Chapter 6

Phage Display: A Powerful Technology for the Generation of High Specificity Affinity Reagents from Alternative Immune Sources

William J.J. Finlay, Laird Bloom, and Orla Cunningham

Abstract

Antibodies are critical reagents in many fundamental biochemical methods such as affinity chromatography. As our understanding of the proteome becomes more complex, demand is rising for rapidly generated antibodies of higher specificity than ever before. It is therefore surprising that few investigators have moved beyond the classical methods of antibody production in their search for new reagents. Despite their long-standing efficacy, recombinant antibody generation technologies such as phage display are still largely the tools of biotechnology companies or research groups with a direct interest in protein engineering. In this chapter, we discuss the inherent limitations of classical polyclonal and monoclonal antibody generation and highlight an attractive alternative: generating high specificity, high affinity recombinant antibodies from alternative immune sources such as chickens, via phage display.

Key words: Chicken, scFv, Phage display, Chromatography

1. Introduction

The rapid expansion of the genomics, proteomics, and biotechnology fields has led to a growing demand for affinity reagents that can specifically recognize proteins, peptides, carbohydrates, and haptens. Affinity reagents of high specificity are routinely required for diverse protein drug targets, members of newly discovered biochemical pathways, posttranslationally modified proteins, protein cleavage products, and even small molecules such as drugs of abuse and toxins. Individual biomedical researchers will often need to monitor, quantify and purify proteins of interest via affinity chromatography, but there may not be any commercially available antibody reagents to allow them to do so (1). Indeed, even in

Dermot Walls and Sinéad T. Loughran (eds.), *Protein Chromatography: Methods and Protocols*, Methods in Molecular Biology, vol. 681, DOI 10.1007/978-1-60761-913-0_6, © Springer Science+Business Media, LLC 2011

situations where there are commercially available antibodies, these reagents are often expensive, poorly characterized, and/or simply not appropriate for demanding applications. Compounding this problem, the technical difficulty of monoclonal antibody generation by the untrained researcher and the high cost (~$15,000) of a commercial monoclonal antibody generation program leads many researchers to the default solution of producing polyclonal hyperimmune sera in hosts such as rabbits. The net result of this is that researchers often settle for reagents that lack the necessary specificity to perform the applications for which they were intended.

In this review, we will outline the limitations of classical antibody generation technologies and illustrate an attractive alternative: the use of phage display libraries of recombinant antibodies built on immunoglobulin repertoires from nonmammalian animals. In particular, we will highlight the advantages of libraries derived from the domestic chicken *Gallus gallus*, which offers a relatively inexpensive and technically accessible route to high-quality monoclonal reagents. If, like many people, you have purchased (or paid to generate) a costly and "specific" antibody, but subsequently found that it is actually polyreactive and of dubious quality, phage display from immunized chickens may offer an attractive alternative.

1.1. Historical Difficulties in Antibody Generation Technology

Hyperimmune sera from rabbits, sheep, or other mammals may be produced in large quantities, but they do not offer the consistency of monoclonal antibodies and need to be regularly replenished and recharacterized. Serum antibodies are also polyclonal and frequently polyspecific, even when purified over an antigen column, rendering them suboptimal for the specific recognition of a single component in a complex matrix. One illuminating study (2) has demonstrated that when used to probe a comprehensive yeast proteome chip, unpurified polyclonal antibody preparations could recognize up to 1,770 different proteins, with some monoclonal antibodies and antigen-column purified polyclonal antibodies also recognizing multiple proteins (related and unrelated).

The arrival of monoclonal antibody technology (3) was a major step forward in generating high-specificity reagents, but the reliance on the murine immunoglobulin system frequently leads to a number of practical difficulties: (1) Monoclonal antibodies are raised on the basis of an inefficient fusion of splenic B-cells to an immortalized mouse myeloma line, followed by limiting dilution of the cell population. Target-specific antibodies are randomly identified, often by a simple direct ELISA, where few preconditions can be set to determine which antibodies are identified and one must "take what one can get" during the screening process. (2) It is often desirable to have multiple monoclonal antibodies with specificity for different epitopes on the same target molecule, but the difficulty in sequencing monoclonals does not allow the rapid identification of unique clones early in the screening process. (3) Humans and rodents are relatively closely related phylogenetically. Many proteins of interest are highly conserved among mammals and this can frequently lead to thymic tolerance restricting the antibody response after immunization. (4) When an immune response to a human protein is raised in mice, the large regions of sequence similarity between murine and human proteins may lead to a restricted number of immunogenic epitopes. (5) To generate antibodies that cross-react with homologs from multiple species of mammal is particularly tricky, as the common epitopes among mammals are the very ones that are unlikely to provoke a strong immunoglobulin response in the mouse. (6) Tolerance issues can become even harder to circumvent when the protein of interest is from a mouse or rat. Creating "knockout" mice, in which the endogenous copy of the gene for the target protein has been disabled, can often break tolerance, but this is a highly laborious and time-consuming process that few laboratories have the resources to undertake. These factors all hinder the generation of high-quality antibody reagents and thereby limit one's experimental options when developing antibodies for purifying or tracking novel proteins.

