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The use of gene-specific IgY antibodies for drug target discovery

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Genomics and gene expression data require interpretation at the protein level to validate the biological or pathological findings. To match with the efficiency and capacity of DNA microarray application, methods and approaches of protein analysis in multiplex and high-throughput manner are required for effectively discovering disease-related proteins. Two-dimensional gel and MS-based technologies provide researchers with such approaches, but neither of them can directly and selectively detect target proteins *in situ*. Antibodies are one of the most crucial tools for meeting this need. Efficient generation of antibodies based on genomics and gene-expression information is an important strategy for producing gene-specific antibodies to link genome to proteome. Avian immunoglobulins isolated from egg yolk (so-called IgY) have several attractive advantages over conventional IgG antibodies.

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▼ The completion of human genome sequencing and its initial annotation have greatly facilitated the application of DNA microarrays for gene expression studies, particularly to disease markers and potential drug targets. Previous cytogenetics and current DNA chip-based methods have generated an enormous quantity of data that require interpretation and validation at the protein level [1–3]. By contrast, several approaches designed to study diseases directly at the protein level have concurrently been developed [4–6]. These methods include 2-D gels coupled with MS, MS combined with liquid chromatography (LC–MS–MS), and surface enhanced laser desorption-ionization time-of-flight MS (SELDI-TOF MS) [7]. One of the common limitations of these methods is that they do not have direct access to proteins *in situ* in cell and tissue samples because proteins must be in solution or in the form of cell lysates for analysis. The development of protein and tissue microarrays provides possibilities for measuring groups of disease-related proteins directly in body fluids, tissue extracts and tissue biopsy samples [8–11]. However, without specific antibodies or other affinity reagents, the usefulness of these methods could also be limited.

Antibodies bridging genome to proteome

It has been recognized that the development of libraries of protein-specific antibodies is needed to profile the human proteome and to understand proteins in groups and their networks of interactions (referred to as a 'protein compendium'). The ideal situation would be to produce antibody libraries based on the available data of genomics and gene expression studies, so that gene expression profiles can be linked directly to proteomic profiles or protein compendia resulting from antibody-based detection and quantification of groups of proteins.

In this sense, to use the information of genomics and gene expression to generate antibodies will comprise a unique approach to link genomic information to proteomic profiles. A gene-specific antibody means that the antibody is produced via immunization with a gene-expression vector that can produce a corresponding antigen in the host animal to induce antibodies [12,13]. This approach (Fig. 1) can make use of the vast amount of data that is being generated from genomics and gene expression studies, particularly from DNA microarray technology. The major advantage of this approach is to avoid the increased cost and longer times usually required to prepare antigens by conventional methods; to produce antigens via direct gene expression in animals eliminates the intermediate steps of recombinant protein expression and purification, thus simplifying the process and increasing its effectiveness. Antibodies produced by this approach carry gene sequence information; these so-called gene-specific antibodies

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can be used to detect and screen protein targets in tissue or cell samples. The data of identification and quantification of target proteins can effectively be traced back to their encoding gene sequences because each antibody contains intrinsic corresponding gene sequence information. In this way, gene-specific antibodies have a crucial bridging function linking genomic profiling to proteomic mapping.

The gene-specific antibody approach is one of the methodologies of affinity-mediated protein discovery. The success of this approach depends on having the capability and throughput to produce large numbers of antibodies with high specificity and affinity. Conventional methods primarily use rodents, rabbits or larger mammals (i.e. goats) to produce antibodies, in either polyclonal or monoclonal forms. These antibodies are IgG and have been used successfully in various immunoassays and clinical applications for diagnosis and therapy.

There is another class of immunoglobulins called IgY, which can be isolated from egg yolks of the lower vertebrates, such as birds, reptiles and amphibia [14,15]. IgG antibodies have undergone extensive development in many immunoassay applications; however, the usefulness of IgY antibodies is not well known, despite their initial report 110 years ago [16]. IgYs, distinct from IgGs in molecular structure and biochemical features, have many attractive advantages over IgGs and are suitable for further development in immunoassays and clinical applications. This article discusses the special characteristics of IgY antibodies and their potential application as polyclonal gene-specific antibodies for screening and validating biomarkers and drug targets.

