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Chapter 7

Phage Display-Derived Antibodies: Application of Recombinant Antibodies for Diagnostics

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Additional information is available at the end of the chapter

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Abstract

Antibodies are produced by the human body in response towards infections as a means of protection. The *in vivo* production of antibodies by B-cells involves a series of intricate gene editing processes resulting in a highly diverse pool of antibodies. However, this diversity can be replicated *in vitro* using phage display. Phage display offers the potential to present the antibody phenotype together with the cloned genotype of the specific antibody in a single-phage particle. Antibodies are highly sought after for diagnostic applications owing to its specificity and affinity towards a target antigen. The advent of recombinant antibody (rAb) technology allows for a faster and more cost-effective solution for antibody generation. It also provides diagnostic developers with the possibility to customize the antibodies. Antibodies have been utilized successfully in various diagnostic platforms ranging from standard immunoassays to lateral-flow assays, nanoparticles, microfluidics, DNA-integrated assays and others. The limitless application of antibodies in the field of diagnostics has made it a critical component in any diagnostic development platform. This chapter focuses on the processes involved in antibody discovery including the various forms of antibody libraries for phage display and panning processes. We also highlight some diagnostic platforms that apply recombinant antibodies.

Keywords: diagnostics, monoclonal antibodies, phage display, scFv, Fab, VHH

1. Introduction

The human body is made up of a series of complex networks or systems that involve different tissues and cells to work in harmony to regulate different functions in the human body. The

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immune system is one of the vital systems in the human body as its main function is to protect the body from infectious agents and pathogens. The immune system is divided into two main forms of immunity, namely the innate and acquired immunity. The innate immunity is the physical barrier that prevents foreign invasion. If the innate immunity fails to inhibit the entrance of these foreign molecules, the second line of defence being the adaptive immunity will then come into play. This response will take place with the help of both T- and B-cell [1]. B-cell activation to secrete antibodies can work through either the T-cell dependent or the T-cell independent pathway. The T-cell dependent activation would require T-helper cells to trigger the processes required for antibody production through B-cell proliferation [2]. This will then lead to the secretion of antibodies in the body.

Over decades, the application and function of antibodies has expanded from being an immunologically important protein to an essential research tool. The basic application of antibodies surrounds the natural feature of antibodies being high affinity and specific binders against target molecules. This feature has allowed antibodies to be successfully applied for diagnostic and therapeutic applications. In general, diagnostic kits are likely to apply antibodies with superior affinities and specificity against a target antigen for detection [3] via different orientations. This includes either the detection of antibodies by antigens, detection of serum antibodies by the corresponding antigens or by competition [4, 5].

Antibodies are a form of recognition protein [6], which is ubiquitously found in serum and body fluid of vertebrates. The diverse antibody repertoire is important for the identification of antibodies against a specific target. Antibodies undergo gene rearrangement processes to generate different gene segment combinations that result in antibodies with different gene sequences. The complexity of antibody diversity is mainly attributed to the combinatorial joining of multiple V, D, J segments of the heavy chain and the V, J segment for the light chain. This process involves multiple gene-editing enzymes to produce numerous combinations of gene segments. After gene segmentation, another process named somatic hypermutation takes place to further diversify the antibody repertoire [7, 8]. Taken together, these processes are mainly responsible for the highly diverse repertoire of antibodies found in the human body. This variation is the basis of the existence of different antigen-binding specificities and affinities of immunoglobulins (Igs) [2, 9].

The story of antibodies can be dated back to 1890, with the first report detailing the presence of antibodies and its function by Emil von Behring and Shibasaburo Kitasato. They used serum from animals immunized against diphtheria for administration to other animals infected with diphtheria and subsequently curing the infected animals [10, 11]. However, Paul Ehrlich in 1900 proposed the side-chain theory based on his hypothesis that the binding ability of a receptor is based on the side chains available for binding [12]. The side-chain theory was then supported by the 'lock and key' hypothesis by Emil Fischer that focused the hypothesis mainly on enzyme functions [12]. The constant evolution and understanding of immunology has helped open new avenues of antibody application and function.

Another major breakthrough in antibody technology development is the introduction of hybridoma technology. Traditionally, antibody production for diagnostic applications involved the use of animals. The immunization of animals with an immunogenic protein with

and without an adjuvant would generally produce a collection of polyclonal antibodies [13]. A polyclonal pool of antibodies is defined as a set of heterogeneous antibodies targeting a specific antigen at multiple epitopes. The ability to identify monospecific antibodies only came about after Kohler and Milstein introduced the hybridoma technology in 1975. Hybridoma technology allows for monoclonal antibodies (MAbs) to be produced by fusing myeloma cells with antibody-producing spleen cells to create a hybrid exhibiting both characteristics [11]. This resulted in the formation of an immortal cell line with characteristics from both the spleenocytes and myeloma cells known as a hybridoma. The hybridomas are then screened until a single clone is obtained and the production of it is up-scaled. It is well known that antibodies generated via this manner are likely to have high affinities due to the maturation process that it underwent. Although successful, the process may be cumbersome and time consuming as researchers have found it at times difficult to generate antibodies using this method against toxic antigens, self-antigens and sensitive antigens such as membrane proteins or DNA. For all the benefits attributed to hybridoma technology, a major pitfall lies in the fact that for every new antigen a new animal host is required for immunization. Thus, it is difficult to predict or predefine the genetic information and epitope of the clone [11, 14]. This increases the difficulties, cost and time required to make antibodies, making it not the idle solution for antibody production [14].