2. Display Technologies as an Alternative Source of Specific Antibodies

To bypass the limitations in polyclonal and monoclonal antibody generation, several groups have turned to in vitro display technologies such as phage display, ribosome display, or yeast display libraries to generate recombinant antibodies. The more recent technologies of yeast and ribosome display are becoming highly established, but phage display is currently the most robust, well characterized, and reliable of these methods. Antibodies derived from these technologies are cloned in microbial hosts and are therefore monoclonal from the start, with their production being easily scaled up. Critically, selection and screening efforts can be directed toward specific epitopes or species cross-reactivity and away from polyspecific binding. Phage display allows a researcher to do in a single tube what would be unfathomable in traditional hybridoma work: interrogate libraries of millions to tens of billions of antibodies on the basis of their binding specifically to a target of interest.

All of this is possible due to the ingenious concept of "genotype-to-phenotype linkage," which is exemplified by phage display. Phage display was originally described as a rapid method for cloning gene fragments that encode a specific protein (4). By cloning gene fragments into the genome of a filamentous *E. coli* bacteriophage, Smith et al. were able to generate libraries of gene fusions with the key phage coat protein p3. After transformation into *E. coli*, the viral replication system packaged the genome (and therefore the cloned gene) into a highly stable complex carrying the gene product on the tip of the phage particle, as a fusion protein with p3. By subsequently selecting expressed virions on an immobilized antibody with specificity for a known protein that had been cloned in the phage genome, they were able to show 1,000-fold enrichment of the gene product. This set of experiments showed that "genotype-to-phenotype linkage" could be achieved and thereby defined the basis of all display technologies developed since.

The subsequent development of a method for the effective cloning of antibody V-gene sequences via PCR allowed the capture of antibody sequences in a recombinant form (5), removing the need to immortalize B-cells via hybridoma fusion as required in traditional monoclonal antibody generation. This discovery was combined with the phage display process to make an efficient method of isolating antibodies and their corresponding gene sequences simultaneously (6). In the antibody phage display process, libraries of diverse V-gene sequences are cloned into an appropriate expression vector in *E. coli*, creating an in-frame fusion with the p3 protein or, as favored in most recently described libraries, a truncated form of p3.

The phage display of protein libraries was originally performed using "phage" vectors (i.e., built upon the phage genome itself), but due to practical difficulties in handling these libraries, more recent phage methods have mostly used so-called phagemid expression vectors. In this case, the DNA backbone is a stable, small plasmid such as pUC, and the p3 gene plus f1 phage packaging origin are the only phage-derived DNA sequences (7). These phagemid libraries are more easily handled and more stable than phage libraries, as the plasmid is incapable of causing phage production by itself. The libraries can therefore be more simply cloned, expanded, and controlled than phage libraries.

By infecting "helper" phage (based on M13) into a growing culture of *E. coli* harboring a phagemid library, the phage propagation machinery is provided, but due to a mutation introduced in the origin of replication on the helper phage genome, preferential packaging of the phagemid DNA and p3 fusion occurs during phage replication (7). Phage production is thereby induced, genotype-to-phenotype linkage is created, and the expressed phage particles are interrogated for the presence of useful protein sequences via target binding. This selective step may be performed by simply immobilizing the target protein on, for example, a protein-binding plastic surface such as an ELISA plate, adding phage, and allowing binding to occur via the antibody-p3 fusion proteins. Nonbinding phage are removed by washing the immobilized surface, and the remaining bound phage are eluted. The eluted phage is then reinfected back into a fresh culture of *E. coli* to retrieve the selected gene sequences. This process is not perfect, however, and two to four rounds of selection/reexpression/selection are normally performed iteratively to remove all unwanted clones and enrich the binding population from the background of the library. Nonetheless, this process can be spectacularly powerful, massively enriching specific antibodies in a single selection round (8). Furthermore, the use of multiple forms of the target antigen in sequential selection rounds and the inclusion of competitor proteins can drive the selected pool toward a highly specific set of epitopes.

Much of the evolution and progression of phage display technology has been driven by the remarkable discovery that this method can be used to mine large libraries of combinatorial human antibody diversity, theoretically removing the need for animals in antibody production (9). By random recombination of V_H and V_L sequences from human lymphocyte cDNA (9), or, by using degenerate oligonucleotides to create diversity of DNA sequence in the loops associated with target binding (10), several groups have created very large libraries of antibody gene sequences for phage display. These libraries are analogous to the naïve antibody repertoire in an animal, and selecting from them can result in the identification of antibody fragments that exhibit high specificity and occasionally high affinity for the target protein. Several major studies have proven that with appropriate application of this technology, specific antibodies can be raised to proteins, peptides, haptens, and even carbohydrates. In the most exemplary studies, antibodies have been raised with equivalent affinities to those associated with a strong humoral immune response (11–14).

2.1. Recombinant Antibody Formats Used in Phage Display

Recombinant antibodies are typically displayed and expressed in "fragment" forms. The simplest and most commonly used fragment is the single-chain fragment variable (scFv) where a flexible peptide sequence links the V-regions of antibodies between the C terminus of one domain and the N terminus of the other, thereby combining both V-domains into a single polypeptide (15). The scFv may be assembled in V_L–V_H or V_H–V_L orientations, with V_H–V_L being the most heavily used format historically. The flexible linker helps to make the scFv simple to express, but must be sufficiently long and flexible to allow effective association of the V-regions to form a functional antigen-combining site. As long as this is true, the classical hydrophobic pairing of the V-regions will stabilize the structure. By far the most common linkers are based on glycine–serine repeat structures such as GGGGS × 3.

