Screening technologies for recombinant antibody libraries

Mieko Kato[1] and Yoshiro Hanyu[2]

[1]Bio-Peak Co., Ltd., 584-70 Shimonojo, Takasaki, 370-0854 Japan
E-mail: mieko.kato@bio-peak.com
[2]Structure Physiology Research Group, Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, 305-8566 Japan

Corresponding author: Yoshiro Hanyu
Tel. +81-29-861-5542, Fax: +81-29-861-2706
E-mail: y.hanyu@aist.go.jp

Abstract

Monoclonal antibodies have become increasingly important in research, diagnosis, and therapeutics. Many antibody preparations are commercially available and this market has grown to an astounding size. The use of antibodies has enabled early diagnosis of a wide variety of illnesses; and the ability to establish antibodies for various types of antigens has accelerated proteomic research. Antibodies with higher affinity and specificity are required in all fields; however, these specifications cannot be met by conventional monoclonal production technology, thus necessitating the generation of monoclonal antibodies using recombinant technology. Recombinant technology also enables the production of antibodies that could not be produced using conventional production technology. In vitro selection techniques enable screening in the environment in which the antibodies are used, thus facilitating the establishment of antibodies suited to specific needs. The production of recombinant monoclonal antibodies requires the creation of highly diverse libraries and subsequent identification of positive clones using a screening technology with low background signals. This review describes the current screening technologies used in recombinant monoclonal antibody production and the possible problems in production and discusses the future outlook of this technology.

Key words: recombinant antibody, library, screening, single-chain variable fragment, phage display, panning.

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1. The increasing importance of antibodies

Monoclonal antibodies are proteins that function to specifically recognize antigens. In addition to being an essential tool in research and diagnosis, they also play a major role in therapeutics (Schirrmann, Meyer, Schütte, Frenzel, & Hust, 2011; Weiner, 2015). The monoclonal antibodies created in the 1970s were mouse antibodies (Köhler & Milstein, 1975), and therefore could not achieve the clinical effects initially anticipated. The development of recombinant technology in the 1990s enabled the humanization of mouse antibodies and the expression of immunoglobulin genes in CHO and other cells, once again raising expectations regarding the potential use of antibodies and sparking new avenues of research and development (Dübel, 2007). Phage display has been used to construct large antibody libraries leading to the further development of monoclonal antibody screening technology (Schirrmann et al., 2011) and the consequent significant expansion of antibody-related technology. This technology has shown particularly remarkable growth in the field of antibody production, as demonstrated by the development of a wide array of antibody preparations (Reichert, 2015).

Monoclonal antibodies that recognize target antigens with high affinity and selectivity are becoming increasingly important in medical research and diagnosis. This importance extends not only to the recognition of antigens, but also to functional antibodies that activate or inhibit antigens, recognize minute structural changes in antigens, and can discern post-translational modifications in antigens. Technology for efficiently and rapidly establishing such highly functional and useful antibodies is of great importance. However, conventional antibody production technology is not capable of sufficiently meeting these requirements.

2. Establishment of antibodies

2.1. Limitations of conventional establishment technologies

Monoclonal antibody production must be rapid and the generated antibodies must have the desired affinity, specificity, and functionality (e.g., inhibition and promotion of antigen function). Currently, antibodies are established using two methods: hybridoma technology, which employs cell fusion, and recombinant antibody technology. In hybridoma technology, antibodies are established by fusing myeloma cells and antibody-producing cells taken from a mouse or other animal immunized against the antigen, thereby immortalizing and cloning the antibody-producing cells. Although this technology has a proven track record for consistent antibody production, it has a number of drawbacks: it requires the use of animals and cell culture techniques; it is time-consuming and laborious; it yields a low number of positive clones, which makes it difficult to obtain antibodies with high specificity and affinity; and it involves post-production steps such as humanization and recombination. In addition, because of the low efficiency of cell fusion (Kato, Sasamori, Chiba, & Hanyu, 2011), the number of clones that can be screened at a time is approximately 10^4.

2.2. Advantages of recombinant antibody establishment

In contrast to conventional technologies, the establishment of monoclonal antibodies using recombinant technology involves the use of an antibody gene library to screen for clones with an affinity for the desired antigens. This method can be adapted to antigens for which antibodies cannot be produced in animals, such as toxic antigens. Furthermore, the antibodies are screened in vitro, which enables the use of assays specifically tailored

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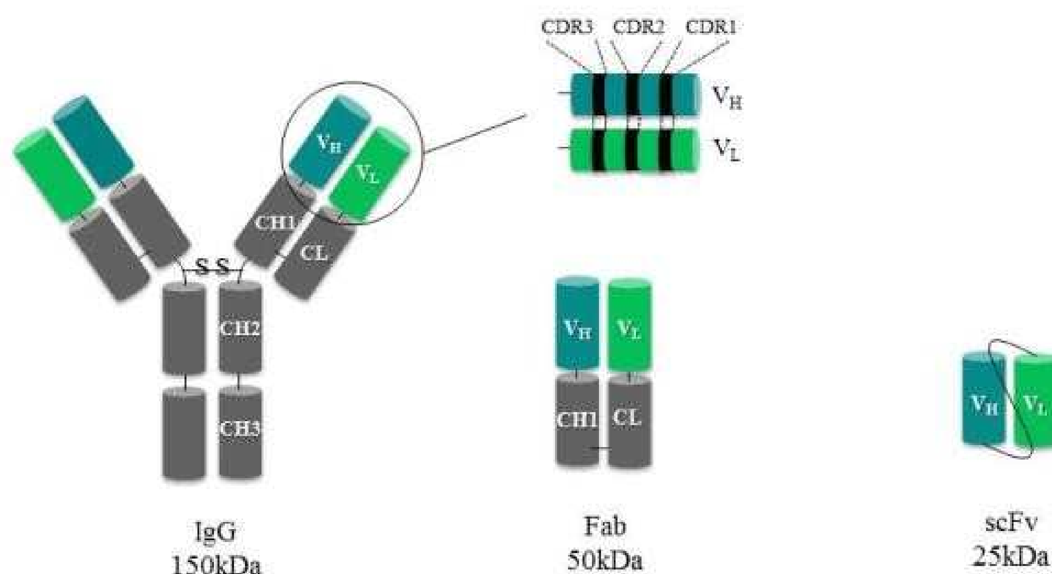