This brought about a string of alternative methods for antibody production. The turn of the century saw the booming of molecular biology due to the success of recombinant DNA technology. Researchers were hard at work to develop the next alternative method for monoclonal antibody generation. This brought about various methods including phage display and other multiple display methods such as yeast display, ribosome display and mammalian cell display methods [14]. In addition, the use of transgenic animals was also introduced mainly with the xeno-mouse technology [11, 13, 14]. Even so, phage display is the preferred choice for recombinant antibody (rAb) production in most laboratories. Phage display allows for a faster and cost-effective solution towards antibody generation using Ff filamentous phage. In general, rAb production involves several steps including the generation of an antibody library, selection and enrichment of phage-displaying antibodies against specific antigens through the panning process, screening of monospecific antibodies and recombinant production of the antibodies via expression systems [15]. As phage-derived rAb may suffer from lower affinities, an additional stage of affinity maturation may be introduced to improve the antibodies produced. A major advantage to the use of phage display for rAb generation in contrast to conventional animal-derived methods is clearly the omission of animal use in the process. Another advantage of phage display is the lower downtime required for antibody production in between antigens. Conventional methods require immunization that may take up to weeks if not months to yield sufficient immune response for antibody production. This makes phage display rather efficient in the long term for antibody production process. However, one must acknowledge that phage-derived antibodies suffer from lower affinity when compared to conventional antibodies. This is due to the absence of affinity maturation in phage-derived antibodies as animal-derived antibodies are produced post maturation.

This chapter highlights monoclonal antibody development for diagnostic applications via phage display technology. This includes the different types of antibody libraries associated with antibody phage display. The chapter also highlights the different methods used to isolate antibodies against target antigens. The application of recombinant antibodies in different diagnostic platforms is also discussed briefly.

1.1. Phage display section

Phage display makes use of the natural replication cycle of bacteriophages to fuse a specific peptide or protein with the coat protein on the surface of the filamentous phage particles for selection. This design allows the presentation of a predefined foreign phenotype on the phage surface with the genotype being retrievable in the phage. This allowed a physical linkage between the phenotypic characteristics with the genotypic information to be established [11, 16–22].

There are two main methods for the display mechanism on phage. The first is with the use of a phage vector system, which allows the expression of coat protein III (pIII) to the foreign protein, in this case the antibodies are driven by the natural phage promoter [23, 24]. Additional helper phages are not required for phage packaging with phage vector systems. Unlike the phage vectors, the expression of the antibody-pIII protein by phagemid systems requires an artificial promoter such as the lac promoter. In addition, phagemid systems also require helper phage for phage packaging. As phagemid vectors do not carry the phage genome, complete phage packaging can only be done with the presence of the helper phage that carries the genetic information for the other proteins required for phage packaging. Therefore, a competition between wild-type pIII with the mutant pIII will occur. This difference in design of both phage and phagemid vectors also contributes to the display efficiency as phage systems are able to provide a multivalent display of the antibodies on pIII, whereas phagemid systems only allow monovalent display.

The isolation of antibody-presenting phages post binding with a target antigen allows simple identification of the clones by standard sequencing. Therefore, this approach has been utilized to introduce a collection of different antibody sequences into the phagemid vector to produce a collection of varying clones known as an antibody library [25]. The generation of antibody libraries will bring together a different set of considerations that is outlined in the following section.

2. Antibody library

For antibody isolation with phage display technology, a collection of antibodies has to be first made available. This involves the initial investment to generate a library of antibody clones to be presented on the surface of bacteriophages. The choice of library to be generated is rather dependent on its application, which would influence the subsequent decision-making process. This is because the type of library required would determine the source required and the minimum library size required ranging from 10^6 to 10^{10} [26]. In general, there are four main types of antibody libraries, namely naïve, immunized, synthetic and semi-synthetic library.

Naïve and synthetic antibodies are known as 'single-pot' libraries, which can be screened against any antigen [22, 26]. **Figure 1** shows the overall summary of all the libraries and their differences. However, each different library has its own particular characteristic that makes it preferred for certain applications. The application of phage display for antibody generation is not confined only to human antibodies but can also be applied to animal-derived antibodies.