The second most commonly used recombinant antibody format is the Fab (fragment antigen binding) molecule. This structure is a complete binding "arm" of an antibody and comprises the full immunoglobulin light chain, expressed in conjunction with the V_H–C_H1 region from the heavy chain (16). Fabs obligately form predominantly monomeric, monovalent fragments. They are the most "natural" of the recombinant antibody fragments and it has been shown that the presence of the constant regions can often help to stabilize antibody variable regions (17). The Fab format is the less commonly used of the two main recombinant antibody formats, however, as its dual polypeptide structure is generally more difficult to express and display in *E. coli* than the scFv (17).

2.2. Why Is Phage Display not more Heavily Used?

Much of the underuse of phage display may be due to experiences with the early libraries derived from naïve or synthetic human antibody diversity, which were donated to academic laboratories that were not specifically invested in antibody engineering. Unfortunately, these forays into display technologies have often left investigators somewhat disappointed. Many people have accepted the viewpoints of recombinant antibody technology experts that these libraries can yield useful high-affinity antibodies to any form of antigen. In general (mostly to those highly skilled in the field), this is indeed true, but the average antibody generated from these libraries is often of disappointingly low affinity to those who are used to high-sensitivity antibodies from immunized sources. Human recombinant antibodies from naïve library sources can require technically challenging in vitro molecular evolution, if they are to perform the demanding "real world" functions required of many reagent antibodies (8). Molecular evolution is far from trivial to perform and is usually beyond both the scope and interest level of the average researcher. Small wonder then that most people either ignore, or at worst disparage, phage display technology itself.

Nevertheless, phage display can be a relatively simple technology to use and when employed to harness natural repertoires of antibodies from immunized animals, it can offer a rapid path to highly specific, high-affinity antibodies against problematic antigens. While the most successful naïve antibody libraries contain over 10^{10} members and are often the domain of biotechnology companies, typical immune libraries are in the 10^7–10^8 range and are easily assembled by a single investigator (18, 19). When an immunized rabbit or sheep has raised a significant serum immunoglobulin titer, the common endpoint to the experiment is to exsanguinate the animal and harvest the serum. However, harvesting B-cell-rich lymphoid tissues from the animal, such as the spleen and bone marrow, allows the isolation of total RNA and the subsequent generation of cDNA (18). This is a simple method with which many biomedical researchers are familiar, and commercial kits are available to simplify most steps of the process.

The immunoglobulin gene sequences of many animals are now known and the cDNA from immune tissues can subsequently be used for the RT-PCR amplification and cloning of the animal's variable region sequence repertoire (18). These cloned variable region sequences can then be assembled into a display library format such as scFv or chimeric Fab (using human C_H1 and $C_{\kappa/\lambda}$ regions) (20). These targeted immune libraries thereby offer a potentially huge advantage over monoclonal antibodies, as libraries of $>10^8$ variants may be built, allowing the effective sampling of a much broader range of antibodies than the hundreds (occasionally thousands) of clones usually examined in a monoclonal antibody screen.

The resulting library can be interrogated for specific binding proteins via phage display and the retrieved antibody fragments expressed very simply in bacteria (20). This process has been used to successfully harness the antibody repertoires of a large number of immune host species, including mice (18), rabbits (19), sheep (21), camelids (22), and sharks (23). Of greater interest to us, however, is to exploit this approach to harvest the novel immunoglobulin repertoires of the domestic chicken (*G. gallus*), which is as simple to use as mice and rabbits, but also highly phylogenetically distant from mammals.

3. Why Chickens (G. gallus)?

Gallus, Scottish dialect for (1) self-confident, daring, cheeky. (2) Stylish, impressive. Origin derogatory, meaning wild; deserving to be hanged (from the Gallows). *Gallus*, the Latin word for "cockerel."

Avians can circumvent many of the common problems encountered with mammalian immunizations described above. As a fully domesticated small animal, chickens are an attractive host for immunization as they are highly accessible, very affordable and easily housed in a generic animal house. Most importantly, however, the amino acid homology between the mammalian and avian orthologs of a given protein is typically lower than between the mammals commonly used for antibody generation, and indeed, some mammalian proteins may not even exist in avians. The immunoglobulin response of chickens to highly conserved mammalian proteins is reliably robust, generally exhibits high avidity, and potentially targets a broad spectrum of epitopes on protein immunogens (24–26).

Chickens therefore have a potentially major advantage over other common immune hosts: they can produce a high affinity cross-reactive antibody response targeting an epitope that is conserved across multiple orthologs of a mammalian protein.

This can lead to significant savings in time and resources as, if a single, broadly applicable cloned reagent can be identified, it can then be used to generate a single affinity column for the capture of the target protein from multiple species. Chicken immunoglobulins have also shown beneficial biophysical properties: they exhibit high stability to changes in pH and temperatures up to 70°C (27, 28), provide functional coating on latex microspheres (29) and demonstrate functional direct covalent coupling to a dextran layer for the detection of serum proteins by surface plasmon resonance (30). Furthermore, as chickens are small animals, very little protein immunogen is required to raise a strong immunoglobulin response. Approximately 200 μg/bird of purified protein is sufficient to carry out a full-immunization regime (31, 32).