Figure 1. Schematic illustration of the IgG molecule and the recombinant antibody fragments

to the properties of the antigen and the desired antibody. Thus, this method enables the establishment of antibodies that cannot be generated by conventional hybridoma technology. The efficient establishment of antibodies with high affinity requires the selection of monoclonal antibodies using genetic engineering techniques such as phage display (McCafferty, Griffiths, Winter, & Chiswell, 1990). Phage display involves the insertion of antibody fragments into single-chain variable fragments (scFv) and fragment antigen-binding (Fab) forms (Holliger & Hudson, 2005), which allows for IgG conversion using recombinant technology. This method has several advantages: it does not require cell culture facilities and technology; it allows for easy addition of functions using engineering techniques; the libraries used are larger than those used for hybridoma technology; antibodies can be produced from an artificial library without the use of animals; and human antibodies can be generated directly (Griffiths et al., 1994; Rothe et al., 2008).

2.3. Recombinant antibodies used for establishment

Recombinant antibodies can provide conventional antibodies with a range of properties, such as increased affinity and stability (Demarest & Glaser, 2008), fusion with other proteins (de Bruyn, Bremer, & Helfrich, 2013), intracellular expression (Kaiser, Maier, Traenkle, Emele, & Rothbauer, 2014), miniaturization (R. E. Kontermann, 2010), and oligomerization (Schaefer, Lindner, & Plückthun, 2010). These recombinant antibodies have many forms, including those identical to IgG, $F(ab')_2$, Fab, and scFv. IgG is the most frequently used mammalian monoclonal antibody. As shown in Figure 1, IgG is comprised of four chains, two heavy chains and two light chains. The molecular weights of the light and heavy chains are approximately 25 kDa and 50 kDa, respectively. The chains are connected via a disulfide bond. The light chains are comprised of two structural domains, VL and CL. VL is located at the N-terminus and recognizes antigens. The heavy chains

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comprise four subunits: VH, CH1, CH2, and CH3. The VH domain recognizes antigens; it has high sequence variation and is located at the N-terminus. Antigen recognition is facilitated via both VH and VL that contain complementarity determining regions (CDRs), which possess extremely high variation, thus enabling the recognition of various antigens. These antibodies can be used with only the antigen-recognizing region intact. Fab, which consists of a light chain and the N-terminus of the heavy chain (Fd: VH and CH1) joined by a disulfide bond, can be obtained from IgG following papain treatment. scFv, which is comprised of antigen-recognizing VH and VL joined by a linker, is the most frequently used for the establishment and display of recombinant antibodies.

scFv is a small molecule with a molecular weight of approximately 25 kDa, one-sixth of the molecular weight of IgG. Its antibody binding function is completely identical to that of IgG, while its small size makes it very easy to manipulate, offering great potential for routine usage in research and testing (Hagemeyer, von Zur Muhlen, von Elverfeldt, & Peter, 2009). In addition, scFV is capable of penetrating tumors, thus making it a promising tool for cancer treatment (Accardi & Di Bonito, 2010). scFvs can be expressed in *Escherichia coli(E.coli)* and produced in large quantities (Miethe et al., 2013; Schirrmann, Al-Halabi, Dübel, & Hust, 2008). When scFvs are expressed in mammalian cells, the inhibiting intracellular antigen function allows scFvs to be used to elucidate the physiological functions of the antigen in cells (Kaiser et al., 2014). scFvs are predicted to be used in a variety of applications, such as fusion with other proteins to induce function (Morino et al., 2001) and increasing avidity via multimerization (Schaefer, Lindner, et al., 2010).

