Chapter 5

Colony Assay for Antibody Library Screening: Outlook and Comparison to Display Screening

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Abstract

Recombinant monoclonal antibodies are established by screening the antibody libraries. To obtain antibodies with a high specificity and affinity, an efficient screening process with a highly diverse library including low background signals is necessary. One of the most extensively used methods is the phage display method. Although phage display screening is a powerful tool for enriching clones from vast libraries, it is not easy to identify single clones with an antigen recognition function only through several rounds of biopanning. The application of colony assays for screening antibody libraries can identify clones with a high reliability by a direct observation of the antibody-antigen binding during the screening process; however, the size of the library that can be dealt with is limited. This chapter describes the colony assay as a current screening technology used in recombinant monoclonal antibody production, the possible problems in this method, and discusses the outlook for this technology.

Keywords: colony assay, screening, antibody library, scFv, *E. coli*, phage display

1. Introduction

The use of recombinant technology for antibody selection offers several advantages over conventional antibody selection strategies, such as the selection of antibodies against toxic or non-immunogenic antigens unattainable using conventional methods [1, 2], the ability to select positive clones from vast libraries [3], the realization of in vitro screening [4], and the bypass of animal usage [5]. The selection and production of recombinant monoclonal antibodies require the creation of highly diverse libraries [6] and the subsequent identification of positive clones using a screening technology with low background signals [7]. In particular, the variable domains of the antibody heavy and light chain (V_H and V_L) are isolated

from the lymph tissue of immunized animals and linked together for creating a single-chain variable fragment (scFv) library, and Fab libraries are constructed too. In general, the antibody fragments used for screening are the scFvs. Currently, entirely synthetic libraries [8–11] and naïve libraries [12] are being used. These antibody gene libraries are incorporated into a phagemid or plasmid and expressed in phage or *Escherichia coli* (*E. coli*). Further, panning [13] or colony assays [14] are performed to isolate scFvs possessing affinity to the antigen, thereby establishing monoclonal antibodies. This step, the screening of antibody libraries, is critical for establishing monoclonal antibody fragments with a high affinity and specificity against the antigen. One of the most extensively used methods is the phage display method [15, 16]. The display of the antibody repertoires on the surface of bacteriophages and their selection through panning enables the isolation of monoclonal antibodies [17]. Phage display is also widely used for affinity maturation [18, 19], in which mutations are introduced into the variable domains of an antibody gene mainly into CDRs to produce antibodies with a higher affinity as the original clone [20]. In addition, cell surface panning techniques [1, 21, 22] are being developed to establish antibodies recognizing membrane proteins on living cells that are difficult to produce using the conventional methods. Technologies that enable liquid panning rather than immobilizing the antigens to a solid phase have also been proposed for phage display to establish antibodies that recognize protein conformation [23]. Screening with a colony assay induces the actual expression of the scFvs themselves and involves a direct confirmation of the antigen-antibody binding, lending it the advantage of a low false-positive rate. In addition, the method can be easily used to screen libraries in the order of magnitude larger than those that can be screened with the hybridoma technology. However, this method poses several problems: it requires extensive and complex manipulation of assay steps, the expression of antibody fragments could be at times nonexistent or very low, and the extensive manipulation during the assay can lead to contamination and death of the *E. coli* cells, potentially preventing gene retrieval. Although this technique is not complete and not widely applied, further development and improvement can render it highly beneficial.

2. Antibody library screening

A critical step in the establishment of antigen-specific monoclonal antibody fragment clones is the screening of the recombinant-antibody libraries [6, 7]. Methods for screening the antibody libraries can be largely divided into two strategies [24]: the display and repertoire cloning strategies (**Figure 1**).