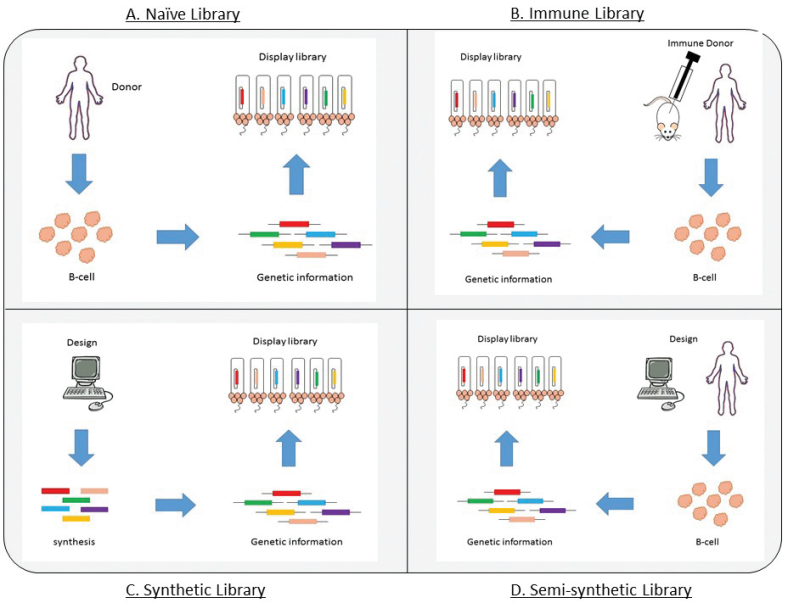

Figure 1. Types of antibody library design.

2.1. Naïve antibody library

Naïve antibody libraries are by definition a collection of antibody genes that are naïve in nature. In other words, the V-gene repertoire originates from IgM isotype of unimmunized or healthy donors [21]. The main characteristic of a naïve library is the unbiased nature of the repertoire. The antibody repertoire of healthy donors would mean no prior exposure or infection of the donors to any form of infection that could skew the immune response. As antibody production by the immune system is a direct response to the exposure of the individual to any pathogen, it is necessary for naïve libraries to obtain its repertoire from truly healthy donors.

The main advantage of a naïve or single-pot library is its large repertoire [22] for monoclonal antibody identification against numerous targets such as self, non-immunogenic or toxic substances. A technical bottleneck associated with naïve libraries is the sheer number of donors required as well as the number of theoretical diversity required. This would involve a large number of ligation and transformation experiments to achieve such numbers. A common

problem of naïve libraries is the number of unknown and uncontrollable contents, such as the presence of memory cells of past infections that might influence the true nature of the naïve repertoire due to the huge naïve repertoire [27]. Another issue common to naïve libraries is the isolation of antibodies with varying degrees of affinities. It is common to obtain antibodies of modest affinities using naïve libraries, as the repertoire would have not undergone affinity maturation processes as opposed to hybridoma-derived or immunized library-derived antibodies.

The adaptation of naïve antibody libraries for diagnostic applications is not a new concept with several antibodies successfully being isolated for various diagnostic targets. Naïve libraries are useful for diagnostic as it can be used for selection against haptens, foreign and self-antigens. The ErBb2 protein, which is a tumour marker expressed by breast tumour, was selected against a naïve antibody library to obtain antibodies for immunoassay applications [28, 29]. **Table 1** summarizes the application of naïve antibody libraries to generate antibodies against a variety of antigens for diagnostic purpose.

Library	Library size	Source	Format	Target	Ref
Tu, 2016	2×10^7	Alpaca	sdAb (VHH)	*Listeria monocytogenes (LM)*	[27]
Yan, 2015	6.86×10^{11}	Camel	sdAbs	Human procalcitonin (PCT)	[28]
Ying, 2014	1×10^{12}	Human	Fabs	Receptor-binding domain of the MERS-CoV spike glycoprotein	[29]
Zhang, 2013	n/a	Human	Fabs	TES1 domain of the oncogenic LMP1	[30]
Boruah, 2013	n/a	Ilama	dsAbs	Glycoprotein of the Rabies virus (RABV)	[31]
Kirsch, 2008 (human naïve HAL4/7 antibody gene library)	5×10^9	Human	scFv	Alphavirus species (virus)	[32]
Velappan, 2007	n/a	Human	scFv	Sin Nombre Virus nucleocapsid protein (SNV-N)	[33]
Jiao, 2005	2×10^9	Human	Fabs	Receptor tyrosine kinase Met	[34]
McElhiney, 2000	1.47×10^8	Human	scFv	Microcystins	[35]
Sheets, 1998	6.7×10^9	Human	scFv	ErbB2 protein, Malaria (rPfHRP2)	[25, 36]

Table 1. Examples of naïve antibody libraries applied for diagnostic applications.

2.2. Synthetic and semi-synthetic antibody library

The antibody-binding region is located at the three complementarity-determining regions (CDRs) of the variable region in both the light chain and heavy chain. The gene sequences along the CDR are highly heterogeneous as a consequences of gene diversification such as V(D)J gene recombination, class switching and somatic hypermutation. The randomization in

the CDR is responsible for the varying affinities as well as specificity to all target antigens. DNA technology advancement encourages synthetic antibody design and synthesis at a reasonable cost in laboratory with the help of structural bioinformatics. This is because the prediction of antibody-antigen interaction can be done after considering the antibody structural constraints and preference.