3. Recombinant antibody establishment technology

Recombinant monoclonal antibodies are

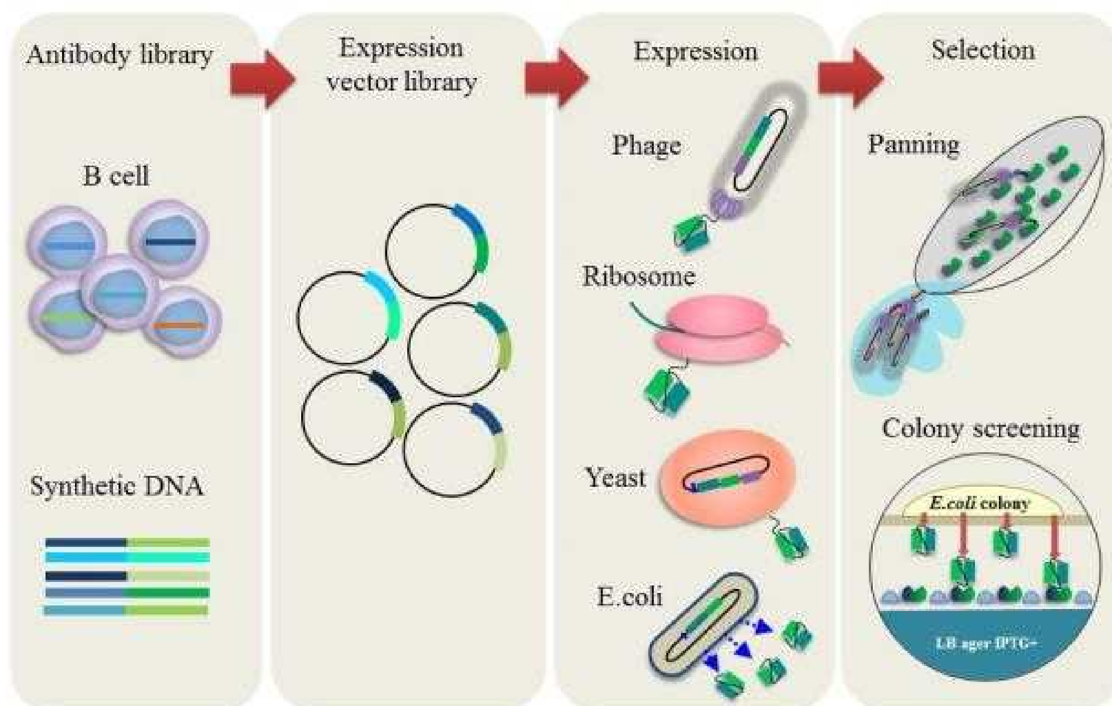

Figure 2. Overview of the methods for the production of recombinant monoclonal antibodies

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established by screening antibody libraries (Hoogenboom, 2005). VH and VL antibody genes are isolated from the lymph tissue of immunized animals and linked to create scFv libraries. At present, completely synthetic libraries are also used (Chen & Sidhu, 2014), as well as naïve libraries, which are derived from the lymph nodes of non-immunized animals (Chan, Chan, Lim, & Hanson, 2011). These gene libraries are incorporated into a phagemid or plasmid and expressed in *E. coli* or phage. Panning or other assays are then performed to isolate scFvs possessing antigen affinity, thereby establishing monoclonal antibodies (Figure 2).

3.1. Construction of antibody libraries

Antibody gene libraries include immune-libraries containing antibody genes derived from the spleen or lymph nodes of immunized mice and rats; and synthetic-libraries, in which the entire antibody gene library is artificially constructed. Immune-libraries are created by PCR amplifying VH and VL genes from cDNA obtained from immune tissue. These are then used to construct an scFv library with tremendous sequence variation that represents the in vivo antibody diversity of the immune cells (Figure 3). In contrast, in synthetic-libraries, the scFv library is artificially created using combinatorial technology. The advantages of synthetic-libraries include the ability to create human antibody libraries and libraries with immense diversity in the order of 10^{11} variants.

3.2. Cloning scFv library expression vectors

To construct an scFv library, antibody heavy chain-derived VH genes and light chain-derived VL genes are amplified, then joined with an appropriate linker and incorporated into an expression vector. When creating an scFv library, it is important to incorporate the VL and VH genes into the vectors in a specific order to prevent mutations, deletions, and frameshifts. In addition, since the genes contain a region

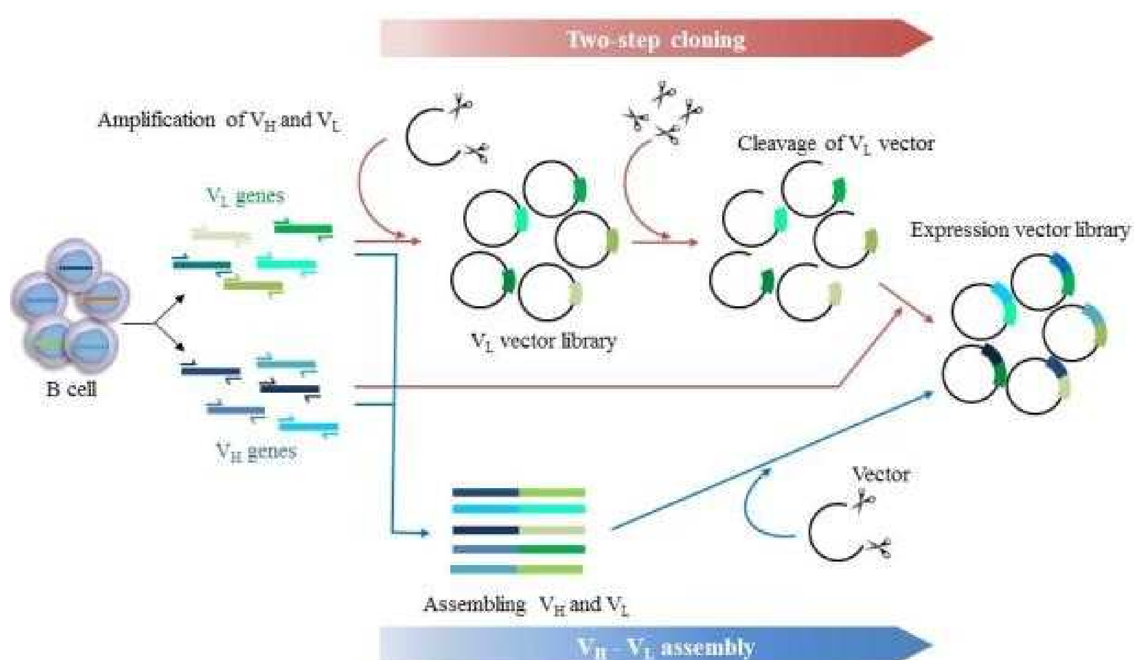

Figure 3. Construction of scFv expression vector libraries

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with great diversity (antigen recognition region: CDR), the scFv library must be constructed so as to maintain this diversity. This is accomplished by the following two methods (Figure 3):

(1) Two-step cloning: subsequent to PCR amplification of VH and VL, VL is first inserted into the vectors that are propagated in *E. coli* to create a VL library. Next, VH is inserted into the vectors to construct the scFv library (Müller, 2010).

(2) VH-VL assembly: VH and VL are PCR amplified and then joined together. This joined product is incorporated into vectors to create the scFv library (Schaefer, Honegger, & Plückthun, 2010).