These observations have led to the regular use of chickens as an immune host for production of the polyclonal antibody termed IgY (egg yolk antibody), in both research and commercial settings. Laying hens will export significant quantities of polyclonal IgY into the egg (~100 mg of IgY per yolk), in a process analogous to mammalian placental IgG transfer, which allows direct screening of their antibody response without the need for serum sampling (33). Once a strong immune response has been raised, large quantities of polyclonal antibody are easily prepared from the yolk. These polyclonal antibodies have been successfully applied in research immunochemistry (34), diagnostics (35), and affinity column purification (36). Indeed, immunodepletion resins based on chicken IgY can be used to remove high-abundance proteins from serum and are now commercially available (Genway Seppro®).

Unfortunately, polyclonal IgY does still suffer from the same issues of ill-defined specificity that all polyclonal antibody preparations do. In addition, there have been several studies describing successful chicken hybridoma monoclonal antibody generation to antigens such as human peptides (37), sporozoite proteins (38), and prion protein (39), but the low antibody expression and instability associated with chicken myeloma cell lines (37, 40) led to the underuse of this species as a source of monoclonal antibodies. Today, however, the progress in chicken antibody phage display has circumvented these problems and made recombinant chicken antibody reagents readily accessible, as we describe below.

3.1. Harnessing the Chicken Immune Response via Phage Display

The chicken immunoglobulin repertoire is almost ideally suited to antibody phage display, as chickens generate their immunoglobulin repertoire from a single set of V_H and V_L germ-line sequences (41). Diversity in the V-regions is created by both V-D-J recombination and somatic hypermutation, with the additional influence of “gene conversion,” where multiple upstream pseudogenes are recombined into the functional sequence. This germ-line V-gene system means that the entire chicken antibody repertoire can be captured using only four PCR primers (42), making chicken libraries highly representative of the induced immunoglobulin response. This is in direct contrast with immune hosts such as mice, which have diverse germ-line V-gene sequences and therefore require complex mixes of PCR primers (18). Additionally, the two V-gene germ-line sequences found in chickens are highly homologous to the human V_λ and V_H3 germ-line families (43), which are both associated with creating V-domains with high stability and solubility. Indeed, chicken scFvs can be stable in crude bacterial culture supernatants for up to 1 month at room temperature (44).

The initial work of Davies et al. (42) showed that a simple recombinant chicken antibody library could be displayed on phage. While this small library was nonimmune and derived from the bursa cells of a single young chicken, the group was able to select target-specific scFv sequences recognizing lysozyme, serum albumin, and thyroglobulin. The potential of chicken recombinant antibodies was further highlighted by a study (45), which used an scFv library derived from the spleens of immunized chickens and successfully generated highly specific scFv antibodies that targeted both mouse and rat serum albumins, where tolerance issues limit the ability to generate murine monoclonal antibodies.

A major study (20) subsequently demonstrated that chickens could be a useful source of scFv and chimeric Fab antibodies with specificity for hapten molecules. However, none of these early studies characterized the antibodies for their affinity or their function as practical reagents. More recent studies have shown that scFv antibodies derived from immunized chickens are highly effective reagents in diverse settings such as diagnostic ELISA for Infectious Bural Disease Virus (46), the diagnosis of prion disease (47), immunodetection of haptenic shellfish toxins (48), immunostaining of SARS-infected cells (49), biosensing of cardiac biomarkers (50), and the measurement of ApoB protein in mouse and human sera (51). Raats et al. (52) have also illustrated that anti-idiotype scFvs from an immune chicken scFv library were of considerably higher sensitivity than those derived from a human antibody library in the same study. The generation of highly selective scFvs towards the PrP protein, which is highly conserved in mammals, further demonstrates the advantage of the chicken as an immune model, as isolated scFvs were shown to react with murine, ovine, and bovine orthologs of the protein (47).

The cloning of chicken antibodies via phage display has also allowed the precise dissection of the specificity and affinity of the chicken immunoglobulin response. High-throughput affinity measurements for panels of chicken scFvs to the inflammatory biomarker C-reactive protein have identified clones that preferentially recognize the multimeric and monomeric forms of the protein (50). In the same study, clones with affinities as high as 350 pM were generated from an immune phage display library of only 3×10^7 total clones. In addition, chicken anti-PrP scFv have been reported to have affinities up to 15 pM, making them among the highest affinity scFvs reported to date (53).

What may be of particular practical interest to many researchers is that chickens can serve as a host for simultaneous immunization with multiple proteins of interest, with as many as eight proteins being used successfully in a single immunization scheme (31, 44, 54). The target proteins of interest are mixed in a single adjuvant preparation and each immunized animal receives all proteins simultaneously. Spleen and bone marrow tissues from the immunized animals are then used to generate relatively small phage display libraries and specific antibodies are derived via selection of the library separately on the individual proteins originally used for immunization (31). The immunized chickens appear to react to the proteins fully independently, as the phage display libraries generate individual scFv antibody clones that are fully specific by western blot and ELISA, showing no reactivity to their co-immunogens (31, 54). This approach has major benefits practically and ethically, as it allows the use of a single library to derive high affinity antibodies to a group of proteins of interest. Multi-immunization methods also simultaneously minimize animal use and raise the likelihood of success in generating an immediately useful reagent (31, 54).