It is with this information that antibody engineers are now able to design, improve and generate customized frameworks for antibody production. The ability to synthesize specific gene sequences and codons has made it possible to introduce randomization at the CDR. In general, CDR3 are found to be the region with the highest diversification other than CDR1 and CDR2 [30]. In addition to gene randomization, antibody-binding site was altered by inserting preferential amino acids that can also be used to introduce specific criteria to the antibody-binding sites. Another key factor is the CDR length that can also be predefined by the user.

In the context of synthetic antibody libraries, the natural immune maturation and somatic hypermutation process can be elevated to generate similar quality antibodies. To construct a synthetic library, the unarranged and randomized V-gene segments are synthetically assembled ex vivo normally by polymerase chain reaction (PCR). However, customization can be done on the genetic sequence, local variability and overall diversity for synthetic libraries unlike naïve libraries. Even specific codon usage can be applied to suit the needs of the user. Modifications to the framework can be carried out to improve features such as solubility and heat stability [31]. The main criterion for synthetic libraries to be beneficial is the theoretical diversity of the library. As the repertoire is largely naïve, the potential combinations generated by synthetic methods are able to rival the process carried out naturally with the large library size. However, the sheer size of the library diversity makes it difficult to re-culture without eventual loss of diversity. Even so, the antibodies enriched from synthetic libraries showed comparable affinities to those derived naturally [32]. Another major bottleneck associated with the use of synthetic libraries is the cost of generating a synthetic library. Even so, the turn of events in genetic engineering over the past few years has seen the cost for oligonucleotide synthesis reduced tremendously. Therefore, now it is a plausible solution for most laboratories to generate their own version of a synthetic antibody library.

A key subset of synthetic libraries is the production of semi-synthetic libraries. The first semi-synthetic library was reported in 1992, in which rearrangement of 49 human VH gene segments with five to eight residues of synthetic CDR was carried out to yield a semi-synthetic single-chain fragment variable (scFv) library [33]. The key difference between semi- and fully synthetic libraries is the source of the diversity. In semi-synthetic libraries, the diversity is largely obtained from natural sources whereby the genes encoding the CDR are isolated. These CDR genes are then inserted to a fixed framework sequence, which encodes the antibody backbone [27, 34]. The diversity is still natural, taking advantage of the maturation processes of antibodies *in vivo*. The application of synthetic antibodies to develop antibodies for diagnostic applications has allowed the generation of antibodies against various antigens of diagnostic value.

This is evident with approximately three billion clones in the ETH-2-Gold library that were used against a wide range of recombinant antigens, such as extracellular glycoprotein of tenascin-C (TNC) [35]. Other versions have been reported such as the Tomlinson I library with 18 amino acid side-chain diversity on the CDR, and the Griffin library [36] was constructed by using six diverse synthetic CDR3 regions [37]. The main difference between Tomlinson I and J libraries is the choice of randomization used before clone into pIT2 phagemid system. The Tomlinson I library uses the DVT degeneracy to introduce diversity at the CDR. The Tomlinson J library makes use of the NNK degeneracy in the CDR design. Nucleotide degeneracy is commonly used to introduce mutations in the codon at the CDR regions that are responsible for antigen-binding specificity and affinity. DVT and NNK degeneracy are generic codons abiding a specific formula for base usage. The base usage of each degeneracy is as follows: D is 33% G/33% A/33% T; V is 33% G/33% A/33% C; N is any nucleic acid and K is 50% G/50% T. When the degeneracy is translated as a codon, it will yield multiple combinations of amino acids to increase the diversity of the gene. The application of different degenerate codons also allows the application of different groups of amino acids in the CDR design. The antibodies isolated from these libraries could then be used as a diagnostic tool [37, 38].

The HuCAL library from Morphosys is a famous fully synthetic antibody library with predefined randomized frameworks [39]. The different versions of the HuCAL libraries, namely HuCAL Gold and HuCAL Platinum, were generated with six trinucleotide-randomized CDRs [40, 41], whereas HuCAL and HuCAL Gold libraries were made with seven VH, four VK and three Vλ germline families. This allows the generation of 49 framework combination of the VH-VL [42]. Structural diversity of the CDR was introduced by randomization of CDR1, CDR2 and CDR3 [39]. Another version of the HuCAL library is the HuCAL Platinum that consists of seven VH, three VK and three Vλ framework sequences. HuCAL Platinum was designed without the use of VH4 and VK4 as they are found to be rare [43]. The HuCAL libraries have been used also for antibody generation for the diagnosis of bovine insulin [39, 40, 42]. **Table 2** shows a list of some known fully-synthetic and semi-synthetic libraries used for diagnostic applications.