Although method (1) is reliable, it requires many steps and is lengthy. In addition, in order to maintain a large antibody library, high *E. coli* transformation efficiency and enzymatic reaction efficiency must be constantly maintained. In method (2), overlap PCR is usually used to join VH and VL genes; a linker domain is added to the 3′-end of VH and the 5′ -end of VL and this linker site is used to join VH and VL with an overlap PCR reaction. Although this method is simple, PCR amplification utilizes single strand DNA; this makes the genes susceptible to deletion, insertion, replacement, or frameshifts, making it difficult to obtain functional scFv antibodies. In addition, VH and VL libraries are comprised of genes containing incredibly diverse antibody recognition sites. Therefore, if a sequence resembling the original primer binding site is expressed in this diversity region, uniform PCR amplification will be inhibited. This poses a drawback in that only clones with certain sequences are selectively amplified, which often effectively results in a significant reduction in library diversity.

Another excellent method involves the use of λ exonuclease (Kato & Hanyu, 2013). During PCR amplification of the VH and VL genes, a linker is added to the 3′ -end of one and the 5′ -end of the other and phosphate is added to both 5′ -ends. The phosphorylated 5′ -ends of the two DNA fragments are digested by λ exonuclease, the linker section becomes a single chain, and VH and VL are joined together. The remaining complementary strand is then unzipped in the 3′ direction by Bst DNA polymerase and a new complementary strand is synthesized (strand displacement synthesis), forming a complete double strand in which VH and VL are connected with a linker. Since the λ exonuclease cannot digest S-oligo (phosphorothioated) DNA, the 5′ -end of the non-phosphorylated primer is phosphorothioated to prevent the non-specific activity of λ exonuclease. In addition, phosphorothioation at the 3′ -end of the phosphorylated primer can regulate the length of the DNA digested by λ exonuclease, which in turn makes prevents the conversion of the highly diverse CDR into a single strand, thus greatly reducing background reactions.

The antibody genes are incorporated into a plasmid or a phagemid in accordance with the subsequent screening system (Figure 2). When cloning into expression vectors, it is important to avoid reducing the size of the library. *E. coli* transformation efficiency is a major factor determining the size of the library, thus high-efficiency transformations using techniques such as electroporation are required. It is also important to optimize restriction enzyme-based reactions and ligase-based vector incorporation reactions and to avoid reducing the size of the library. In addition, solid phase culturing on plates is recommended in order to eliminate library bias caused by the amplification of specific clones due to the post-transformation growth rate of *E. coli*.

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3.3. Recombinant antibody screening methods

Methods for screening recombinant antibodies can be largely divided into display strategies (Hust, Frenzel, Schirrmann, & Dübel, 2014) and repertoire cloning strategies (Persson, Caothien, & Burton, 1991) (Figure 2). In a display strategy, the gene and the protein, i.e. the antibody gene and the antibody (genetic information and antigen recognition function) are joined, and proteins with antigen recognition function are screened to obtain genetic information (Jostock & Dübel, 2005). Phage display systems in which an scFv is joined to the filamentous phage coat protein g3p is displayed on the phage are often used (Geyer, McCafferty, Dübel, Bradbury, & Sidhu, 2012). Other display systems include yeast display (Feldhaus & Siegel, 2004), in which an scFv is displayed on the surface of yeast; and ribosome display (Schaffitzel, Hanes, Jermutus, & Plückthun, 1999), in which a ribosome, mRNA, and an scFv are integrated. In these screening methods, the antigen is fixed and panning is used to remove weakly bound clones, leaving those clones bound intact to the antigen. This method is characterized by repeated selection, proliferation, and enrichment of positive clones to enable the processing of large libraries. Panning with phage display is further discussed in section 3.3.1.

In contrast, in repertoire cloning strategies an scFv gene library is transformed into *E. coli*, scFvs are expressed and secreted as a single separable colony, and scFvs with antigen recognition function are screened to obtain genetic information. Clones are selected based on assays using characteristics such as affinity, thus providing this method with advantages such as a low rate of false positives and the ability to reliably identify clones with high affinity. However, an assay must be performed for each individual *E. coli* clone, only positive clones are selected, and there is no propagation process in the screening method; therefore, it suffers from the drawback of limited libraries. During the initial phase, a clone is sampled from an *E. coli* library expressing scFvs. This clone is then cultured, and its expression is induced. The antibody reactivity of the supernatant is measured and positive clones are gathered. In this method, no more than a few thousand clones can be examined at once. Although this number is greater than that possible using the hybridoma technique, positive clones cannot be obtained when the positive rate is lower than one in a few thousand. The use of solid phase culturing, periplasmic expression, and *E. coli* colony formation provide a method (colony lift screening) for handling large libraries (3.3.2).

3.3.1. Screening with phage display

When using a phage display system (Schofield et al., 2007), panning is used to isolate phage displaying single-chain antibodies exhibiting affinity (Figure 4). Positive clones are established by displaying an antibody fragment (primarily an scFv) on the g3p coat protein of filamentous phage, linking gene information and function, and selecting only phage with affinity for the antigen (Parmley & Smith, 1988). This method has the advantage of processing large libraries ($\sim 10^{11}$). Screening is accomplished with panning. The antigen is fixed, the clone bound to the antigen is left intact, and the weakly-bound clones are washed away. The remaining clones, which possess binding capacity, are detached from the antigen by acid treatment and transformed into *E. coli*. The *E. coli* cells are cultured to propagate the positive clones. Helper phages are then infected and phages displaying scFvs with binding capacity are collected. Panning is performed repeatedly for this group of phages. Repeated selection and propagation of positive clones enriches for clones with