2.1. Display strategies for antibody library screening

In the display strategy [25], the antibody fragment and its gene, i.e., the antigen recognition function and information, are joined, and antibody fragments with an affinity against the antigen are screened. Phage display systems in which an scFv joined to the filamentous phage coat protein, g3p, is displayed on the phage are extensively used [15, 16]. Other display systems include yeast display systems in which scFvs are displayed on the surface of yeast [26]; mRNA display [27]; ribosome display in which a ribosome, mRNA, and an scFv are

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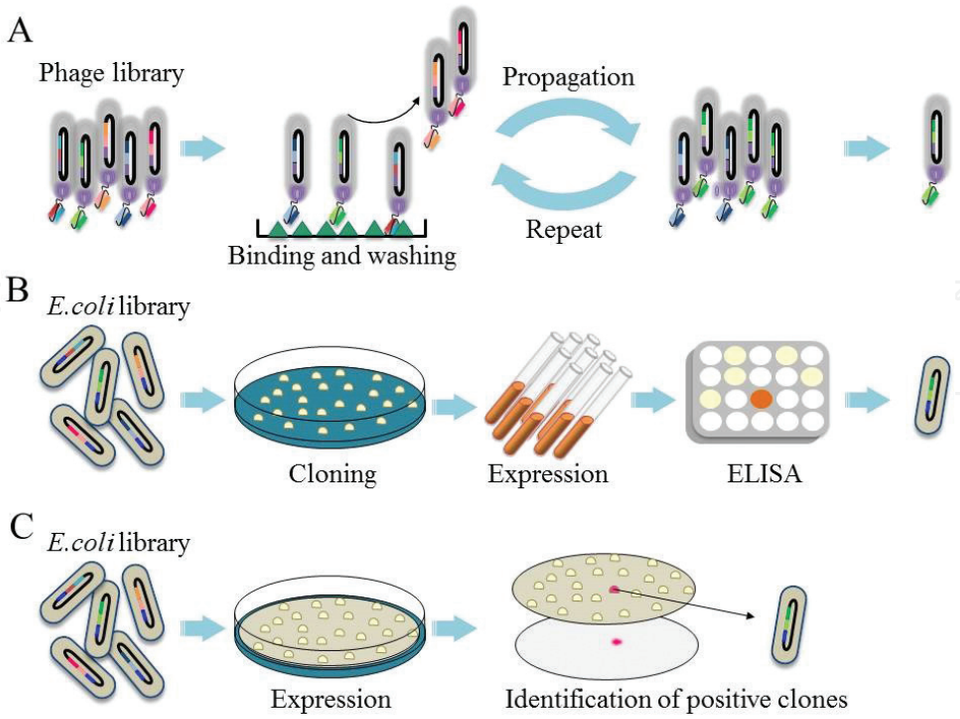

Figure 1. Strategies for antibody library screening. (A) Display strategy: scheme of the phage display panning process. (B) Repertoire cloning strategy: scheme of the cloning and assay process. (C) Detection of antigen-specific antibody fragments released from a bacterial colony by a colony assay.

integrated [28]; bacterial surface display [29]; and mammalian cell surface display [30, 31] for human antibody discovery. In these display systems, panning is applied for screening [13]. The antigen is immobilized on the surface of a microtiter plate, and the scFv library can be screened with phage display and with ribosome/mRNA display. Weakly bound clones are removed by washing, retaining the specific clones bound to the antigen (**Figure 1A**). This panning method is characterized by repeated selection, proliferation, and the enrichment of positive clones for enabling the processing of large libraries. For yeast, bacterial and mammalian cell surface display FACS with the cells displaying the recombinant antibody fragments using labeled antigen is applied.

2.2. Repertoire cloning strategies for antibody library screening

In contrast, in repertoire cloning strategies, the antibody library is transformed into *E. coli*; the scFvs are expressed and secreted from a single clone, and scFvs are screened by ELISA (**Figure 1B**). Clones are selected based on assays, using scFv characteristics such as the affinity; thereby, this method offers advantages such as low false-positive rates and the ability to reliably identify clones with a high affinity. However, an assay must be performed for each individual *E. coli* clone, and only the positive clones are selected. There is no enrichment process in the screening method, and only limited libraries can be used for antibody selection.

Particularly, antibody repertoires from immunized animals with a clone number of approximately 10^6 are suitable for the repertoire cloning but not naïve and synthetic antibody

repertoires with high clone numbers (10^9–10^{12} clones). During the assay, a clone from an *E. coli* library is cultured, and its expression is induced. The reactivity of the expressed scFv against the antigen is measured. Clones exhibiting high reactivity are selected as the positive clones. In this method, only a few thousands of clones can be examined simultaneously, even if a multi-well microtiter plate is used. Although this number is higher than the clones obtained by the hybridoma technique, positive clones cannot be efficiently obtained, when the positive ratio is low.