Multitarget immunization regimes should be designed with one of two objectives in mind. First, the simplest scenario combines multiple unrelated proteins, which leads to unrelated B-cell responses after immunization. To derive antibodies of greatest specificity during a multitarget immunization of this kind, it is important to ensure that each of the protein immunogens is highly purified and that no closely related proteins are co-immunized into a single animal. Secondly, to derive antibodies that are cross-reactive to orthologs of a conserved protein from multiple species, it is likely to be beneficial, but not necessarily essential, to include each ortholog in the mix of immunogens given to each animal. Iterative selection rounds that change ortholog each time can then be used to bias toward the isolation of cross-reactive antibodies.

The generation and selection of chicken recombinant antibodies is extremely reliable using the methods described in detail in the accompanying chapter. The subsequent identification and sequencing of antibodies displaying the characteristics desired can also be performed simply. In general, scFvs isolated from immunized chicken libraries exhibit high affinity and can be assayed via a direct ELISA, using crude periplasmic extracts from the protein expressing *E. coli* clones. The level of further downstream analysis carried out on positive hits identified during the binding ELISA depends on what the end user requires. Specific binding function may suffice for scFvs that are to be used simply as reagent antibodies for in vitro analysis of samples via ELISA or western blotting. For antibodies to be used in affinity chromatography, the antibody fragments must be purified and tested for their function after being coupled to a solid matrix and for their specificity during purification.

4. Toward Affinity Chromatography with Antibody Fragments

Few studies have been performed using antibody fragments in affinity chromatography, but several potential approaches have been described. For the antibody binding site(s) to be fully solvent-exposed and active, the antibody fragment must be directionally captured onto the solid matrix. Even for full-length IgG, nonspecific adsorption or covalent coupling onto solid supports can lead to denaturation, reducing or negating antigen-binding function (55). Antibody fragments may be slightly more prone to chemical or physical denaturation than full-length immunoglobulins, but scFv and Fab have been used successfully in affinity chromatography and in the creation of SPR sensing surfaces, which can go through serial rounds of binding and regeneration (56).

McElhiney et al. (57) created a simple scFv-based affinity column for the concentration and cleanup of microcystin toxins from environmental samples, by transiently coupling the His-tagged scFv to a disposable nickel chelate column. The scFv was thereby coupled directionally, maximizing the functional antibody content on the column, and the analyte for purification was co-eluted with the scFv before quantification by reverse-phase HPLC. However, this method can only be used under a limited number of conditions, as the interaction of the His-tag with nickel is noncovalent and pH dependent. Other possibly useful low-affinity expression tags include *E. coli* maltose binding protein and glutathione S-transferase, which have both been successful in protein purification (58, 59) (see also Chapter 9 for a discussion on protein tagging).

High affinity, highly stable linkage via affinity tagging may also be achieved by site-specific biotinylation of antibody fragments and their immobilization onto a matrix that has been passively or covalently coated with avidin. Bacterial expression vectors are now available that introduce biotin into specific peptide tags (AviTag), which can be produced on the termini of recombinant proteins. A similar method was proven to be efficient in the production of Fabs that are specifically biotinylated in vivo during bacterial expression, via C-terminal fusion of the Fabs to the *E. coli* acetyl-CoA carboxylase (60). Importantly, these biotinylated Fabs were successfully used to purify recombinant TNF-alpha from bacterial lysates, via a streptavidinated column. The peptide tagging method has also been used successfully to label both Fab and scFv antibodies for their oriented immobilization and use as capture antibodies in clinical diagnostic ELISAs (61, 62). These studies suggest that biotin–streptavidin coupling is a simple and rapid method for the stable, directional capture of recombinant antibody fragments.

While the covalent coupling of recombinant antibody fragments via their reactive lysine side chains is likely to be disruptive to their function, some alternative covalent coupling methods have been identified. In the simplest example, the disulfide bonds linking the two constant regions of a Fab can be reduced using a mild agent to expose cysteine thiols. These thiol groups can then be used to covalently couple the fragment to a thiol-activated surface (63). More elegant versions of this approach have expressed antibody fragments with a C-terminal cysteine group, then gently applied the same chemistry, to preferentially reduce the exposed disulphide groups (56). The exposed terminal thiols are again an efficient reactive group for covalent attachment. It is also possible to express scFv fused to the constant regions of human IgG light chains as another source of usable cysteine residues external to the V-regions (64).

Whether any of the above attachment methods are appropriate for a given affinity purification application may be decided upon by the individual investigator. In cases where stable linkage has been achieved and the column is to be reused, it is prudent for the investigator to examine multiple clones for their stability under repeated cycles of elution and regeneration. While chicken scFvs are built upon naturally stable frameworks, the stability of different clones cannot be taken for granted. In cases where stability remains an issue, the appropriate chicken V-regions can be cloned into an Fc-fusion (65) or IgG (66) expression vector to produce full-length antibody in mammalian, yeast, and even plant culture systems (67).

References

1. Kusnezow, W. & Hoheisel, J. D. (2002) Antibody microarrays: promises and problems. *Biotechniques* **Suppl**, 14–23.
2. Michaud, G. A., Salcius, M., Zhou, F., Bangham, R., Bonin, J., Guo, H., Snyder, M., Predki, P. F. & Schweitzer, B. I. (2003) Analyzing antibody specificity with whole proteome microarrays. *Nat Biotechnol* **21**, 1509–12.
3. Kohler, G. & Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–7.
4. Smith, G. P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228**, 1315–7.
5. Orlandi, R., Gussow, D. H., Jones, P. T. & Winter, G. (1989) Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc Natl Acad Sci U S A* **86**, 3833–7.
6. McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552–4.
7. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P. &

Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res* **19**, 4133–7.