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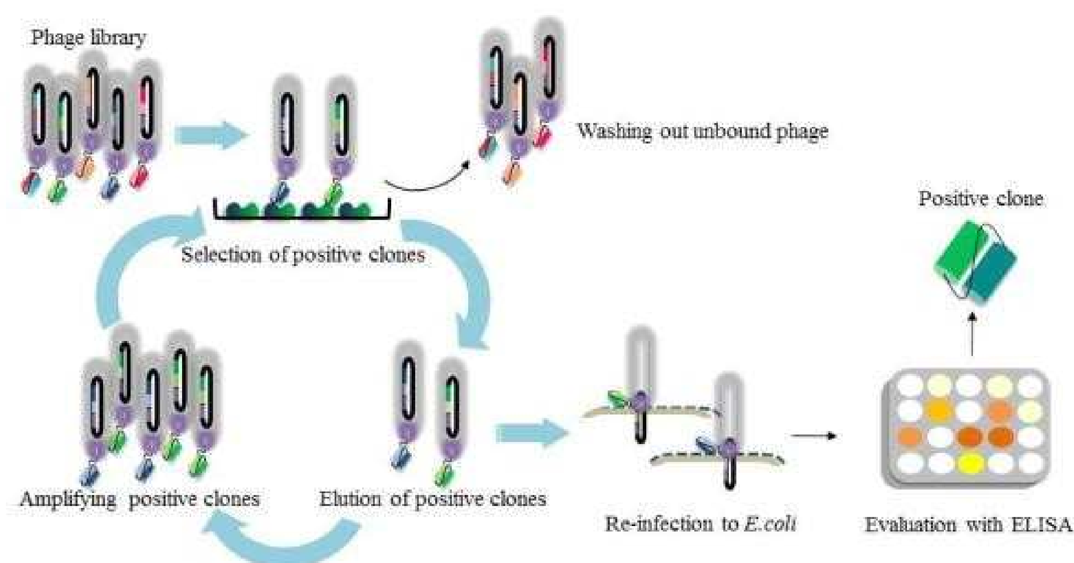

Figure 4. Schematic illustration of the panning process used in phage display systems

binding capacity and, ultimately, establishes positive clones with high binding capacity. Since cloning is not performed until the final step, this method makes it possible to handle large libraries. Repeated panning of scFv libraries with an antigen enables the enrichment of scFv antibodies with high affinity for the antigen, thereby making this method extremely effective for the production of high affinity scFv antibodies. In addition, changing the environment in which the panning is performed enables the selection of clones with specific binding characteristics For instance; screening at high temperatures will identify antibodies with effective binding ability at those conditions. Negative selection is also possible; a clone that binds a certain target is eliminated and subsequently a clone that binds a different target is selected. With this method, it is possible to obtain clones that recognize post-translational modifications and minute structural changes. The antibodies displayed by phages are generally scFvs. The selected antibody- displaying phage can either be mass expressed as a recombinant antibody in *E. coli* (Miethe et al., 2013); or the gene can be isolated and inserted into an appropriate vector to produce IgG from CHO or other cells (Jäger et al., 2013; Nettleship et al., 2008).

One limitation of this method is that the high background in panning selection often results in false-positive clones (Rauth, Schlapschy, & Skerra, 2010). In addition, since several antibody fragments are themselves toxic to *E. coli*, these clones will be lost during panning even if they possess high affinity. Conversely, repeated panning may result in the relatively preferential propagation of clones with reduced *E. coli* toxicity even if the clones do not possess high binding capacity. Toxicity to *E. coli* can increase the background, resulting in a high number of false-positive clones being obtained. Therefore, panning and washing is extremely difficult and is used less frequently than expected. This hindrance stems from the indirect detection of the antigen-antibody bond (Nelson, 2010).

3.3.2. Screening by colony lift assay

In colony screening methods (Figure 5), antibody libraries are expressed in *E. coli* for selection of clones with favorable affinity. An scFv library is transformed into *E. coli* cells,

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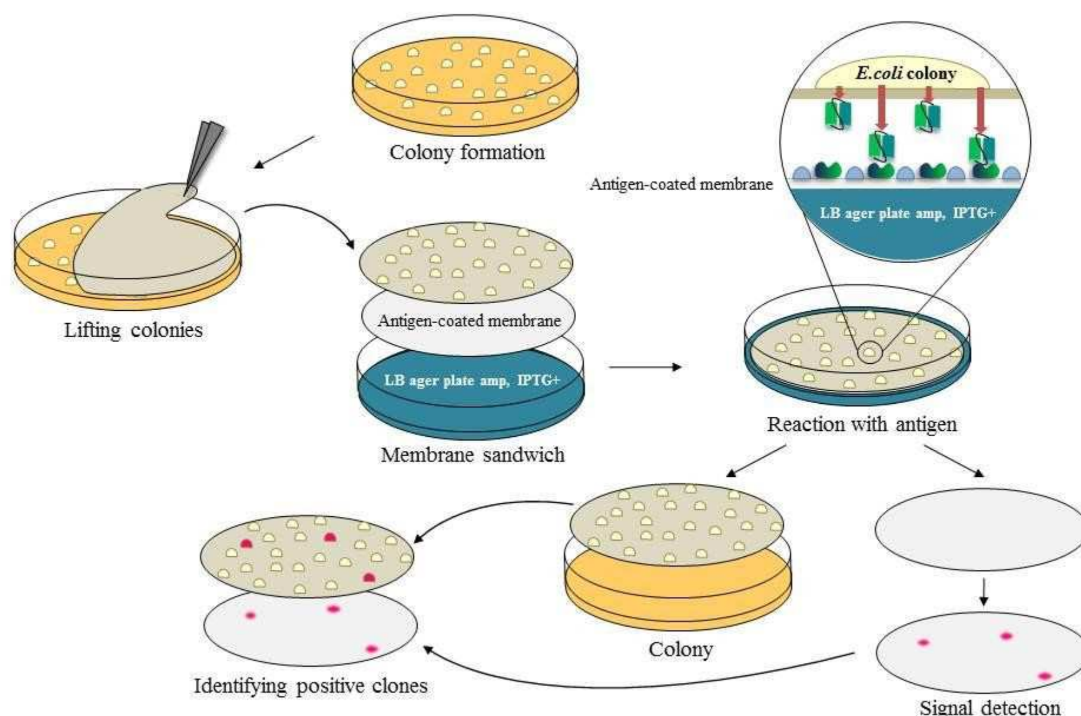