Colony assay in which periplasmic expression and *E. coli* colony formation lifted onto filters are used provides a method for handling large libraries (**Figure 1C**). In colony assay, the clones do not need to be picked up individually before screening; all the colonies on the plate can be assayed simultaneously. Thus, numerous clones can be assayed from a single plate. Antibody fragments released by bacteria were detected by a phage plaque assay in earlier experiments [32, 33]. Libraries of the antibody fragments were expressed in *E. coli* using phage λ vectors [34, 35]. Then, the active fragments secreted from the viable *E. coli* colonies were detected by colony-lift immunoassay [36]. With the colony assay, considerably larger libraries can be dealt with because the number of colonies screened can be easily increased.

2.3. Screening with a phage display

In a phage display system, panning is used to isolate phages that display the antibody fragments exhibiting affinity to the antigen (**Figure 1A**). Positive clones are established by selecting only the phages that displays antibody fragments (primarily scFvs) fused to the g3p coat proteins on the surface of the filamentous phage, which have affinity to the antigen. This method has the advantage of processing large libraries (~10^{11}) [3, 37]. The antigen is immobilized, and the recombinant phage bound to the antigen is left intact; weakly bound recombinant phages are washed away. The remaining recombinant phages, which possess a binding capacity are detached from the antigen by acid treatment and infected into *E. coli*. Further, *E. coli* cells are cultured to propagate positive clones. The *E. coli* clones expressing the phagemids are then infected with a helper phage, and the phages displaying scFvs with binding capacities are collected. Panning is performed repeatedly for the selected group of phages. The repeated selection and propagation of positive clones enrich clones with antibodies comprising binding capacities to the antigen. Then, single clones are isolated at the final step with high binding capacities [38]. This method renders it possible to handle large libraries.

One limitation of this method is that the high background during panning selection often results in false-positive clones. A specific antigen-binding activity is typically not the only driving force exploited during the panning process [39, 40]. Multiple rounds of panning have been documented to frequently cause a strong bias for antibodies directed against immunodominant epitopes and abundant proteins [41], resulting in the loss of the library's diversity and of valuable antibody clones. Several factors influence the selection of the antigen-specific clones and produce undesired effects; these factors include a high efficiency of expression and folding despite poor antigen-binding activity, the nonspecific hydrophobic binding properties of the phage particle itself, and a superior compatibility with the host cells, not related to the antibody fragment affinity.

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However, as several antibody fragments are themselves toxic to *E. coli*, these clones will be lost during panning, even if they possess a 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 a high binding capacity. Toxicity to *E. coli* can increase the background, resulting in several false-positive clones being obtained. This situation renders panning extremely difficult; it is not easy to establish single positive clones only through several rounds of panning [14]. Although the phage display is a powerful tool for establishing monoclonal antibodies, it is used less frequently than expected [39].

3. Colony assay for antibody library screening

As an alternative antibody-screening tool, the colony assay can be used which is sometimes superior to the phage display method [42]. The advantage of this method is that the antibody-antigen binding can be directly observed during the screening process, reducing the selection of false-negative clones [24]. Thus, the colony assay presents notable advantages over the phage display and biopanning method.

3.1. Principle of the colony assay

In the colony assay (**Figure 2**), antibody libraries are expressed in *E. coli* for the selection of clones with a favorable affinity to the antigen. An scFv library is transformed into *E. coli* cells, and afterward transformed *E. coli* cells are plated on appropriate agar plates. After growing of the colonies, they are lifted onto a filter. Further, an expression-inducing reagent such as isopropyl-β-$_D$-thiogalactopyranoside (IPTG) is applied, inducing the expression and secretion of scFvs from the *E. coli* cells (**Figure 2A**). scFvs with the desired affinity will diffuse out and bind the antigen coated on the membrane beneath the colonies. However, scFvs without affinity will not bind the antigen (**Figure 2B**), and the unbound scFvs are washed away. Then, the bound scFvs with an affinity against the antigen are detected using an enzymatic method. The His-tags attached to the scFvs are detected with anti-His antibodies (**Figure 2C**). Positive clones are identified as the colonies corresponding to positive signals (**Figure 2D**).

