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Bacteriophage Display of Combinatorial Antibody Libraries

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Bacteriophage display of combinatorial antibody libraries is one means by which monoclonal antibodies of a desired specificity can be selected without the use of conventional hybridoma technology. Phage display is the expression of proteins and peptides on the surface of a bacteriophage particle, and a combinatorial antibody library is a collection of rearranged variable-region gene segments that have been randomly combined through the use of recombinant molecular biology techniques to yield a large 'library' of antibodies.

Background to the Methodology

The isolation of specific antibodies from a cloned immunological repertoire requires a large library as well as a rapid and efficient selection procedure. The diversity of these libraries should be large in order to contain all possible combinations of the variable domains of heavy chains and light chains which are combined randomly during the construction of these libraries. The requirements of size and a powerful selection process are perfectly met by bacteriophage-displayed antibody libraries.

Originally, phage display was developed in the 1980s by G Smith for selecting peptides (Smith, 1985). The potential of the linkage of genotype and phenotype realized in this methodology for the very rapid and efficient selection of ligands from large pools of candidates was soon recognized and phage display was extended to the display of various types of proteins, including antibody fragments.

Phage display of antibody libraries is based on cloning of antibody genes into phagemid vectors. Phagemid vectors are plasmids that carry parts of a phage genome, and phagemid DNA can be packaged into the coat of a filamentous phage upon infection of the host cell with a helper phage (Figure 1). The phagemid DNA is introduced into *Escherichia coli* cells and the genes coding for the antibody fragments are expressed and directed to the periplasm of the cell via leader sequences. The oxidizing environment of the periplasm allows the assembly and proper folding of the antibody fragments. Since the single-chain antibody fragment or the heavy chain fragment is fused to the C-terminal portion of the coat protein 3, it is included in the phage assembly which takes place in the periplasm. This results in filamentous phage which display antibody fragments monovalently on their surface and

contain the genes that encode those antibody fragments packaged within their phage coat.

Phage that display antibody fragments specific to the antigen of interest can be efficiently amplified and isolated

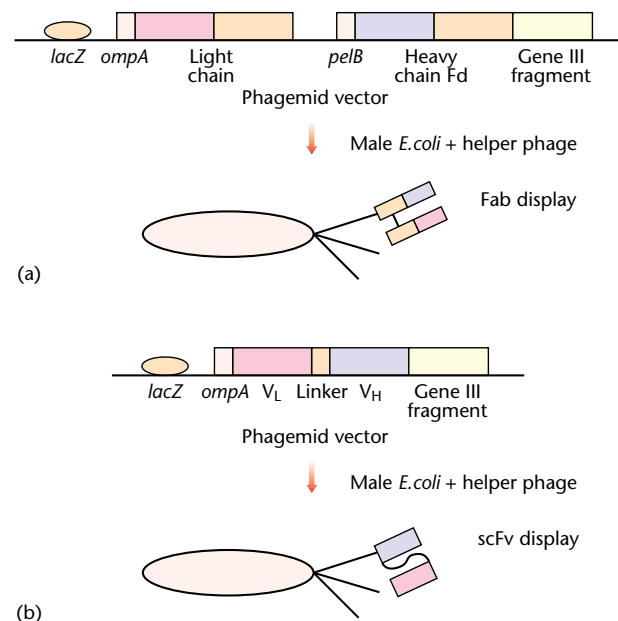


Figure 1 Expression of antibody fragments by phage display. A schematic representation of the expression of Fab and scFv antibody fragments using phage display. The antibody fragments are cloned into the phagemid vector under the control of the *lacZ* promoter as fusion proteins with the C-terminus of the minor coat protein, gene III. The translated proteins are directed to the periplasmic space by the *pelB* signal peptide where they are assembled on the phage particles. (a) Fab display. (b) scFv display.

from the phage library by panning, a procedure where antigen recognition is linked to replication. In the panning procedure the antibody-displaying phage library is incubated with the antigen of interest and nonbinding phage are eliminated by washing. Binding phage are recovered by disrupting the antigen–antibody complex (using acidic conditions or proteases, for example) and reamplified by infecting *E. coli* cells. Typically, a several hundred-fold enrichment of the phage displaying a specific antibody fragment over phage-displaying antibody fragments with other specificities is obtained in one round of panning. Thus, after a few rounds of panning the selected phage pool should mainly consist of phage displaying antigen-specific antibody fragments. Single clones are isolated and can be used for further characterization.