Figure 5. Schematic illustration of the colony screening process

which are then grown on agar plates forming colonies. Following colony formation, a nitrocellulose membrane is adhered to the plate and the colonies are lifted. The nitrocellulose membrane is superimposed on a membrane that has adsorbed the antigen (the antigen-coated membrane) and then placed on an agar plate containing an expression-inducing agent, such as IPTG. The original plate with the colonies is preserved. The expression-inducing agent causes the scFv library to be expressed and secreted from the *E. coli* cells. scFvs with the desired affinity will bind the antigen. The antigen-coated membrane is then collected and bound scFvs are detected using an enzymatic method. Positive spots are then identified by superimposing the membrane on the original plate to select the preserved clones. Dreher et al (Dreher, Gherardi, Skerra, & Milstein, 1991; Skerra, Dreher, & Winter, 1991) further developed the technique for selecting positive clones: *E. coli* colonies are grown on a hydrophilic filter, which is then superimposed on an antigen-coated membrane and placed on an agar plate containing an expression-inducing agent; the presence of antibodies bound to the membrane is then detected and the spot is superimposed on the colony. This method circumvents the difficult technique of lifting the colony (Rauth et al., 2010).

Colony lift assay screening identifies clones with high reliability by directly observing the antibody-antigen bonds. In addition, the method can be easily used to screen libraries an order of magnitude larger than can be screened with hybridoma technology, which can already process very large libraries (Giovannoni, Viti, Zardi, & Neri, 2001). Screening with the colony lift assay induces actual expression and involves direct confirmation of bonds, thus lending it the advantage of a low false-positive rate. However, this method does pose several problems: it requires extensive and complex manipulation; expression is sometimes nonexistent or very low; and the extensive

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manipulation can lead to contamination and death of the *E. coli* cells, thus potentially preventing gene retrieval. In addition, the fact that expression is induced following *E. coli* growth (colony formation) can result in unstable or low levels of expression, which can result in insufficient assay sensitivity. Although this technique has great potential, it has yet to become widespread.

4. Summary and outlook

The use of recombinant technology for antibody production provides many advantages over conventional antibody production strategies, such as the production of antibodies against antigens unattainable using conventional methods, the ability to select positive clones from very large libraries, and bypassing the use of animals. The establishment of a high-quality library and efficient screening are the most important factors for successful antibody production. There is a great need to improve screening technology in order to quickly and reliably establish high-performance antibodies.

Other potential screening methods not covered in this review include inserting a single hybridoma into a droplet (Debs, Utharala, Balyasnikova, Griffiths, & Merten, 2012), which allows for faster screening of more hybridomas than with conventional methods. Several attempts have also been made to trap a single gene in a droplet in order to express antibodies (Buhr et al., 2012). Future technological developments will address the issues of insufficient sensitivity common in assays utilizing antibodies producing a single cell or a single gene (Chattopadhyay, Gierahn, Roederer, & Love, 2014).

Although artificial libraries provide important advantages such as the ability to construct large libraries in the order of 10^{11} antibodies and the ability to establish human antibodies directly, constructing high-quality libraries has proven difficult. However, developments in combinatorial technologies have yielded techniques such as TRIM that are currently being used to construct high-quality libraries (Prassler et al., 2011; Rothe et al., 2008).

Novel technologies have also been proposed for phage display that enable panning in liquid rather than fixing antigens to a solid phase (Haque & Tonks, 2012); new methods for reducing screening background are also being developed. In addition, cell surface panning techniques are being developed for membrane proteins in order to utilize their characteristics to establish antibodies against antigens that are difficult to produce using conventional methods (Akahori et al., 2009; Giordano, Cardó-Vila, Lahdenranta, Pasqualini, & Arap, 2001). Phage display is also widely used for affinity maturation (Steinwand et al., 2014; Thie, Voedisch, Dübel, Hust, & Schirrmann, 2009), in which a mutation is introduced into the antibody gene to produce antibodies with higher affinity (Prassler, Steidl, & Urlinger, 2009).

Colony screening methods, including the relatively simple Dreher colony assay (Dreher et al., 1991), are limited by the transfer of the filter on which the colony grows, which often results in failure. A direct cloning method (Kato & Hanyu, 2015) has been developed in which the filter is first superimposed on the antigen-coated membrane, the colony is formed on the filter, and the assay can then be performed. This method does not require the filter to be transferred and screening can be completed in a single step; this method is anticipated to afford more efficient antibody establishment.

