FR2 consists of an amino acid sequence at least 90%, 95% or 99% identical to the FR2 sequence contained within SEQ ID NO: 5,

VL FR3 consists of an amino acid sequence at least 90%, 95% or 99% identical to the FR3 sequence contained within SEQ ID NO: 5, and wherein

at least one VL complementarity determining region comprises a variably mutated amino acid sequence.

14. A library according to claim 12 or claim 13, comprising a variably mutated amino acid sequence in CDR3 of the VL domain.

15. A library according to claim 14, wherein

VL CDR1 consists of an amino acid sequence at least 90%, 95% or 99% identical to the CDR1 sequence contained within SEQ ID NO: 5, and

VL CDR2 consists of an amino acid sequence at least 90%, 95% or 99% identical to the CDR2 sequence contained within SEQ ID NO: 5.

16. A library according to claim 15, wherein the VL domain amino acid sequence comprises the amino acid sequence SEQ ID NO: 5.

17. A library according to any of claims 14 to 16, wherein the VL CDR3 comprises a sequence of sequence of six amino acid residues containing a five randomised amino acid residues and a proline residue.

18. A library according to claim 17, wherein the proline residue is in a conserved position.

19. A library according to claim 18, wherein the proline residue is at the 5th position in the sequence of six amino acid residues, or at residue 95.

20. A library according to any of claims 12 to 19, wherein the sequence of residues Cys-Gln-Gln immediately precedes the VL CDR3.

21. A library according to any of claims 12 to 19, comprising VL FR4 SEQ ID NO: 6.

22. A library according to claims 12 to 21, wherein the VL domain comprises the sequence:

SEQ ID NO: 5 - R1 - R2 - R3 - R4 - Pro - R6 - SEQ ID NO: 6
wherein

R1, R2, R3, R4 and R6 are amino acid residues.

23. A library according to any of the preceding claims, wherein each antibody molecule comprises a VH domain and a VL domain, forming a VH-VL domain pair.

24. A library according to claim 23, wherein the VH domain is as defined in any of claims 1 to 11 and the VL domain is as defined in any of claims 12 to 22.

25. A library according to claim 23 or claim 24, wherein the antibody molecules are scFv.

26. A library according to any of the preceding claims, wherein the antibody molecules are expressed by bacteria.

27. A library according to any of claims 1 to 26, wherein the antibody molecules are displayed on replicable genetic packages.

28. A library according to claim 27, wherein the antibody molecules are displayed on filamentous bacteriophage.

29. A library of nucleic acid molecules, wherein the library of nucleic acid molecules encodes a library of antibody molecules as defined in any of claims 1 to 25.

30. A library according to claim 29, wherein the nucleic acid molecules are contained in bacteria.

31. A library according to claim 29 or claim 30, wherein the nucleic acid molecules comprise a nucleotide sequence encoding the antibody molecule fused to a coat protein of filamentous bacteriophage.

32. A library according to claim 31, wherein the protein is pill.

33. A library according to claim 31 or claim 32, wherein the nucleotide sequence comprises an amber stop codon between the antibody molecule and the coat protein.
34. A method of selecting an antibody molecule that binds an antigen, comprising
providing a library according to any of claims 1 to 28; and
contacting the library with the antigen, so that the antigen binds to one or more
antibody molecules in the library; and
selecting nucleic acid encoding an antibody molecule that binds to the antigen.

35. A method according to claim 34, comprising providing bacteria secreting the antibody
molecules and performing iterative colony filter screening.

36. A method according to claim 34 or 35, further comprising expressing the nucleic acid to
produce the antibody molecule, and isolating the antibody molecule.

37. A method according to claim 36, further comprising formulating the antibody molecule
into a composition additionally comprising a pharmaceutically acceptable excipient.

38. A method according to any of claims 34 to 37, wherein the antigen comprises an
epitope that is conserved between humans and mice.

39. Use of a library according to any of claims 1 to 28 for screening for an antibody
molecule that binds one or more antigens.