The selection of phage display antibody libraries is not confined to purified antigens immobilized on artificial surfaces but can be extended to other targets like virus preparations or antigens expressed on the surface of cells. Panning of phage display antibody libraries against antigens on cells is advantageous in many cases, for example when the antigen is not available in purified form, when it has yet to be characterized or when the natural confirmation of the antigen is lost during the purification procedure, a phenomenon often seen with integral membrane proteins. If a therapeutic antibody that recognizes a surface protein is the goal, selection of the library on cells will preferentially yield clones that bind well to the native target protein in the context of the cell membrane.

Generation of Libraries

A variety of different methodologies and phagemid vectors have been used for the construction of antibody phage display libraries from many different sources. Besides conventional libraries that consist of cloned immunoglobulin genes, semisynthetic or fully synthetic libraries where certain complementarity-determining regions (CDRs) were replaced by randomized sequences have been described (Barbas, 1995).

Typically, for the generation of an antibody library, fragments of expressed heavy and light chain genes are amplified from lymphatic tissue or peripheral blood lymphocytes (PBLs) by reverse transcription and the polymerase chain reaction (PCR). Information on immunoglobulin genes is available from sequence databases and has been used to design oligonucleotide primers for amplification of immunoglobulin genes from many different species. Apart from human antibody libraries, those from other primates, mice, rabbits and chickens are the most widely used. The PCR fragments are cut by restriction enzymes and cloned into a phagemid vector. This can be done by a two step cloning involving several

restriction enzymes or by fusing the amplified heavy and light chain fragments by PCR. Such an assembly step together with the use of a single rare cutting restriction enzyme, *Sfi*I, that allows for unidirectional cloning, facilitates the construction of antibody libraries. If a humanized version of antibodies from an animal source is the final goal, chimaeric antibody libraries where Fab fragments with variable regions from the immunized animal source and human constant regions are displayed on phage, can be constructed. This facilitates the humanization of the selected antibodies.

The phagemid DNA containing the antibody library is then introduced into *E. coli* cells by electroporation. A high transformation efficiency is of great importance, since this step is usually the limiting factor for the size of a combinatorial antibody library. Superinfection with helper phage leads to the production of recombinant phage that display antibody fragments on their surface which can then be used for the panning procedure.

Naive versus immunized libraries

All newborn animals and humans begin life with a repertoire of naive B lymphocytes that are used to build humoral immunity to a large array of foreign proteins. The naive cells respond *in vivo* to immune challenges and develop into cells which produce high-affinity monoclonal antibodies or become memory cells that are reserved for future exposure to antigen. In addition to maintaining a pool of memory B cells, the adult immune system continues to generate virgin B lymphocytes. The process of immunization for the production of antibodies for diagnostics and therapeutics represents an important time-honoured tradition in immunology. Prior to the development of phage display, researchers were dependent on hybridoma technology and later Epstein–Barr virus (EBV) transformation in order to extract the desired monoclonal antibodies. Both of these options are limiting because they require immunization of the animal and the screening of large numbers of clones to achieve success. With the advent of phage display it became possible to capture antibody gene segments at any stage of development, from naive to memory cell, for use as building blocks for large antibody libraries. In addition, many of the limitations associated with older methodologies became obsolete. However, the ultimate success of selecting antibodies from combinatorial libraries depends, in part, on the size of a given library and the ability of the *in vitro* system to copy natural immune selection and, in some cases, affinity maturation.