8. Hoogenboom, H. R. (2005) Selecting and screening recombinant antibody libraries. *Nat Biotechnol* **23**, 1105–16.
9. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. & Winter, G. (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J Mol Biol* **222**, 581–97.
10. Barbas, C. F., 3rd, Bain, J. D., Hoekstra, D. M. & Lerner, R. A. (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proc Natl Acad Sci U S A* **89**, 4457–61.
11. Fellouse, F. A., Esaki, K., Birtalan, S., Raptis, D., Cancasci, V. J., Koide, A., Jhurani, P., Vasser, M., Wiesmann, C., Kossiakoff, A. A., Koide, S. & Sidhu, S. S. (2007) High-throughput generation of synthetic antibodies from highly functional minimalist phage-displayed libraries. *J Mol Biol* **373**, 924–40.
12. Hoet, R. M., Cohen, E. H., Kent, R. B., Rookey, K., Schoonbroodt, S., Hogan, S., Rem, L., Frans, N., Daukandt, M., Pieters, H., van Hegelsom, R., Neer, N. C., Nastri, H. G., Rondon, I. J., Leeds, J. A., Hufton, S. E., Huang, L., Kashin, I., Devlin, M., Kuang, G., Steukers, M., Viswanathan, M., Nixon, A. E., Sexton, D. J., Hoogenboom, H. R. & Ladner, R. C. (2005) Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. *Nat Biotechnol* **23**, 344–8.
13. Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wolle, J., Pluckthun, A. & Virnekas, B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* **296**, 57–86.
14. Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J. & Johnson, K. S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol* **14**, 309–14.
15. Huston, J. S., Levinson, D., Mudgett-Hunter, M., Tai, M. S., Novotny, J., Margolies, M. N., Ridge, R. J., Bruccoleri, R. E., Haber, E., Crea, R. & et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci U S A* **85**, 5879–83.
16. Barbas, C. F., 3rd, Crowe, J. E., Jr., Cababa, D., Jones, T. M., Zebedee, S. L., Murphy, B. R., Chanock, R. M. & Burton, D. R. (1992) Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus F glycoprotein and neutralize infectivity. *Proc Natl Acad Sci U S A* **89**, 10164–8.
17. Rothlisberger, D., Honegger, A. & Pluckthun, A. (2005) Domain interactions in the Fab fragment: a comparative evaluation of the single-chain Fv and Fab format engineered with variable domains of different stability. *J Mol Biol* **347**, 773–89.
18. Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H. R. & Pluckthun, A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J Immunol Methods* **201**, 35–55.
19. Li, Y., Cockburn, W., Kilpatrick, J. B. & Whitelam, G. C. (2000) High affinity ScFvs from a single rabbit immunized with multiple haptens. *Biochem Biophys Res Commun* **268**, 398–404.
20. Andris-Widhopf, J., Rader, C., Steinberger, P., Fuller, R. & Barbas, C. F., 3rd. (2000) Methods for the generation of chicken monoclonal antibody fragments by phage display. *J Immunol Methods* **242**, 159–81.
21. Charlton, K., Harris, W. J. & Porter, A. J. (2001) The isolation of super-sensitive anti-hapten antibodies from combinatorial antibody libraries derived from sheep. *Biosens Bioelectron* **16**, 639–46.
22. Arbabi Ghahroudi, M., Desmyter, A., Wyns, L., Hamers, R. & Muyldermans, S. (1997) Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett* **414**, 521–6.
23. Dooley, H., Flajnik, M. F. & Porter, A. J. (2003) Selection and characterization of naturally occurring single-domain (IgNAR) antibody fragments from immunized sharks by phage display. *Mol Immunol* **40**, 25–33.
24. Ikemori, Y., Peralta, R. C., Kuroki, M., Yokoyama, H. & Kodama, Y. (1993) Research note: avidity of chicken yolk antibodies to enterotoxigenic *Escherichia coli* fimbriae. *Poult Sci* **72**, 2361–5.
25. Lemamy, G. J., Roger, P., Mani, J. C., Robert, M., Rochefort, H. & Brouillet, J. P. (1999) High-affinity antibodies from hen's-egg yolks against human mannose-6-phosphate/insulin-like growth-factor-II receptor (M6P/IGFII-R): characterization and potential use in clinical cancer studies. *Int J Cancer* **80**, 896–902.