40. Use according to claim 39, wherein the antigen comprises an epitope that is conserved
between humans and mice.
Figure 1

A

scFv sequence

\[ V_\text{H} - GSGSGG_x - V_\text{K} - \text{AAEQKLISEEDLNGAA-amber-} \text{pplI - linker - myc-tag} \]

\[ V_\alpha \text{ (based on 98-3G)} \]

\[ EVQLVESGYCAR \]

\[ \text{linker} \]

\[ V_\alpha \text{ (based on 186)} \]

\[ QVQLQPSGYCAR \]

\[ \text{linker} \]

\[ V_\beta \text{ (based on dv-36)} \]

\[ \text{linker} - \text{DVQLQPSGYCAR - XXXXXXFP - TFGAGKLEIKR - tag} \]

\[ V_\zeta \text{ (based on V1C)} \]

\[ \text{linker} - \text{DVQLQPSGYCAR - XXXXXXFP - TFGAGKLEIKR - tag} \]

\[ V_\gamma \text{ (based on kv4-72)} \]

\[ \text{linker} - \text{QVQLQPSGYCAR - XXXXXXFP - TFGAGKLEIKR - tag} \]

B

\[ a \rightarrow \text{Ncol} \rightarrow \text{b1-3} \rightarrow c \rightarrow \text{d} \rightarrow \text{NotI} \]

\[ 1) \text{ PCR assembly of } V_\alpha \text{ and } V_\zeta \]

\[ 2) \text{ Cloning into the vector pHENI} \]

\[ \left\{ \begin{array}{c}
\text{pelB} \\
\text{Ncol} \\
\text{NotI} \\
\text{myc-tag} \\
\text{gene plII} \\
pHENI \\
colE1 ori \\
Amp \\
M13 ori
\end{array} \right. \]
Fig. 2A - MuLib1 (PCR screening)
M = Molecular weight standard
+ = Positive control
- = Negative control

Fig. 2B - MuLib1 (Dot-Blot)
+ = Positive control
- = Negative control

Figure 2
Fig. 2C - MuLib1 (Western Blot)

102 kD
76 kD
52 kD

1 2 4 8 16
Dilution factor

scFv - plIII
plIII

Figure 2 (continued)
Fig. 3A – MuLib2 (PCR screening)
M = Molecular weight standard
+ = Positive control
- = Negative control

Fig. 3B – MuLib2 (Dot-Blot)
+ = Positive control
- = Negative control

Figure 3
Fig. 3C – MuLib2 (Western Blot)

102 kD
76 kD
52 kD

scFv - pIII
plll

1 2 4 8 16

Dilution factor

Figure 3 (continued)
Fig. 4A - PHILOtop (PCR screening)
M = Molecular weight standard
+ = Positive control
- = Negative control

Fig. 4B - PHILOtop (Dot-Blot)
+ = Positive control
- = Negative control
Fig. 4C - PHILOtop (Western Blot)

102 kD  
76 kD  
52 kD  

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Dilution factor

Figure 4 (continued)
A

i) QVQLQGPALVKPGASVKSCKASGYFTSYWMHWVKQRPGRRGWISGRGRDSNPGTYNEKFKSATLTVDKPSSTAYMQLSL1SSTEDSAYYCAR
ii) QVQLQGPALVKPGASVKKSCASGYFTSYMHWVKQTPGRLEWIGA1YPGNGYTFNQRFKSATLTDSSSTANMQLSL1SSTEDSAYYCAR

i) germline gene VH 186.2
ii) PHILOtop VH

= position 52a
* position 82a
^ position 82b
` position 82c

B

iii) QIVLSQPSAILSAFPSGEKVTMCRASSSASYWYQQKPGSSPKPIYATSNLASSGVAPSFSGSGSGLTISRVEAEDAAFYYCQ
iv) QIVLSQPSAILSAFPSGEKVTMCRASSSASYWFQKPGSSPKPIYATSNLASSGVVPFSGSGSGSGLTISRVEAEDAAFYYCQ

iii) Germline gene Vk4-72
iv) PHILOtop VL

Figure 6
A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/10  C07K16/00  C40B40/06  C40B50/08