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References

Accardi, L. and Di Bonito, P. (2010).Antibodies in single-chain format against tumour-associated antigens: present and future applications. *Curr Med Chem 17*(17),1730–55. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20345346

Akahori, Y., Kurosawa, G., Sumitomo, M., Morita, M., Muramatsu, C., Eguchi, K., … Kurosawa, Y. (2009). Isolation of antigen/antibody complexes through organic solvent (ICOS) method. *Biochem Biophys Res Commun 378*(4), 832–5. doi:10.1016/j.bbrc.2008.11.129

Buhr, D. L., Acca, F. E., Holland, E. G., Johnson, K., Maksymiuk, G. M., Vaill, A., … Kiss, M. M. (2012). Use of micro-emulsion technology for the directed evolution of antibodies.*Methods 58*(1), 28–33. doi:10.1016/j.ymeth.2012.07.007

Chan, C. E. Z., Chan, A. H. Y., Lim, A. P. C., and Hanson, B. J. (2011). Comparison of the efficiency of antibody selection from semi-synthetic scFv and non-immune Fab phage display libraries against protein targets for rapid development of diagnostic immunoassays. *J Immunol Methods 373*(1-2), 79–88. doi:10.1016/j.jim.2011.08.005

Chattopadhyay, P. K., Gierahn, T. M., Roederer, M., and Love, J. C. (2014). Single-cell technologies for monitoring immune systems. *Nat Immunol 15*(2), 128–35. doi:10.1038/ni.2796

Chen, G. and Sidhu, S. S. (2014). Design and generation of synthetic antibody libraries for phage display. *Methods Mol Biol 1131*, 113–31. doi:10.1007/978-1-62703-992-5_8

De Bruyn, M., Bremer, E., and Helfrich, W. (2013). Antibody-based fusion proteins to target death receptors in cancer. *Cancer Lett 332*(2),175-83.doi:10.1016/j.canlet.2010.11.006

Debs, B. E., Utharala, R., Balyasnikova, I. V., Griffiths, A. D., and Merten, C. A. (2012).Functional single-cell hybridoma screening using droplet-based microfluidics. *Proc Natl Acad Sci USA*. doi:10.1073/pnas.1204514109

Demarest, S. J.and Glaser, S. M. (2008). Antibody therapeutics, antibody engineering, and the merits of protein stability. *Curr Opin Drug Disc Dev 11*(5), 675–87. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18729019

Dreher, M. L., Gherardi, E., Skerra, A., and Milstein, C. (1991). Colony assays for antibody fragments expressed in bacteria. *J Immunol Methods 139*(2), 197–205. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2045660

Dübel, S. (2007). Recombinant therapeutic antibodies. *Appl Microbiol Biotechnol 74*(4), 723–9. doi:10.1007/s00253-006-0810-y

Feldhaus, M. J.and Siegel, R. W. (2004). Yeast display of antibody fragments: a discovery and characterization platform. *J Immunol Methods 290*(1-2), 69–80. doi:10.1016/j.jim.2004.04.009

Geyer, C. R., McCafferty, J., Dübel, S., Bradbury, A. R. M., and Sidhu, S. S. (2012). Recombinant antibodies and in vitro selection technologies. *Methods Mol Biol 901*, 11–32. doi:10.1007/978-1-61779-931-0_2

Giordano, R. J., Cardó-Vila, M., Lahdenranta, J., Pasqualini, R., and Arap, W. (2001).Biopanning and rapid analysis of selective interactive ligands. *Nat Medi 7*(11), 1249–53. doi:10.1038/nm1101-1249

Giovannoni, L., Viti, F., Zardi, L., and Neri, D. (2001). Isolation of anti-angiogenesis antibodies from a large combinatorial repertoire by colony filter screening.*Nucl Acids Res 29*(5), E27. Retrieved fromhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=29740&tool=pmcentrez&rend ertype=abstract

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Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L.,… Allison, T. J. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J 13*(14), 3245–60. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=395221&tool=pmcentrez&ren dertype=abstract

Hagemeyer, C. E., von Zur Muhlen, C., von Elverfeldt, D., and Peter, K. (2009). Single-chain antibodies as diagnostic tools and therapeutic agents. *Thromb Haemost 101*(6), 1012–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19492141

Haque, A. and Tonks, N. K. (2012). The use of phage display to generate conformation-sensor recombinant antibodies. *Nat Protocols 7*(12), 2127–43. doi:10.1038/nprot.2012.132

Holliger, P. and Hudson, P. J. (2005). Engineered antibody fragments and the rise of single domains. *Nat Biotechnol 23*(9), 1126–36. doi:10.1038/nbt1142

Hoogenboom, H. R. (2005). Selecting and screening recombinant antibody libraries. *Nat Biotechnol 23*(9), 1105–16. doi:10.1038/nbt1126

Hust, M., Frenzel, A., Schirrmann, T., and Dübel, S. (2014). Selection of recombinant antibodies from antibody gene libraries. *Methods Mol Biol* 1101, 305–20. doi:10.1007/978-1-62703-721-1_14

Jäger, V., Büssow, K., Wagner, A., Weber, S., Hust, M., Frenzel, A., and Schirrmann, T. (2013).High level transient production of recombinant antibodies and antibody fusion proteins inHEK293 cells. *BMC Biotechnol* 13, 52. doi:10.1186/1472-6750-13-52

Jostock, T. and Dübel, S. (2005). Screening of molecular repertoires by microbial surface display. *Comb Chem High Throughput Screen 8*(2), 127–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15777176

Kaiser, P. D., Maier, J., Traenkle, B., Emele, F., and Rothbauer, U. (2014). Recent progress in generating intracellular functional antibody fragments to target and trace cellular components in living cells. *Biochim BiophysActa*, *1844*(11), 1933–42. doi:10.1016/j.bbapap.2014.04.019

Kato, M. and Hanyu, Y. (2013). Construction of an scFv library by enzymatic assembly of VL and VH genes. *J Immunol Methods 396*(1-2), 15–22.

Kato, M. and Hanyu, Y. (2015). Direct cloning from antibody libraries. to be submitted to *J Immunol Methods*.

Kato, M., Sasamori, E., Chiba, T., and Hanyu, Y. (2011). Cell activation by CpG ODN leads to improved electrofusion in hybridoma production. *J Immunol Methods 373*(1-2), 102–10.