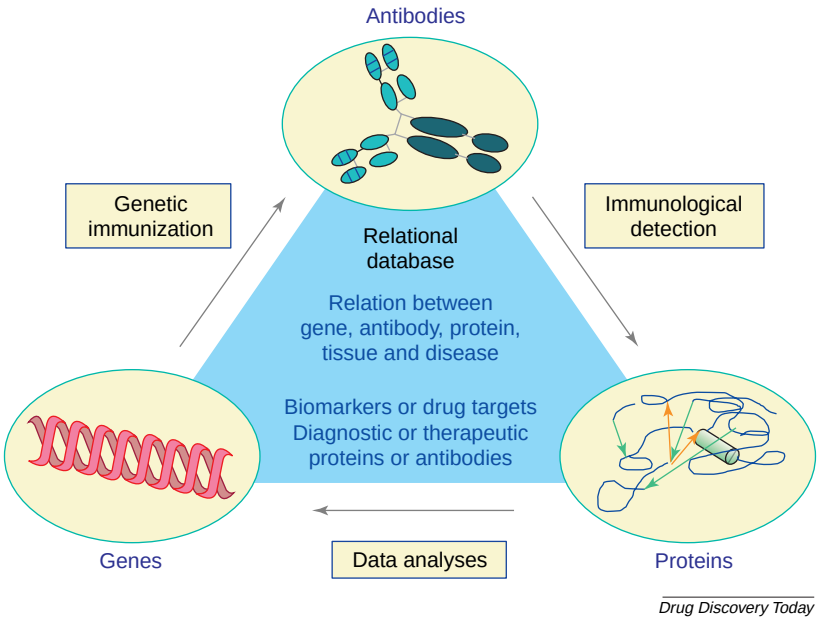

Figure 1. Approach and application of gene-specific antibodies for protein discovery and validation. The strategy of this approach is to avoid current difficulties encountered in interpreting gene expression data at the protein level and instead use antibodies as the bridge to directly link the genome to the proteome. This is done by generating antibodies from genes and applying the antibodies to detect specific gene products in tissues or cells, as well as the target proteins' relationship to diseases. The proteins and the corresponding antibodies identified can be used for various biomedical applications.

Overview of IgY antibodies

IgY antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibia, and are transferred in the female from serum to egg yolk to confer passive immunity to embryos and neonates [17]. This process corresponds to placental IgG transfer in mammals, which confers passive immunity to the fetus. The nomenclature of IgY was initially proposed by Leslie and Clem because of its enrichment in egg yolk [14]. IgY is the functional equivalent of IgG. From an evolutionary perspective, IgY antibodies were considered to be the ancestor of mammalian IgG and IgE antibodies [18].

There are several attractive advantages of using chickens as the immunization host and their eggs as the sources for antibody isolation, as described below.

Better immune responsiveness to mammalian antigens

One of the important advantages of using IgY is an enhanced immunogenicity against conserved mammalian proteins because of the phylogenetic distance between donor and recipient organisms [19]. This makes the production of antibodies against conserved mammalian proteins generally more successful in chickens than in other mammals. In addition, IgY antibodies tend to recognize the same protein in several mammalian species, making them more widely applicable.

High affinity antibodies with persistent titer

Chicken IgY antibodies were found to have high affinity (avidity): antibodies with high avidity against bacterial or human proteins have been developed [20,21]. The avian immune response was also shown to be persistent: 20–30 μg of a highly conserved mammalian antigen induced high and long-lasting IgY titers in the yolk from immunized hens [19,22].

Non-invasive collection of antibodies

Distinct from bleeding immunized animals each time for the production of antiserum, collecting eggs from immunized hens for isolation of IgY antibodies is a non-invasive, non-stressful process for the animal and for the human handler. This is not only a much easier and more reliable procedure, but also a method of biological production that is favorable to animal welfare [23].

A simple and economical isolation process

IgY antibodies are concentrated in egg yolks. The isolation process involves separation of yolks from egg whites, followed by the purification of antibodies in yolks from lipids and other materials. Different materials have been used and various methods were developed, including polyethylene glycol (PEG) precipitation, DEAE fractionation, chloroform extraction, water dilution, precipitation with dextran sulphate or dextran blue or xanthan gums, separation in a two-phase system (phosphate and Triton X-100), a freeze-thaw cycle coupled with gel filtration on Biogel P150, and so on [24–32]. The isolation procedures are generally efficient and economical, although the various methods generate IgY antibodies with different yields, purity, stability and activity.