The generation of large combinatorial libraries is aided by the fact that a given B cell repertoire is naturally combinatorial. The use of multiple gene segments, junctional diversity, and the random association of heavy and light chains results in a large B cell pool. Most antibody libraries utilize the rearranged gene segments from this

natural repertoire to form a recombinant pool of antibodies, while some libraries need only part of the rearranged gene segments (see below). A combinatorial antibody library can be made from the gene segments of essentially any animal whose immunoglobulin gene structure and sequences are known. Depending on the host, the source of B cells can be derived from peripheral blood lymphocytes, whole spleen, bone marrow and tonsil. Other secondary lymphoid tissues are used more rarely (e.g. peritoneal cells). The most commonly used hosts are humans, mice, rabbits, and, less frequently, chickens.

A naive combinatorial library refers to an antibody library that is derived from the pool of antibody genes in an animal or human that has not been intentionally immunized with a specific antigen. More specifically, these libraries are often obtained from the immunoglobulin M (IgM)-producing B cells, as the naive cells usually express antibodies of the IgM isotype. The most abundant source of naive B lymphocytes is the bone marrow, although the spleen is also used. In some cases it is possible to obtain fetal tissue or cord blood, both of which are abundant in immature cells. Using the molecular biology techniques described in the previous section, the heavy and light chain antibody genes are extracted and randomly combined to form a library of molecules that can be used in the selection against almost any antigen. The use of naive libraries has several advantages. First, one is not limited by the need for prior immunization. This is especially helpful when trying to obtain human antibodies because medical ethics prevent the intentional immunization of humans with unapproved, unsafe or untested immunogens. Second, one is provided with the opportunity to perform further manipulations on selected molecules that mimic affinity maturation. Although this can be done with any antibody, naive molecules are not subject to the constraints that are often found in naturally high-affinity antibodies from immunized libraries. Naive libraries can also offer the opportunity to select antibodies that bind to antigens which are difficult immunogens, i.e. highly conserved with limited epitopes that may only be weakly immunogenic. However, there are also disadvantages. In some instances extraordinarily large libraries ($> 10^{10}$) are required before successful selection is achieved. The use of naive libraries may increase the possibility of selecting polyspecific antibodies, as the natural immature repertoire is abundant in these molecules.

A second type of naive library is the semisynthetic combinatorial library. This refers to a library of antibody molecules that are derived in part from immunoglobulin genes and in part from synthetically synthesized oligonucleotides. Most often this is accomplished by combining one or more 'germline' variable-region genes with a large assortment of randomly synthesized oligonucleotides that serve as one or more of the complementarity-determining regions (CDRs), usually heavy chain CDR3. However,

using a number of molecular biology techniques, a semisynthetic library could be built by other means as well.

An immunized library is an antibody library derived from an animal or human that has been immunized with a specific antigen. The immunization can be intentional, e.g. a given antigen that is repeatedly used to vaccinate an animal over the course of several weeks or months, or the immunization can be natural, e.g. an individual infected with human immunodeficiency virus (HIV). Immunized libraries are most commonly constructed using the rearranged antibody genes from IgG-producing cells. However, libraries can be made from any class of immunoglobulin-producing cells, particularly if the antigen of choice elicits a response that is predominated by one isotype of immunoglobulin. Using immunized libraries to select antibodies is advantageous because the starting pool has an increased concentration of antigen-specific molecules. Since the library is produced from an immunized animal, *in vivo* affinity maturation has taken place, leading to an increased chance of obtaining high-affinity antibodies. Limitations of immunized libraries are primarily encountered with human antibody libraries because they are restricted to individuals who have received approved vaccines or who have been naturally infected or exposed to foreign antigens. It is possible to use libraries from immunized animals, such as rabbits or mice, and later humanize the selected antibodies.

scFv and Fab libraries

The use of naive, semisynthetic or immunized combinatorial antibody libraries is greatly dependent on the circumstances, i.e. the nature of the antigen or the proposed use of the selected molecules. Regardless of the overall goals of the selection, there are numerous applications for each type of library and any of the libraries can be constructed with different antibody fragments. An antibody molecule is composed of two identical heavy and two identical light chains to form a disulfide-linked, bivalent molecule (**Figure 2**). The molecule can be subdivided into different fragments, some of which result from specific protease cleavage of the natural full-length immunoglobulin. Bacteriophage display is limited to those fragments that can be successfully expressed in bacterial cells. This includes Fv, scFv, Fd, light chain and Fab. Larger molecules often are not expressed due to the inability of the bacteria to properly fold and/or glycosylate the proteins.