Library	Type	Diversity generation	Format	Target	Ref
Synthetic phage display nanobody (Nb) library	Synthetic	Degenerate codon NNK	scAbs (VHH)	Human prealbumin (PA) and neutrophil gelatinase-associated lipocalin (NGAL)	[50]
Synthetic	Synthetic	–	scFv	Coronary artery disease (CAD)	[51]
ETH-2-Gold library	Synthetic	–	scFv	Extracellular glycoprotein C-domain of tenascin-C (TNC)	[42]
Ylanthia Library	Synthetic	Fixed VH/VL framework pairs selected on biophysical characteristics	Fab	Recombinant human (rh) ErbB4, rhFZD4/Fc, rhTNFalpha, M-CSF, eGFP	[49, 52]

Library	Type	Diversity generation	Format	Target	Ref
HuCAL Gold	Synthetic	7 VH, 4 VK and 3 Vλ germline family	scFv, Fab	IL18R-Fc, -Gal, Est-BSA	[46, 48]
HuCAL Platinum	Synthetic	7 VH, 3 VK and 3 Vλ framework	Fab	Human TRAIL R2/Fc and rhIL-2Rαalpha;/Fc fusion protein	[47]
Tomlinson I Library	Synthetic	DVT side chain	scFv	Microcystins, ferritin, SARS-associated corona virus	[35, 51, 53]
Griffin Library	Semi-synthetic	–	scFv	Microcystins	[43, 44]
Tomlinson J Library	Semi-synthetic	NNK side chain	scFv	SARS-associated corona virus	[53]

Table 2. A list of fully-synthetic and semi-synthetic libraries and application in diagnostics.

2.3. Immunized library

The immunized library repertoire originates from V-gene of immunized donors [27] or disease-infected donors. In immunized libraries, the immunized repertoire would be specific for one antigen or a collection of antigens specific for a particular disease. This limits the use of the libraries when compared to naïve and synthetic libraries. Even so, the V-genes are collected from donors that have been exposed to the target antigen allowing isolation of higher-affinity antibodies using such libraries. These antibody libraries mainly produce antibodies of good affinities with high clonal diversity due to *in vivo* somatic hypermutation that contributes towards affinity maturation. This would influence the library diversity in a way that the final library size required does not necessarily need to be as high as naïve libraries [26]. This is useful to study disease immune responses, vaccination strategies and human immunity. The major setback to such libraries is the need to generate a new library to study every different disease [22, 26, 27].

Several different libraries have been developed for various diseases such as hepatitis B [44] and those listed in **Table 3**. These libraries contain a plethora of useful antibodies that are specific to the disease making it a valuable asset for infectious diseases. The generation of immunized libraries is not restricted to humans but can also be carried out in animals such as mice. Immunization of mice with the target antigen would likely yield a library of clones against the specific target protein. Although this may not differ much from the conventional hybridoma technology, however, conversion to a recombinant version would allow easy up-scaling for production and also for modification.

Library	Source	Format	Target for diagnosis	Ref
Gong, 2015	Camel	sdAbs (VHH)	H7N2 virus	[61]
Prantner, 2015	Human	sdAbs	Mesothelin cancer biomarker	[62]
Zhou, 2015	Chicken	scFv	Bursal disease virus (VP2 protein)	[63]

Library	Source	Format	Target for diagnosis	Ref
Hosking, 2015	Buffalo	scFv	Schistosome infection	[64]
Zhou, 2015	Rainbow trout	scFv	Viral haemorrhagic septicaemia, VHS (viral haemorrhagic septicaemia virus, VHSV)	[65]
Unger, 2015	Ilamas/camelid	scAbs (VHH)	*Clostridium difficile* toxin (CDT)	[66]
Li, 2015	Camel	scAbs (VHH)	Coronary artery disease, CAD (Apolipoprotein B-100)	[67]
Sabir, 2014	Camel	sdAbs (VHH)	Tuberculosis (TB)	[68]
Shahi, 2014	Camel	scAbs (VHH)	Botulinum neurotoxin E (BoNT/E)	[69]
Mohammadzadeh, 2014	Mice	scFv	HIV-1 capsid protein p24	[70]
Li, 2014	Chicken	scFv	Newcastle disease virus (NDV)	[71]
Yang, 2014	Camel	scAbs (VHH)	Porcine circovirus type 2 (PCV2)	[72]
Zhu, 2014	Camel	scAbs (VHH)	Influenza H3N2	[73]
Chiliza, 2008	Chicken	scFv	Malaria	[74]
Cumming, 1998	Mice	scFv	*Mycobacterium tuberculosis* (65-kDa Mtb antigen)	[75]

Table 3. A list of immunized antibody libraries and application in diagnostics.

Immunized libraries have been used successfully in developing antibodies against diagnostic biomarkers. This can be seen with the application of this concept for epitopes of immunogenic antigen, such as carcinoembryonic antigen (CEA) [32, 45]. Anti-CEA antibodies can be applied for targeting and to image colorectal tumours. This transcends the conventional diagnostic platforms allowing *in vivo* cell staining or even with clinical imaging technologies. Besides, immune Fab antibody libraries were used to isolate antibody clones against human immunodeficiency virus type 1 (HIV-1), respiratory syncytial virus (RSV) and herpes simplex virus (HSV) [46]. Additionally, hepatitis A antibodies were isolated from hepatitis A immune library against CR236 via serodiagnostic immune adherence test (IA) or complement-fixation (CF) test [47]. Diagnosis of heparin-induced immune thrombocytopenia (HIT) was made possible by using HIT antibodies from immune libraries. This is crucial as patients who have been diagnosed with HIT are required to stop heparin and start using alternate anticoagulant for treatment [48, 49]. Diagnosis of anthrax was done via detection against antigen *Bacillus anthracis* spores with immunized scFv antibody phage display library [50].