Large yield and scalable production

A chicken usually lays ~280 eggs in a year and an egg yolk contains 100–150 mg of IgY antibodies. This can result in 28–42 g of IgY per year from each chicken [33]. It was shown that antigen-specific IgY antibodies were between 2% and 10% of the total IgY harvested [34]. The industrialized scale of hosting and caring for millions of chickens has been well developed in industry, therefore, the production of IgY antibodies can be readily scalable.

Comparison of IgY with IgG

Despite the similarities between IgY and IgG antibodies, there are some profound differences in their chemical structures. The IgY heavy chain is 65–70 kDa, whereas the molecular mass of the mammalian IgG heavy chain is ~50 kDa. The IgY light chain is 19–21 kDa; the IgG is 22–23 kDa [22,35]. The greater molecular mass of IgY is a result of an increased number of heavy-chain constant domains and an extra pair of carbohydrate chains. In addition, the hinge region of IgY is shorter and less flexible than mammalian IgG. Recently, it has also been suggested that IgY is a more hydrophobic molecule than IgG [36], which matches the lipid-rich environment of the egg yolk. The structural and amino acid sequence differences determine the differences between the two types of antibodies in their biochemical features and immunological functions. The various parameters of the two classes of antibodies are compared in Table 1. The major differences observed between IgG and IgY are described below.

No binding to bacterial or mammalian Fc receptors

IgY antibodies do not bind to bacterial Fc receptors, such as staphylococcal protein A or streptococcal protein G [37,38], indicating the immunological difference of the Fc region from that of IgG. However, it has been shown that the protein A-reactive site was generated after IgY bound antigen [37].

No reaction to mammalian immunoglobulins and complement factors

Chicken egg-yolk immunoglobulins do not react with mammalian IgG or IgM [39], nor with human anti-mouse IgG antibodies (HAMA) [40,41], nor binding to the rheumatoid factor (RF), which is an anti-immunoglobulin autoantibody found in many different diseases [42]. IgG molecules often give false positive results by interaction with RF in immunoassays [36]. The lack of cross-reactivity between IgY and IgG can be used in many ways to reduce unwanted reactions in assays using anti-IgG antibodies. IgY does not activate mammalian complement factors [43], which also helps to reduce the assay interference by complement factors in mammalian serum samples [44].

Remarkable affinity and avidity

IgY antibodies were shown to have high avidity against bacterial or human proteins [20,21,45]. This could result from either the evolution divergence between mammal and avian species, or from the different affinity maturation process of IgY [18,38].

Different immunoprecipitation characteristics

The immunoprecipitation characteristics of IgY are different from that of IgG, presumably because of the different structure of their hinge regions [18].

Resistance to extreme pH and temperature

IgY is stable at pH 4–9 and up to 65°C in aqueous condition; this compares with IgG, which is stable at pH 3–10 and up to 70°C [22,46]. However, the resistance of IgY to the more extreme pH ranges increases if high salt conditions or stabilizing reagents such as sorbitol are present [47]. IgY was reported to be stable at 4°C for an extended period [25].

Suitable for surface applications

IgY was shown to have good stability after being applied to latex microspheres because of its more hydrophobic surface