3.2. Filter-sandwich assay

Dreher et al. [43, 44] improved the colony assay by developing the filter-sandwich colony-screening assay (hereafter, the filter-sandwich assay) for selecting positive clones; *E. coli* colonies are grown directly on a hydrophilic filter, which is then transferred to an antigen-coated membrane soaked with IPTG solution and placed on an agar plate containing IPTG to induce antibody fragment production. The antibody fragments produced by the colonies diffuse out and bind to the antigen on the membrane. The presence of antibody fragments 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 [14, 36]. In addition, the filter-sandwich assay was further optimized. The procedure can now be performed by a single step [45] under tightly controlled IPTG concentration for expression of the scFvs.

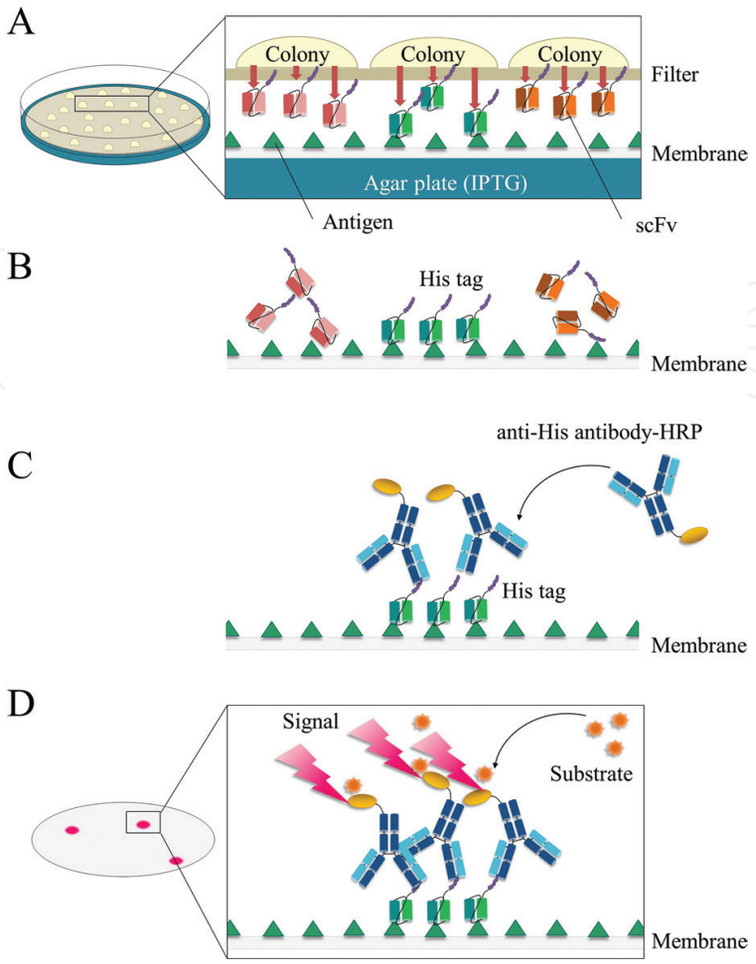

Figure 2. Scheme of the colony assay principle. (A) scFvs are expressed and secreted from *E. coli*. (B) scFvs with the desired affinity bind the antigen beneath the colonies. (C) Bound scFvs with an affinity against the antigen are detected using an enzymatic method. (D) Positive clones are identified as the colonies corresponding to positive signals.

3.3. Procedure for filter-sandwich assay

The procedure used in the filter-sandwich colony assay is depicted schematically in **Figure 3**.

In particular, the RNAs are isolated from the lymph tissue of immunized animals, and the corresponding cDNA is synthesized; this cDNA is used as the template for the polymerase chain reaction (PCR) amplification of the V_L and V_H domains. Further, the variable domains are assembled to an scFv and cloned into an expression vector to create the scFv libraries [46]. As expression vector, for example, pET22b (+), containing a *pelB* signal sequence for periplasm expression and a His-tag sequence for the detection of the scFv expression driven by the T7 promoter, is used. The antibody repertoire is transformed into *E. coli*, and the filter sandwich assay is performed as described in **Figure 3**.