3. Selection and screening of antibody libraries

Panning is an *in vitro* selection method that functions to isolate antibody fragments based on their affinity towards the antigen from a diverse collection of clones. This approach is common for antibody development for diagnostics and therapeutic application [51, 52]. Before selection of antibodies can be carried out, the antibody libraries must be developed first. The constructed library must then be packaged as fusion protein on bacteriophage particles. The basic

principle involves accessibility of phage-presenting antibodies to bind to a target antigen based on affinity.

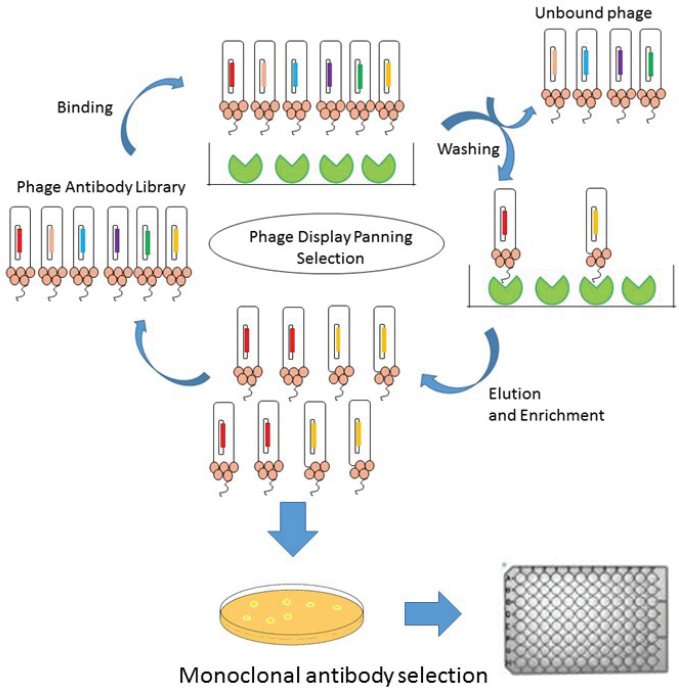

Figure 2. Antibody selection bio-panning protocol.

In this respect, (**Figure 2**) shows that the target antigen must first be immobilized on solid surfaces such as nitrocellulose, magnetic beads, column matrices or plastic surfaces in the form of polystyrene tube or 96-well microtitre plates [22]. In fact, the major difference among all the panning selection strategies is the immobilization surface where antigens are coated on. Other than this, parameters such as washing, elution and enrichment steps can be optimized for a successful selection process. As for evaluation, polyclonal and monoclonal antibody phage enzyme-linked immunosorbent assay (ELISA) is usually used to determine the presence of a positive clone after panning [53].

3.1. Conventional panning strategy

To date, there have been a number of different panning strategies reported for the isolation of MAbs against various antigens. Traditionally, the favoured method for most researchers is to immobilize or coat antigens on solid supports such as polystyrene immunotube and polystyrene immunoplate [53].

The attachment of antigen to the polystyrene surface can either be direct using surface-treated plates or by using intermediate capture mechanisms. This includes streptavidin-coated plates to capture biotinylated ligands (protein or peptide). The biotin-streptavidin mechanism is useful to avoid epitope disconformation during antigen immobilization on a plastic surface [54]. After the immobilization of antigen, phage-displayed libraries are incubated with the bound antigens for affinity capture. This was then continued with the washing of unbound phages before phage elution for rescue. Then, the eluted phage was subjected to amplification and precipitation steps for the following panning round until a positive clone is obtained [55]. Here, the wash step can be modified to introduce different degrees of stringency to specify certain characteristics required for the antibodies. This way, customization of the strategy helps to determine the final output characteristics.

3.2. High-throughput panning strategies

To increase the screening effectiveness, streptavidin magnetic beads can be used to coat the biotinylated antigens. A major advantage in using nanoparticles is the higher surface area to volume ratio for the capture of higher amount of targets. Magnetic-based panning allows multiple antigen screening at the same time. A semi-automated panning process includes manipulation of magnetic beads by multi-pin method, robotic arm or a robotic system during the panning process to increase the panning efficiency [56]. The main concept of panning using the semi-automated process applies a similar concept of affinity-based selection as the conventional method. The incubation, wash and elution steps are carried out with automation to improve reproducibility and accuracy. However, the phage rescue process is still carried out offline by manual infection. Even so, the process still utilizes significant automation to lower labour involvement and allows for high-throughput screening to be carried out.

The concept of full automation refers to a pipeline process without the involvement of any human, whereas semi-automation requires human involvement at some point of the process [53]. The main benefit that high-throughput panning brings to the process is a higher efficiency in selection with minimum labour as all the parameters can be easily programmed for repetitive steps. This also increases reproducibility of the incubation time, temperature, washing and elution condition. This allows for easy handling of multiple targets at the same time. Such protocols can help to reduce from 2 days to a single day for one selection cycle, which means only a week is required for the entire panning process. This method is easily automated with the use of magnetic particle processors such as the Kingfisher Flex system [56].