Table 1. Comparison of IgY and IgG antibodies

Features of comparison	IgG	IgY	Refs
Animal	Mammal	Birds, reptiles, amphibia	[16,18]
Sources	Blood plasma	Egg yolk	[14,17]
Molecular Weight (by SDS–PAGE)	Whole: 150 kDa	Whole: 180 kDa	[22]
	Light chains: 22 kDa × 2	Light chains: 21 kDa × 2	
	Heavy chains: 50 kDa × 2	Heavy chains: 70 kDa × 2	
Molecular weight (by MALDI-TOF MS)	Whole: 150 kDa	Whole: 167 kDa	[35]
	Light chains: 23 kDa × 2	Light chains: 19 kDa × 2	
	Heavy chains: 50 kDa × 2	Heavy chains: 65 kDa × 2	
Basic structure differences	Flexible hinge region, shorter Fc stem with 2 pairs of carbohydrate groups	Shorter and less flexible hinge, longer Fc region with 3 pairs of carbohydrate groups	[18]
Immune response to mammalian antigens	Adversely affected by phylogenetic homology	Enhanced by phylogenetic differences	[19]
Affinity maturation mechanism	Somatic hypermutation	Pseudo-V gene conversion	[18,38]
Affinity or avidity	Good (10^{-8}-10^{-10} M)	Comparable	[20,21]
Quantity (yield per month per animal)	Milligrams with 1–10% specific antibodies if mice or rabbits used	Grams with 2–10% specific antibodies	[34]
Cross reactivity	Reaction to mammalian immunoglobulins and complement factors	No binding to mammalian immunoglobulins and complement factors	[39,64]
Non-affinity isolation	Need to remove various plasma components	Need to mainly to remove the lipid components in egg yolk	[24–32]
Affinity purification	Proteins A or G, or antigen-based purification	Limited to antigen-based purification	[33]
Stability	Good, stable at pH 3–10, up to 70°C	Good, stable at pH 4–9, up to 65°C	[22,46,47]
Hydrophobicity	Less hydrophobic than IgY	Fc region is hydrophobic	[36]
Immunoprecipitation	Good	Less effective due to short hinge structure	[18]
Productivity	Limited in quantity if mice and rabbits are hosts	High with greater quantity and long duration	[19,22]
Scalability	Relatively difficult	Feasible and practical	[33]
Monoclonal antibodies	Have been well developed	Two cases reported, more development needed	[67,68]
Immune suppression	Several products are under development	May be useful for xenotransplantation	[52]
Diagnosis	Widely used, especially monoclonal antibodies	Useful and practical for various applications	[41,53]
Therapeutics	Well developed	To be further developed such as in antibiotic-alternative therapy	[33,43,65,66]

compared to mammalian antibodies [48]. Immobilized IgY antibodies were also shown to improve the detection of serum antigens with surface plasmon resonance [44].

Amenable for various applications

IgY has been successfully used in conventional immunoassays, such as ELISAs, western blots, immunoprecipitation, immunohistochemistry, immunocytochemistry, cell sorting, cell and tissue staining, [49–51]. The quality of these assays using IgY antibodies is either comparable to, or better than, using conventional IgG antibodies. IgY antibodies have also been shown to have advantages in different applications, such as xenotransplantation [52], diagnostics [53] and antibiotic-alternative therapy [43].