26. Stuart, C. A., Pietrzyk, R. A., Furlanetto, R. W. & Green, A. (1988) High affinity antibody from hen's eggs directed against the human insulin receptor and the human IGF-I receptor. *Anal Biochem* **173**, 142–50.
27. Hatta, H., Tsuda, K., Akachi, S., Kim, M. & Yamamoto, T. (1993) Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared with rabbit IgG. *Biosci Biotechnol Biochem* **57**, 450–4.
28. Shimizu, M., Nagashima, H., Sano, K., Hashimoto, K., Ozeki, M., Tsuda, K. & Hatta, H. (1992) Molecular stability of chicken and rabbit immunoglobulin G. *Biosci Biotechnol Biochem* **56**, 270–4.
29. Davalos-Pantoja, L., Ortega-Vinuesa, J. L., Bastos-Gonzalez, D. & Hidalgo-Alvarez, R. (2001) Colloidal stability of IgG- and IgY-coated latex microspheres. *Colloids Surf B Biointerfaces* **20**, 165–175.
30. Vikinge, T. P., Askendal, A., Liedberg, B., Lindahl, T. & Tengvall, P. (1998) Immobilized chicken antibodies improve the detection of serum antigens with surface plasmon resonance (SPR). *Biosens Bioelectron* **13**, 1257–62.
31. Finlay, W. J., deVore, N. C., Dobrovolskaia, E. N., Gam, A., Goodyear, C. S. & Slater, J. E. (2005) Exploiting the avian immunoglobulin system to simplify the generation of recombinant antibodies to allergenic proteins. *Clin Exp Allergy* **35**, 1040–8.
32. Gassmann, M., Thommes, P., Weiser, T. & Hubscher, U. (1990) Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J* **4**, 2528–32.
33. Polson, A., Coetzer, T., Kruger, J., von Maltzahn, E. & van der Merwe, K. J. (1985) Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol Invest* **14**, 323–7.
34. Larsson, A., Karlsson-Parra, A. & Sjoquist, J. (1991) Use of chicken antibodies in enzyme immunoassays to avoid interference by rheumatoid factors. *Clin Chem* **37**, 411–4.
35. Tsen, Y. C., Kao, G. Y., Chang, C. L., Lai, F. Y., Huang, C. H., Ouyang, S., Yu, M. H., Wang, C. P. & Chiou, Y. N. (2003) Evaluation and validation of a duck IgY antibody-based immunoassay for high-sensitivity C-reactive protein: avian antibody application in clinical diagnostics. *Clin Chem* **49**, 810–3.
36. Qian, W. J., Kaleta, D. T., Petritis, B. O., Jiang, H., Liu, T., Zhang, X., Mottaz, H. M., Varnum, S. M., Camp, D. G., 2nd, Huang, L., Fang, X., Zhang, W. W. & Smith, R. D. (2008) Enhanced detection of low abundance human plasma proteins using a tandem IgY12-SuperMix immunoaffinity separation strategy. *Mol Cell Proteomics* 7, 1963–73.
37. Michael, N., Accavitti, M. A., Masteller, E. & Thompson, C. B. (1998) The antigen-binding characteristics of mAbs derived from in vivo priming of avian B cells. *Proc Natl Acad Sci U S A* **95**, 1166–71.
38. Sasai, K., Lillehoj, H. S., Matsuda, H. & Wergin, W. P. (1996) Characterization of a chicken monoclonal antibody that recognizes the apical complex of *Eimeria acervulina* sporozoites and partially inhibits sporozoite invasion of CD8+ T lymphocytes in vitro. *J Parasitol* **82**, 82–7.
39. Matsushita, K., Horiuchi, H., Furusawa, S., Horiuchi, M., Shinagawa, M. & Matsuda, H. (1998) Chicken monoclonal antibodies against synthetic bovine prion protein peptide. *J Vet Med Sci* **60**, 777–9.
40. Nakamura, N. A. Y., Horiuchi, H., Furusawa, S., Yamanaka, H. I., Kitamoto, T. & Matsuda, H. (2000) Construction of recombinant monoclonal antibodies from a chicken hybridoma line secreting specific antibody. *Cytotechnology* **32**, 191–8.
41. McCormack, W. T., Tjoelker, L. W. & Thompson, C. B. (1993) Immunoglobulin gene diversification by gene conversion. *Prog Nucleic Acid Res Mol Biol* **45**, 27–45.
42. Davies, E. L., Smith, J. S., Birkett, C. R., Manser, J. M., Anderson-Dear, D. V. & Young, J. R. (1995) Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *J Immunol Methods* **186**, 125–35.
43. Tsurushita, N., Park, M., Pakabunto, K., Ong, K., Avdalovic, A., Fu, H., Jia, A., Vasquez, M. & Kumar, S. (2004) Humanization of a chicken anti-IL-12 monoclonal antibody. *J Immunol Methods* **295**, 9–19.
44. Chiliza, T. E., Van Wyngaardt, W. & Du Plessis, D. H. (2008) Single-chain antibody fragments from a display library derived from chickens immunized with a mixture of parasite and viral antigens. *Hybridoma (Larchmt)* **27**, 413–21.
45. Yamanaka, H. I., Inoue, T. & Ikeda-Tanaka, O. (1996) Chicken monoclonal antibody isolated by a phage display system. *J Immunol* **157**, 1156–62.
46. Sapats, S., Gould, G., Trinidad, L., Parede, L. H., David, C. & Ignjatovic, J. (2005) An ELISA for detection of infectious bursal disease virus and differentiation of very virulent strains based on single chain recombinant chicken antibodies. *Avian Pathol* **34**, 449–55.
47. Nakamura, N., Shuyama, A., Hojyo, S., Shimokawa, M., Miyamoto, K., Kawashima, T., Aosasa, M., Horiuchi, H., Furusawa, S. &

Matsuda, H. (2004) Establishment of a chicken monoclonal antibody panel against mammalian prion protein. *J Vet Med Sci* **66**, 807–14.