The next-generation phage display allows differentiation of unselected and selected phage after enrichment rounds [57] against a target antigen for both large combinatorial peptide and antibody libraries through DNA sequence analysis of the phenotype-bearing phage [51]. There are some similarities of this platform with conventional phage panning, where both includes laborious colony picking and functional ligand screening. The sheer number of clones to be analysed is easily overcome by the use of next-generation sequencers (NGSs). This strategy is cost-effective, fast and less labour intensive as compared to conventional phage display selection. Moreover, this technology improves the overall accuracy for large quantification. In terms of coverage, DNA deep sequencing through NGS offers a high coverage for full

repertoire of ligand particles. In short, high-throughput DNA sequencing through NGS method is cost-effective, provides higher accuracy and high coverage for large quantification especially for library screening [57].

Another method utilizing the mass spectrometry immunoassay (MSIA™) system was introduced where the separation of antibodies and antigen for mass spectrometry (MS) analysis is done via affinity. Previously, the MSIA™ method is an immune affinity method used in protein analyte purification for MS detection purposes. The MSIA™ tip was successfully used as a solid phase to carry out semi-automated panning for antibody enrichment. The MSIA™ tips that contain streptavidin that are covalently linked to a porous monolithic solid support will function as the capture molecule. The streptavidin capture molecules are best known for the easy capture of biotinylated target through biotin-streptavidin interaction. The MSIA™ tip method only requires the use of a standard electronic multichannel pipettor and an adjustable pipette stand. This method is also cost-effective for phage display panning as it does not require investments on instrumentation. However, the method can also be incorporated to larger pipetting instruments for antibody panning also. The panning protocol uses the similar concepts as conventional panning that includes incubation, washing and final elution. Then, bound phages are then amplified and used in the following panning rounds to obtain clonal enrichment. This method was reported to successfully identify antibodies against the hemolysin E antigen of *Salmonella typhi* [58]. In short, this method is also an attractive alternative for high-throughput screening.

3.3. Cell panning

Cell panning is an innovative screening method using whole fixed or live cells, tissue section or live animals expressing the antigen of interest for panning [22]. In other words, whole live cells serve as an antigen carrier to screen phage antibodies [59, 60]. All the selection methods mentioned were panned against purified antigens, but whole cell antigen is used in whole cell panning. This panning strategy is usually an alternative method for complex and difficult antigens which cannot be purified with similar properties, for example, cell surface receptor or antigen [60] is only functional when retained in lipid bilayers [59]. There are several parameters to consider when carrying out cell panning such as (1) quality of antibody library, (2) display manner, (3) antigen concentration and (4) cell-surface antigen density. This is to ensure the success of the panning process.

4. Antibody formats

Antibodies are normally identified in a Y-shape configuration. The variability is mainly due to the V-region (two arms of Y-end) as this is where the antigens bind [61]. Thus, smaller versions of antibody formats have been developed to take advantage of the binding specificities of the V-region. Due to advancement in recombinant DNA technology, a number of new antibody formats such as domain antibodies, single-chain fragment variable, tandem scFv,

diabody, tetrabody, minibody and single-chain fragment antigen binding (scFab) have been introduced (**Figure 3**).

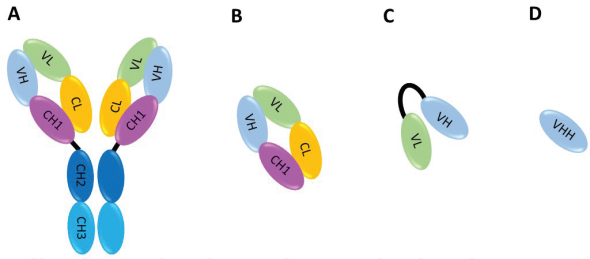

Figure 3. Antibody fragment design. (A) Full antibody; (B) fragment antibody binding (Fab); (C) single-chain fragment variable (scFv); and (D) single-domain antibody (sdAbs).

A main consideration of antibody formats is the size; smaller fragments have an advantage that they are able to retain the antigen-binding specificity and can be produced economically [26, 62]. This is important for application in diagnostics, as this will in turn contribute to lowering the production cost during manufacturing. The main consideration for any antibody to be used on a diagnostic platform is mainly the specificity and affinity of the antibody against the target antigen. These two characteristics are not lost with the use of smaller fragments albeit there will be no avidity effect with the monomeric smaller fragments.

Both scFv and Fab formats are commonly used in research and industrial applications, and are the preferred formats for presentation on phage [16, 20, 34, 62, 63]. This is because the expression of complete IgG is not suitable for *Escherichia coli* as the large size and post-translational modifications available are not designed for bacteria [64]. In the context of antibody formats for application in phage display, the smaller-sized formats are preferred due to the ease of expression and presentation efficiency. Larger native structures may not be tolerated well by the bacterial host required for the packaging of new phage particles. The following section will mainly discuss the antibody formats commonly used in phage display. This includes the domain antibodies, single-chain fragment variable and fragment antigen binding, which are truncated versions of the full immunoglobulin.