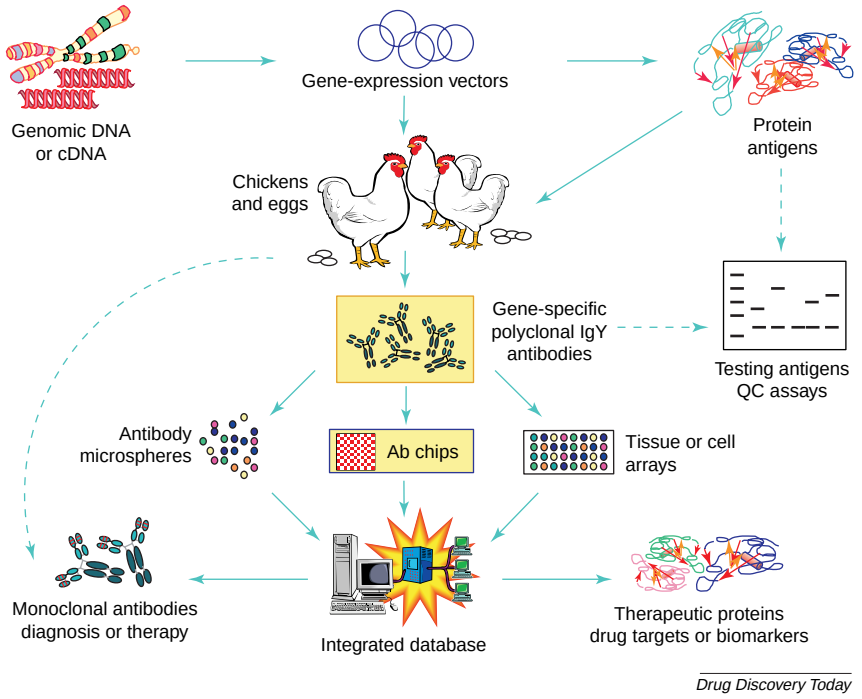

Figure 2. Gene-specific antibody approach and its potential applications. The approach employs DNA or protein antigen-based immunization in chickens to generate gene-specific IgY antibodies. The antibodies can be used for multiplex HTS of unknown proteins in tissue or cell samples, for example, through tissue arrays, antibody chips or cell sorting. This process can localize protein targets in tissues or cells, determine their potential function and analyze their expression profiles. Large quantities of data can be generated and organized into an integrated and relational database. The new proteins identified can be further developed as therapeutic targets or agents or as diagnostic markers. Monoclonal or therapeutic antibodies can also be further developed based upon the IgY antibody and its target antigen information.

Gene-specific IgY antibodies

The approach of gene-specific antibodies employs gene expression vectors to immunize animals for the production of antibodies. Immunization with plasmid DNA encoding target proteins efficiently induced both humoral and cell mediated immune responses in host animals [54–56]. In this method, the production of specific antibodies is initiated by the gene expression vector injected into the host animal. The gene product endogenously produced in the injected animal becomes the immunogen to induce the response of the host immune system. Polyclonal antibodies produced by this method recognized both linear and conformational antigens, as assayed by western blots and ELISAs, respectively [57].

Gene-specific antibodies also take advantage of bioinformatics from genomic and immunogenicity analyses to select gene fragments for protein domains as the specific antigens. This process can largely eliminate the homologous regions between proteins to make the antibodies specific to the target proteins. Therefore, gene-specific antibodies could be considered as mono-specific or pseudo-monoclonal antibodies. Depending upon the needs of application, the specific antibodies in the total IgY isolated from egg yolk might need to undergo antigen-based affinity purification for highly-concentrated and more-specific products (Fig. 2).

The antibodies produced this way can be used to screen and identify unknown proteins in biological samples, to localize their specific protein targets in tissues or cells, to determine their potential function and to analyze their expression profiles. Large quantities of proteomic data can be generated and organized into an integrated and relational database. The new proteins identified can be further developed as diagnostic markers or therapeutic targets of important diseases, such as cancer. Monoclonal or therapeutic antibodies can also be further developed based upon the IgY antibody and its target antigen information [12].

DNA immunization-based gene-specific antibody production has also been successfully applied for IgG antibodies [58]. Figure 2 indicates that the gene-specific antibody approach does not exclude using protein antigens. In fact, the recombinant protein antigens are needed for immunization enhancement, in combination with DNA immunization, antibody testing and affinity purification. The approach described in Fig. 2 is more focused on using genomic or gene-expression information to make antibodies in chickens. This strategy has several practical benefits, as described below.

Expedites the antibody production process

By using gene expression directly in the immunization host, the process will simplify the operations of antigen preparation by expressing target protein and purifying *in vitro* (in *Escherichia coli* or other protein expression systems). This process is particularly good for avoiding the potential contaminations of unwanted antigens associated with protein purification.

Avoids the need for protein antigens for priming

The success of using gene expression in the immunization host largely depends on the protein expression level. Thus,

to ensure generation of high titer, recombinant protein antigens can be used as boosters to further stimulate the immune response. Using a gene expression vector as the primary immunization step can replace using protein antigens to prime the animals, which avoids the delay in commencing immunization that can be caused by the preparation time of protein antigens. However, this process might increase the cost and complexity of the immunization process, which could make the process difficult to scale up to high-throughput levels.

Meets special requirements: membrane protein antigens

Genetic immunization is useful for some special requirements where protein antigen preparation has proven difficult, such as membrane proteins and protein antigens that require post-translational modifications [58]. The antigens generated in host animals via gene expression are expected to be close to their native form and to undergo certain post-translational modifications. Those requirements are difficult to achieve via *in vitro* or bacterial protein expression systems. The challenge is to determine if post-translational modifications in avian species are similar to those of native mammalian (murine) genetic immunization [58].