The most commonly used fragments in library construction are scFv and Fab. scFv is an antibody heavy chain variable region tethered to an antibody light chain variable region through a flexible linker sequence. Prior to the development of scFv fragments, Fv fragments were shown to be functionally expressed in *E. coli*. An Fv is essentially the same molecule as an scFv but without the linker. The

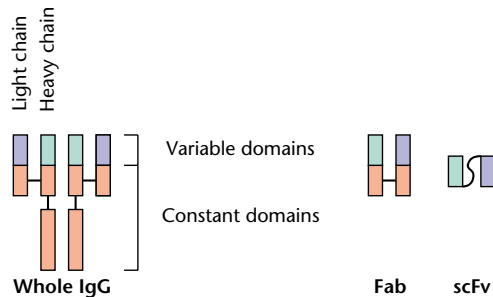


Figure 2 Immunoglobulin structure. A schematic representation of the structure of an antibody molecule. The intact IgG molecule contains two identical heavy chains and two identical light chains that are covalently linked through disulfide bonds. The Fab molecule consists of a single light chain and the heavy chain Fd ($V_H + C_H1$) covalently linked by a single disulfide bond. The scFv fragment consists of a light chain variable region linked to a heavy chain variable region through a synthetic peptide linker.

Fv antibody fragments are dependent on the natural association between the variable regions of heavy chains and light chains and there is a tendency for the fragment to dissociate. The covalent linkage that distinguishes the scFv from the Fv fragment eliminates many of the problems associated with Fv fragments. An scFv is assembled using the molecular biology techniques described above and can be expressed in one of two ways, V_H -linker- V_L or V_L -linker- V_H . A given configuration may work better in the three-dimensional structure for binding to different antigens or may be expressed more efficiently by the bacteria. In addition to the gene arrangement the linker can be varied in length, depending on the application. Shorter linkers promote dimer formation, and thus higher avidity, while long linkers primarily promote expression of monomers.

One advantage of using scFv antibody fragments is that they are small and generally easier to work with. Since the V_H and V_L polypeptides are covalently linked, there is less formation of extraneous molecules, such as V_H - V_H or V_L - V_L . scFv can also be humanized if the library is made from a nonhuman host. On the other hand, if the goal of antibody selection is therapeutic application, scFv may not be the molecule of choice, as they exhibit a shorter half-life than Fab or IgG *in vivo*. Although scFv can be converted to Fab, it is possible that the resulting molecule will not bind with the same affinity or fit as the originally selected molecule. scFv fragments are also dependent on peptide tag addition for detection and purification.

The other commonly used antibody fragment is the Fab. This molecule consists of the entire light chain in association with the heavy chain Fd ($V_H C_H1$). The two chains are produced as separate proteins and are post-translationally linked through a disulfide bond. The resulting antibody fragment is monovalent. The two chains

are usually transcribed as a dicistronic message, while translation occurs separately, resulting in two polypeptides. Often the two chains are preceded by different bacterial signal sequences. Similar to the scFv, the gene arrangement within the expression vector can either be $V_H C_H1 / V_L C_L$ or $V_L C_L / V_H C_H1$. This order may or may not affect protein expression. However, since the two chains are not covalently linked like scFv it is possible to produce extraneous molecules, such as heavy or light chain homodimers, though the heterodimer is the predominant product.

Fab fragments are more suitable for some therapeutic applications, as they have a longer half-life *in vivo* and are readily converted to even longer lived IgG molecules. Unlike scFv, detection and purification does not depend on the addition of a peptide tag, but instead can utilize antibodies that are specific for the constant regions. Fab libraries afford an opportunity to link the first step of humanization with selection by constructing chimaeric Fab fragments consisting of nonhuman variable regions fused to human constant regions. Chimaeric Fab libraries can be made from essentially any animal by recombining the animal's V_H and V_L genes with human C_H1 and C_L .