48. Finlay, W. J., Shaw, I., Reilly, J. P. & Kane, M. (2006) Generation of high-affinity chicken single-chain Fv antibody fragments for measurement of the *Pseudonitzschia pungens* toxin domoic acid. *Appl Environ Microbiol* **72**, 3343–9.
49. Lee, Y. C., Leu, S. J., Hung, H. C., Wu, H. H., Huang, I. J., Hsieh, W. S., Chiu, W. T., Hsieh, M. S., Cheng, T. F. & Yang, Y. Y. (2007) A dominant antigenic epitope on SARS-CoV spike protein identified by an avian single-chain variable fragment (scFv)-expressing phage. *Vet Immunol Immunopathol* **117**, 75–85.
50. Leonard, P., Safsten, P., Hearty, S., McDonnell, B., Finlay, W. & O'Kennedy, R. (2007) High throughput ranking of recombinant avian scFv antibody fragments from crude lysates using the Biacore A100. *J Immunol Methods* **323**, 172–9.
51. Sato, Y., Nishimichi, N., Nakano, A., Takikawa, K., Inoue, N., Matsuda, H. & Sawamura, T. (2008) Determination of LOX-1-ligand activity in mouse plasma with a chicken monoclonal antibody for ApoB. *Atherosclerosis* **200**, 303–9.
52. Raats, J., van Bree, N., van Woezik, J. & Pruijn, G. (2003) Generating recombinant anti-idiotypic antibodies for the detection of haptens in solution. *J Immunoassay Immunochem* **24**, 115–46.
53. Nishibori, N., Horiuchi, H., Furusawa, S. & Matsuda, H. (2006) Humanization of chicken monoclonal antibody using phage-display system. *Mol Immunol* **43**, 634–42.
54. Hof, D., Hoeke, M. O. & Raats, J. M. (2008) Multiple-antigen immunization of chickens facilitates the generation of recombinant antibodies to autoantigens. *Clin Exp Immunol* **151**, 367–77.
55. Nielsen, U. B., Cardone, M. H., Sinskey, A. J., MacBeath, G. & Sorger, P. K. (2003) Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proc Natl Acad Sci U S A* **100**, 9330–5.
56. Torrance, L., Ziegler, A., Pittman, H., Paterson, M., Toth, R. & Eggleston, I. (2006) Oriented immobilisation of engineered single-chain antibodies to develop biosensors for virus detection. *J Virol Methods* **134**, 164–70.
57. McElhiney, J., Drever, M., Lawton, L. A. & Porter, A. J. (2002) Rapid isolation of a single-chain antibody against the cyanobacterial toxin microcystin-LR by phage display and its use in the immunoaffinity concentration of microcystins from water. *Appl Environ Microbiol* **68**, 5288–95.
58. Pavlickova, P., Schneider, E. M. & Hug, H. (2004) Advances in recombinant antibody microarrays. *Clin Chim Acta* **343**, 17–35.
59. Lichty, J. J., Malecki, J. L., Agnew, H. D., Michelson-Horowitz, D. J. & Tan, S. (2005) Comparison of affinity tags for protein purification. *Protein Expr Purif* **41**, 98–105.
60. Weiss, E., Chatellier, J. & Orfanoudakis, G. (1994) In vivo biotinylated recombinant antibodies: construction, characterization, and application of a bifunctional Fab-BCCP fusion protein produced in *Escherichia coli. Protein Expr Purif* **5**, 509–17.
61. Saviranta, P., Haavisto, T., Rappu, P., Karp, M. & Lovgren, T. (1998) In vitro enzymatic biotinylation of recombinant fab fragments through a peptide acceptor tail. *Bioconjug Chem* **9**, 725–35.
62. Warren, D. J., Bjerner, J., Paus, E., Bormer, O. P. & Nustad, K. (2005) Use of an in vivo biotinylated single-chain antibody as capture reagent in an immunometric assay to decrease the incidence of interference from heterophilic antibodies. *Clin Chem* **51**, 830–8.
63. Vikholm-Lundin, I. & Albers, W. M. (2006) Site-directed immobilisation of antibody fragments for detection of C-reactive protein. *Biosens Bioelectron* **21**, 1141–8.
64. Raats, J. M. & Hof, D. (2005) Recombinant antibody expression vectors enabling double and triple immunostaining of tissue culture cells using monoclonal antibodies. *Eur J Cell Biol* **84**, 517–21.
65. Ono, K., Kamihira, M., Kuga, Y., Matsumoto, H., Hotta, A., Itoh, T., Nishijima, K., Nakamura, N., Matsuda, H. & Iijima, S. (2003) Production of anti-prion scFv–Fc fusion proteins by recombinant animal cells. *J Biosci Bioeng* **95**, 231–8.
66. Akamatsu, Y., Pakabunto, K., Xu, Z., Zhang, Y. & Tsurushita, N. (2007) Whole IgG surface display on mammalian cells: application to isolation of neutralizing chicken monoclonal anti-IL-12 antibodies. *J Immunol Methods* **327**, 40–52.
67. Wieland, W. H., Lammers, A., Schots, A. & Orzaez, D. V. (2006) Plant expression of chicken secretory antibodies derived from combinatorial libraries. *J Biotechnol* **122**, 382–91.