Uses available genomic and gene-expression information

In the post-genomic era, the tremendous amount of genomics and gene-expression information needs to be used appropriately and effectively. It has been recognized by scientists in the field of genomics and proteomics that genomics and gene expression data can not be readily and directly interpreted at the protein level because of transcription and translation control, post-translational modifications and the different half-lives between mRNA and proteins [59]. Using genomics and gene-expression data to make gene-specific antibodies can overcome this difficulty and provide scientists with tools or reagents to access proteins and analyze the vast number of gene products at the protein level.

Provides biological linkage between genome and proteome

The gene-specific antibody approach provides antibodies that are developed specifically with gene-coding information; therefore, in principle any protein target discovered in various types of assays or in different biological samples can be traced back to their gene sources, which is particularly useful for identifying unknown proteins. This approach is called Antibody-Mediated Identification of Genes and Proteins (AMIGAP), which uses antibodies to bridge the gap between genes and proteins. If a gene-specific antibody library can be developed that corresponds to all the protein components of a given organism, the proteome and genome can be linked.

Gene-specific polyclonal IgY antibodies have enormous potential for various applications. By rapidly developing the technology of affinity reagents for meeting the needs of proteomics and for screening and validating drug targets and biomarkers, polyclonal IgY antibodies will become more popular. However, gene-specific polyclonal IgY antibodies should be viewed as a complementary technology approach to other affinity reagent methodologies, particularly the relatively mature technology of monoclonal IgG antibodies.

Monoclonal IgG antibodies have the recognized advantages of single epitope-based specificity, selected high affinity and continual supply, making them good reagents for diagnosis and therapeutics, in addition to their wide use in conventional immunoassays. The limitations of IgG monoclonal antibodies, besides their non-specific cross-reactivity to human immunoglobulins and complement factors [40,60–63], are the time and effort requried to find good pairs in multiplex assays, such as antibody chips or microsphere arrays. The relatively higher cost and longer time needed to prepare highly selected monoclonal antibodies make them less suitable for HTS.

By contrast, polyclonal gene-specific IgY antibodies can be produced more quickly and at less cost. It should be feasible to use polyclonal antibodies both for capturing or detecting antibodies and for self-pairing. Self pairing can be used for releasing – to a certain extent – the rate-limiting bottleneck in developing paired antibodies for sandwich-based immunoassays. These features make polyclonal antibodies a better fit for conducting initial HTS. Although polyclonal antibodies do not provide an indefinitely sustainable supply – as monoclonal antibodies can – the large yield of initial IgY antibody enables the production of large batches of each individual antibody to form a library for target screening purposes. The key is to develop an approach that is simple and fast, and that directly links gene expression information to gene product (protein) status in tissue or cell samples for protein expression profiling.

Concluding remarks

Immunization of hens represents an excellent alternative for efficiently generating polyclonal antibodies because: (1) chicken housing is inexpensive, (2) egg collection is non-stressful to hens, (3) isolation and affinity purification of IgY antibodies is extremely high-yielding, fast and simple, and (4) there are extensive applications of IgY. In addition, the yolk immunoglobulin has several intrinsic biochemical advantages, particularly no cross-reaction with mammalian immunoglobulins [64]. Because they do not activate mammalian complement and show no interaction with mammalian Fc receptors or other polyvalent antibodies

that could mediate an inflammatory response in the gastrointestinal tract, IgY antibodies are attractive for peroral or aerosol immunotherapy [43,65,66]. Other direct clinical applications of IgY, such as inhibition of xenograft rejection [52], are also being developed. Considering the benefits of IgY technology in polyclonal antibody production and the universal application of these antibodies in research and medicine, IgY technology will have an increasing role in research, diagnostics and immunotherapy.

Here, the approach of gene-specific polyclonal IgY antibodies and their applications for unknown protein and antibody screening have been introduced and discussed. Direct gene expression in host animals using DNA as the primary antigen for immunization, known as genetic immunization, has the merit of simplifying the conventional immunization process using protein antigens, although this approach does not necessarily exclude its combination with protein antigens. For example, the primary immunization could be genetic, with booster immunizations using recombinant protein antigens. The antibodies developed in such an approach will not only be useful in various types of immunoassays but will also prove helpful in linking protein target information back to their cognate genes. It is foreseen that gene-specific polyclonal IgY antibodies will be further developed and widely used for screening and validating drug targets and biomarkers, as well as for the production of novel antibodies.

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