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  C07K  C40B

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

Electronic database consulted during the international search (name of database and, where applicable, search terms used)
EPO-Internal, BIOSIS, CAB Data, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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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
  
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  
  "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
15 October 2010

Date of mailing of the international search report
19/11/2010

Name and mailing address of the ISA/
European Patent Office, P B 5818 Patentlaan 2
NL - 2280 HV Rijkwiik
Tel (+31-70) 340-2040,
Fax (+31-70) 340-3016

Authorized officer
Hornig, Horst
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<td>MANEERAT KOOHAP ITAGTAM ET AL: &quot;Efficient amplification of light and heavy chain variable regions and construction of a non-immune phage scFv library&quot;&lt;br&gt;MOLECULAR BIOLOGY REPORTS ; AN INTERNATIONAL JOURNAL ON MOLECULAR AND CELLULAR BIOLOGY,&lt;br&gt;vol. 37, no. 4, 25 June 2009 (2009-06-25), pages 1677-1683, XP019793283&lt;br&gt;KLUWER ACADEMIC PUBLISHERS&lt;br&gt;ISSN: 1573-4978&lt;br&gt;cited in the application the whole document</td>
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<td>IMAI SUNAO ET AL: &quot;Quality enhancement of the non-immune phage scFv library to isolate effective antibodies.&quot;&lt;br&gt;BIOLOGICAL &amp; PHARMACEUTICAL BULLETIN,&lt;br&gt;vol. 29, no. 7, July 2006 (2006-07), pages 1325-1330, XP002605231&lt;br&gt;ISSN: 0918-6158&lt;br&gt;cited in the application the whole document</td>
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<td>OKAMOTO T ET AL: &quot;Optimal construction of non-immune scFv phage display libraries from mouse bone marrow and spleen established to select specific scFvs efficiently binding to antigen&quot;&lt;br&gt;BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS,&lt;br&gt;vol. 323, no. 2, 15 October 2004 (2004-10-15), pages 583-591, XP004562767&lt;br&gt;ACADEMIC PRESS INC. ORLANDO, FL, US&lt;br&gt;ISSN: 0006-291X DOI: 10 .1016/J .BBRC .2004 .08 .131&lt;br&gt;cited in the application the whole document</td>
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<td>EP 2 000 149 A1 (BIOGEN IDEC INC [US]) 10 December 2008 (2008-12-10) figures 4,5</td>
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<td></td>
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<td>AT 421335 T</td>
<td>15-02-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 5603294 A</td>
<td>08-06-1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BR 1100622 A</td>
<td>18-04-2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2626445 A1</td>
<td>26-05-1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1094965 A</td>
<td>16-11-1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1607006 A</td>
<td>20-04-2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 101007850 A</td>
<td>01-08-2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 1912111 A</td>
<td>14-02-2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CN 101007851 A</td>
<td>01-08-2007</td>
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<td>DE 122009000070 T11</td>
<td>11-02-2010</td>
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<td>DK 2000149 T3</td>
<td>03-08-2009</td>
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<td>DK 1005870 T3</td>
<td>18-05-2009</td>
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<tr>
<td></td>
<td></td>
<td>ES 2326144 T3</td>
<td>01-10-2009</td>
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<tr>
<td></td>
<td></td>
<td>ES 2321567 T3</td>
<td>08-06-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HK 1125574 A1</td>
<td>31-12-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HU 72914 A2</td>
<td>28-06-1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL 107591 A</td>
<td>17-02-2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LU 91620 A9</td>
<td>11-01-2010</td>
</tr>
<tr>
<td></td>
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<td>NL 300424 T1</td>
<td>04-01-2010</td>
</tr>
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<td></td>
<td>PL 175557 B1</td>
<td>29-01-1999</td>
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<td>PT 2000149 E</td>
<td>14-07-2009</td>
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<td></td>
<td>PT 1005870 E</td>
<td>28-04-2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SG 45294 A1</td>
<td>16-01-1998</td>
</tr>
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<td></td>
<td></td>
<td>US 5736137 A</td>
<td>07-04-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZA 9308466 A</td>
<td>20-06-1994</td>
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