Like selecting a library type, the choice of scFv or Fab is partly dependent on the ultimate goal of the project. Regardless, the multiple combinations that can be formed between library types and antibody types give a greater flexibility to the bacteriophage display system.

Applications of the Technology

Antibodies from various sources are widely used in research and for diagnostic and therapeutic purposes. The demand for modified antibodies that are more specific and have higher affinities or that are otherwise improved as well as for antibodies recognizing new epitopes is high. Phage display technology is increasingly important in supplying this demand.

Phage display of antibody fragments allows for the selection of specific antibodies from immunized libraries from various sources. To date this methodology has been used for the selection of antibodies from several different species like humans, other primates, chickens, rabbits and mice. Furthermore it has been shown, that it is possible to select high-affinity binders to virtually any antigen from large naive libraries or from semisynthetic or synthetic libraries using phage display (Barbas *et al.*, 1995; Vaughan *et al.*, 1996). Catalytic antibodies that are able to catalyse a variety of chemical reactions were selected and studied and improved in their catalytic activity by means of phage display. Phage display selection mimics to a certain degree the generation of specific antibodies by the immune system and is useful to study the immune response and antibody-antigen interactions. Antibodies involved in autoimmune

disease like Graves ophthalmopathy, thyroid disease as well as the autoimmune antibody repertoire in HIV infection have been isolated and studied using the phage display technology. An interesting new approach based on magnetically activated cell sorting was used to isolate human antibodies to the Rh(D) antigen on red blood cells from an antibody library constructed from a Rh(D)-negative individual (Siegel *et al.*, 1997).

Many laboratories have reported the isolation of antibodies with therapeutic potential by antibody phage display. This includes antibodies to the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120, that were isolated from a library constructed from bone marrow derived from an asymptomatic HIV-1-infected individual. Some isolated gp120-specific antibodies proved to be very potent neutralizers of the virus *in vitro* (Barbas *et al.*, 1992). Isolation of human antibodies specific to other viral targets like respiratory syncytial virus (RSV), and herpes simplex viruses (HSV-1 and HSV-2) were also reported (Barbas and Burton, 1996). Tumour antigens have also been targeted by this approach and antibodies specifically recognizing antigens expressed on carcinoma cells like melanoma, colon and ovarian cancer have been isolated by antibody phage display. Immunization of prion knockout mice with the prion protein led to a specific immune response to the prion protein which is recognized as a self antigen in normal mice. Using phage display, antibodies with high affinity to mouse prions were isolated by this approach (Williamson *et al.*, 1996).

The application of this technology goes beyond the rapid and efficient selection of specific antibodies from large libraries constructed from immunized sources. It has also great potential for modification of already available promising antibodies selected from a phage display library or isolated by conventional hybridoma technology. Affinity maturation of existing antibodies is often desired because it should improve their therapeutic and diagnostic potential. Increasing the affinity of antibodies that are already good binders to a certain antigen was shown to be feasible by phage display using an approach called CDR-walking. Libraries based on randomizing of CDRs of the parental antibody are constructed and selected for improved binding to the antigen of interest. Greater than 400-fold improvement of affinity as well as broadening the specificity was shown to be feasible by this technology (Barbas *et al.*, 1994; Yang *et al.*, 1995).

Antibodies derived from animals are very immunogenic and therefore usually not suitable to be used for therapeutic purposes in humans. Various strategies for humanization of animal antibodies that have therapeutic values were successfully pursued using bacteriophage display. A strategy that is based on sequential replacement of the variable light and the heavy chains of a mouse antibody with selected human sequences, while conserving the CDR3 sequences of the original antibody, has been described. The resulting humanized antibodies recognized

the same epitope with the same or even higher affinities than the parental antibody (Rader *et al.*, 1998).

Phage display allows the potential of the immune system to be exploited to produce ligands to virtually any given structure and its combination with modern protein engineering technology makes the generation of custom designed antibodies with high affinity and specificity to any diagnostic or therapeutic target feasible.

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