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature 256*(5517), 495–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1172191

Kontermann, R. E. (2010). Alternative antibody formats. *Curr Opin Mol Ther 12*(2), 176–83.Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20373261

McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature 348*(6301), 552–4. doi:10.1038/348552a0

Miethe, S., Meyer, T., Wöhl-Bruhn, S., Frenzel, A., Schirrmann, T., Dübel, S., and Hust, M. (2013). Production of single chain fragment variable (scFv) antibodies in Escherichia coli using the LEX™ bioreactor. *J Biotechnol 163*(2), 105–11. doi:10.1016/j.jbiotec.2012.07.011

Morino, K., Katsumi, H., Akahori, Y., Iba, Y., Shinohara, M., Ukai, Y., …

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Kurosawa, Y. (2001).Antibody fusions with fluorescent proteins: a versatile reagent for profiling protein expression. *J Immunol Methods 257*(1-2), 175–84. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11687251

Müller, D. (2010). scFv by Two-Step Cloning. In R. Kontermann and S. Dübel (Eds.), *Antibody Engineering*. Springer Berlin Heidelberg. pp. 55–9.

Nelson, A. L. (2010). Antibody fragments: Hope and hype. *mAbs*. doi:10.4161/mabs.2.1.10786

Nettleship, J. E., Ren, J., Rahman, N., Berrow, N. S., Hatherley, D., Barclay, A.N., and Owens, R. J. (2008). A pipeline for the production of antibody fragments for structural studies using transient expression in HEK 293T cells. *Protein Expr Purif 62*(1), 83–9. doi:10.1016/j.pep.2008.06.017

Parmley, S. F. and Smith, G. P. (1988). Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene 73*(2), 305–18. Retrieved from. http://www.ncbi.nlm.nih.gov/pubmed/3149606

Persson, M. A., Caothien, R. H., and Burton, D. R. (1991). Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. *Proc Natl Acad Sci USA 88*(6), 2432–6. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=51246&tool=pmcentrez&rend ertype=abstract

Prassler, J., Steidl, S., and Urlinger, S. (2009). In vitro affinity maturation of HuCAL antibodies: complementarity determining region exchange and RapMAT technology. *Immunotherapy*, *1*(4), 571–83. doi:10.2217/imt.09.23

Prassler, J., Thiel, S., Pracht, C., Polzer, A., Peters, S., Bauer, M., … Enzelberger, M. (2011). HuCAL PLATINUM, a Synthetic Fab Library Optimized for Sequence Diversity and Superior Performance in Mammalian Expression Systems. *J Mol Biol 413*(1), 261–278. doi:10.1016/j.jmb.2011.08.012

Rauth, S., Schlapschy, M., and Skerra, A. (2010). Selection of Antibody Fragments by Means of the Filter-Sandwich Colony Screening Assay. In R. Kontermann and S. Dübel (Eds.), *Antibody Engineering*. Springer Berlin Heidelberg. pp. 255–6.

Reichert, J. M. (2005). Antibodies to watch in 2015. *mAbs. 7*(1), 1–8. doi:10.4161/19420862.2015.988944

Rothe, C., Urlinger, S., Löhning, C., Prassler, J., Stark, Y., Jäger, U., … Urban, M. (2008). The human combinatorial antibody library HuCAL GOLD combines diversification of all six CDRs according to the natural immune system with a novel display method for efficient selection of high-affinity antibodies. *J Mol Biol 376*(4), 1182–200. doi:10.1016/j.jmb.2007.12.018

Schaefer, J. V., Honegger, A., and Plückthun, A. (2010). Construction of scFv Fragments from Hybridoma or Spleen Cells by PCR Assembly. In R. Kontermann and S. Dübel (Eds.), *Antibody Engineering*. Springer Berlin Heidelberg. pp. 21–44.

Schaefer, J. V., Lindner, P., and Plückthun, A. (2010). Miniantibodies. In R. Kontermann and S.Dübel (Eds.), *Antibody Engineering*. Springer Berlin Heidelberg. pp. 85–99.

Schaffitzel, C., Hanes, J., Jermutus, L., and Plückthun, A. (1999). Ribosome display: an in vitro method for selection and evolution of antibodies from libraries. *J Immunol Methods*, *231*(1-2), 119–35. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10648932

Schirrmann, T., Al-Halabi, L., Dübel, S., and Hust, M. (2008). Production systems for recombinant antibodies. *Front Biosci 13*, 4576–94. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18508530

Schirrmann, T., Meyer, T., Schütte, M., Frenzel, A., and Hust, M. (2011). Phage

Copyright 2015 KEI Journals. All rights reserved

display for the generation of antibodies for proteome research, diagnostics and therapy. *Molecules16*(1),412–26.doi:10.3390/molucules16010412

Schofield, D. J., Pope, A. R., Clementel, V., Buckell, J., Chapple, S. D., Clarke, K. F., … McCafferty, J. (2007). Application of phage display to high throughput antibody generation and characterization. *Genome Biol 8*(11), R254.doi:10.1186/gb-2007-8-11-r254

Skerra, A., Dreher, M. L., and Winter, G. (1991). Filter screening of antibody Fab fragments secreted from individual bacterial colonies: specific detection of antigen binding with a two-membrane system. *Anal Biochem 196*(1), 151–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1888028

Steinwand, M., Droste, P., Frenzel, A., Hust, M., Dübel, S., and Schirrmann, T. (2014). The influence of antibody fragment format on phage display based affinity maturation of IgG. *mAbs*, *6*(1), 204–18. doi:10.4161/mabs.27227

Thie, H., Voedisch, B., Dübel, S., Hust, M., and Schirrmann, T. (2009). Affinity maturation by phage display. *Methods Mol Biol 525*, 309–22, xv. doi:10.1007/978-1-59745-554-1_16

Weiner, G. J. (2015). Building better monoclonal antibody-based therapeutics. *Nat Rev Cancer* *15*(6), 361–370. doi:10.1038/nrc3930

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