The hydrophilic PVDF filter is placed on an agar plate. Transformed *E. coli* with the scFv libraries is spread onto the filter and incubated. After the formation of the bacterial colonies on the filter surface, the filter harboring the colonies is transferred to an antigen-coated nitrocellulose membrane on the agar plate containing IPTG and incubated to induce scFv expression.

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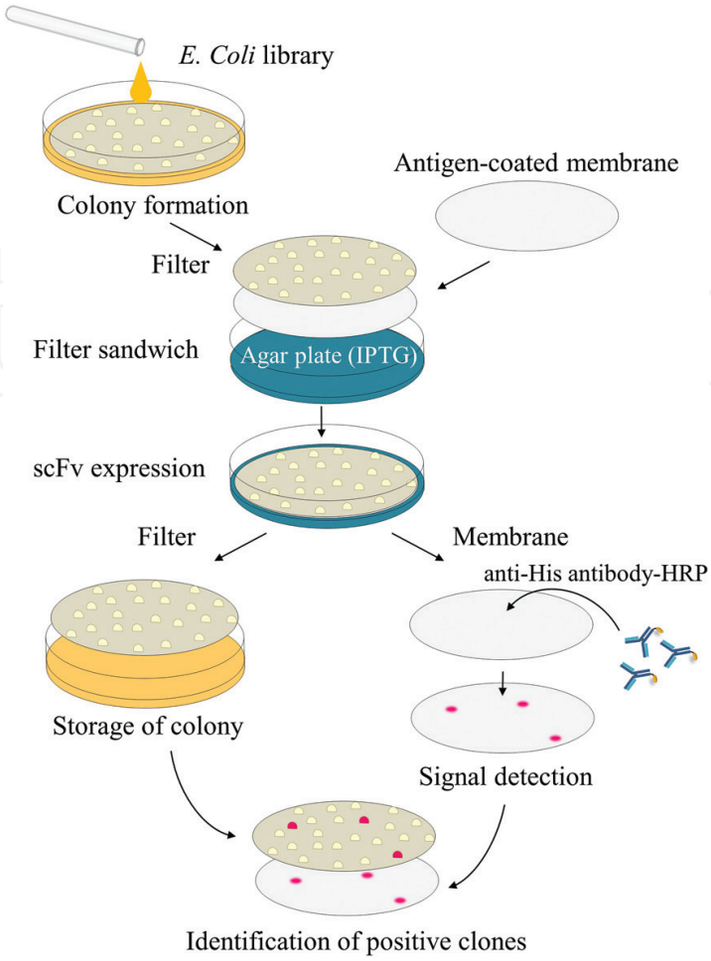

Figure 3. Procedure for filter-sandwich colony assay.

Then, the filter harboring the colonies is removed, placed on a fresh plate, and stored for the later recovery of the bacteria. Subsequently, antigen-bound scFvs on the nitrocellulose membrane are detected with chemiluminescence from a horseradish peroxidase (HRP)-conjugated anti-His antibody. The filter harboring the colonies and the image presenting the chemiluminescence data are superimposed, and positive colonies corresponding to the chemiluminescence signals are identified. These positive clones are transferred to a medium and incubated. The plasmid encoding the scFv gene with an affinity against the antigen is purified, and the antibody coding sequence is determined.

3.4. Establishing monoclonal antibody fragments by colony assay

A colony assay is used for screening the antibody fragments against a variety of antigens, with optimizations for each specific purpose. The recombinant antibody fragments against EspA and the intimin of *E. coli* O157:H7 were established by colony filter screening [47]. Colony-lift assay was combined with phage display, using cell-coated filters to screen the phage libraries for cell-binding clones [48]. Robert et al. developed subtractive colony filter screening to select scFvs that recognize atherosclerotic but not the normal aorta [49]. Giovannoni et al.

isolated antiangiogenesis antibodies from combinatorial libraries by iterative colony filter screening: colonies located around the positive signals were selected, and the screening step was repeated; monoclonal scFvs were established after several rounds of the assay [50]. Neumann-Schaal et al. developed a colony-screening method in which *E. coli* colonies producing the required scFv were selected in the presence of ampicillin conjugated to the antigen of interest; this method relies on the neutralization of the conjugate by the produced scFv. The scFvs were identified against biotin by the growth of the scFv library-expressing *E. coli* in the presence of a biotin-ampicillin conjugate [51]. Kumada et al. improved the sensitivity of the colony assay utilizing antibody-coupled liposome encapsulating HRP [52].

4. Summary

It is possible to screen 3–5 × 10^3 clones on a 10-cm diameter plate in a filter-sandwich assay, whereas in the hybridoma method, dozens of 96-well microtiter plates are required for screening these clones. Further, the filter-sandwich assay can be readily upscaled by increasing the number of plates. Therefore, the number of positive clones from the filter-sandwich assay can be higher than that from the hybridoma method. This would increase the chance of obtaining monoclonal antibody fragments with the desired affinity, specificity, and function.

However, the filter-sandwich assay needs to be improved further for the selection of positive clones, particularly with respect to the reliability of the antibody fragment expression and the handling of the colonies during the assay. For the colony assay, the control of the expression level is critical. Because the scFv expression by itself is considerably toxic to *E. coli*, an excess induction of expression, namely, exposure to an excess of the expression-inducing reagent (IPTG), leads to cell death and prevents the selection of antigen-specific scFvs. Conversely, exposure to insufficient IPTG induces inadequate antibody expression for the detection of signals from positive clones. In the filter-sandwich assay, expression induction is not stringently controlled because the concentration of the IPTG added to the cells cannot be precisely controlled. IPTG reaches the colonies by diffusing through the filter from the antigen-coated membrane and the agar plate. Quantitative control of the expression level is required for superior screening. This uncertainty in the IPTG concentration in the filter-sandwich assay also causes a problem in the induction timing. For appropriate induction, the colony size is a critical factor [14, 44]; however, the colony continues to grow during the assay. Hence, the timing of the expression induction is crucial for proper expression. If the ITPG diffusion is delayed, an initially small colony would grow too large for proper induction to occur; however, if the colonies are too small, the signal from each colony is inadequate for detecting the antigen binding. The induction strength cannot be accurately determined, particularly during the step, when the filter is transferred to the IPTG-containing plate to initiate the induction of expression. These induction-related uncertainties in the filter-sandwich assay lead to unstable expression and failure in isolating the antibody-encoding genes. Stringent control of the expression level is critical. Various factors related to the expression vector, such as the promoter, strength of the ribosomal binding site, fusion tags, and the copy number, must be optimized [53–57]. The incubation temperature is also an important factor in controlling the

expression strength [58]. For inducing expression, additional methods such as the cold-shock system [59] should be examined. Expression-inducing reagents that are less toxic than IPTG to *E. coli*, such as rhamnose [60], should also be tested.

In the filter-sandwich assay, before the induction of antibody expression, the filter harboring the colonies must be transferred without disturbance. This transfer requires delicate manipulation of the filter and frequently produces unwanted stress on the filter, occasionally disturbing the colonies themselves. A method that does not require the transfer of the filter should be developed for more efficient antibody establishment. Recently, a single-step colony assay was established by us using a tightly controlled IPTG concentration for scFv expression [45]. One advantage is also that no transfer of the filter on which the colonies are grown to the antigen-coated membrane is necessary.

The establishment of a high-quality antibody library and efficient screening are the most important factors for successful recombinant antibody selection and production. Improvements in the screening technology are critical for quickly and reliably establishment of high-performance antibodies. Phage display screening is a powerful tool for this purpose; however, it has certain disadvantages such as the frequent selection of false-positive clones, but it can easily deal with a vast library. On the other hand, screening with a colony assay could identify the positive clones reliably; however, it cannot deal with a large complex library. Thus, screening methods using a display panning system and a colony assay have certain advantages and disadvantages, respectively. They should be utilized cooperatively, depending on the purpose of the experiments. Hence, condensing the library by phage display and then cloning the positive clones by colony assay would be advantageous. To efficiently establish high-quality antibodies, the adequate choice of these technologies and their combination would be crucial.

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