4.1. Domain antibody (dAb)

Domain antibodies are small (11–15 kDa) and consist only of either the VH or the VL domain [34]. Thus, they will only have three out of a possible six CDR from a full variable region consisting of both VH and VL. It was found that single-domain antibodies (sdAbs) are able to provide better stability as well as solubility when specific families such as the VH3 framework are used. The stability of the human VH domain antibody can be further enhanced by extending the length of CDRH3 loop [65], much like the hypervariable region of camelid VHH that are longer than the human VH. According to Ponsel and Neugebauer [34], camelid and

cartilage fish's single-domain antibodies are more stable, with a high resistance towards aggregation and temperature due to the framework sequence [34, 66].

Domain antibodies can also penetrate tissue efficiently as compared to full-length IgG due to their smaller size. VHHs are commonly used as detection units on biosensor or immune-adsorbent to identify the presence of lysozyme, carbonic anhydrase, alpha-amylase [67], beta-lactamase or even act as a cancer-imaging agent. It was also used to detect the surface antigen of different hepatitis serotypes [66]. In addition, single-domain antibody is used due to an easier production system when compared to conventional antibodies because of the size and folding [56, 68]. As domain antibodies are devoid of any quaternary structure, production and stability of domain antibodies allows it to be used at extreme conditions. This provides great benefit for diagnostics, as the improved heat stability would allow easy transportation of antibodies without cold chain. This is especially beneficial for diagnostic kit development for in-field diagnosis of infectious diseases in areas with limited resources.

4.2. Single-chain antibody variable fragments of ~27 kDa

Single-chain antibody variable fragment is made up of VL and VH domain with a glycine-serine flexible linker in between to hold the domain in proximity to form the binding cavity upon folding [3]. In addition, GS linkers are known to improve the folding, flexibility and stability of the single-chain fragment variable as compared to proline-rich linker. This is because pro-rich sequence exhibits rigid and stiff conformation due to the absence of hydrogen at the amine, which forms hydrogen bonds with other amino acids [69]. Therefore, the scFv fragments are designed with six CDRs, thus increasing the diversity and repertoire of the antibodies. The scFv has been shown to bind to a variety of antigens, such as hapten, protein, carbohydrate, receptor, tumour antigen and viruses [63]. Moreover, small scFvs are easily folded and producible in *E. coli* for diagnostic applications too [3, 63, 64, 70].

As a linker joins the VH and VL domain physically, the single polypeptide composition helps to facilitate the production and folding in *E. coli* that allows for efficient presentation of antibody repertoire on phage surface [70, 71] due to better expression in *E. coli* [14, 71]. Thus, many libraries generated use the scFv fragment as the preferred format for selection [64, 72]. The scFv format has been used in whole blood agglutination assay for rapid diagnostic test for HIV-1 [71]. The scFv fragment was detected by tags in ELISA besides fusing with reporter enzyme to act as secondary antibody [73].

4.3. Fragment antibody-binding fragments of ~57 kDa

As a comparison to scFv, Fab fragments are composed of the VH, CH1 and entire VL fragment held together by interchain disulphide bonds. The Fab fragments have a tendency to form dimers which could cause problems with binding and affinity as it exerts the avidity effect [13]. *In vitro*, Fab expression vector is more complex than the scFv system as it consists of two separate polypeptide chains linked by the formation of a disulphide bridge. Thus, it is important to ensure a balanced expression of both antibody chains in order to allow good presentation of Fab on phage.

There are several designs used for Fab presentation that includes either the monocistronic or the bicistronic arrangement of antibody. The gene arrangement of a Fab fragment for phage display requires fusion of either VL or VH to the phage pIII coat protein. The production of the accompanying chain will be carried out simultaneously as an independent protein allowing it to locate each other at the periplasmic cavity to form disulphide bonds between them for proper presentation. This challenge is overcome by alternative approaches using molecular chaperones to improve the presentation of full Fab fragments during phage display and protein expression [74].

In diagnostic applications, Fab-peptide epitope and Fab-Fab bifunctional reagent were used to detect the HIV-1, HIV-2 and hepatitis B surface antigen, which were known as antibody-based reagent [71] in agglutination assays. **Table 4** shows the broad application of different antibody formats for diagnostic applications. There is no actual best format for diagnostics but is mainly subjected to the preference of the users and the diagnostic platform set-up.

Antibody fragment format	Source	Antigen	Disease	Ref
Single Domain antibody (sdAb)	Camel	Prostate-specific antigen (PSA)	Prostate cancer	[98]
	Semi-synthetic	*Mycobacterium tuberculosis* (MTb) α-crystalline	*Mycobacterium tuberculosis*	[99]
	Ilama	Marburg virus variants	Haemorrhagic fever	[100]
	Human	Protein C3a, cancer antigen, carcinoembryonic antigen, MUC family glycoproteins, autoantibodies, sialyl-LewisX and cytokines	Breast cancer	[101]
	Alpaca (VHH)	*Listeria monocytogens (LM)*	Food-borne pathogens	[27]
	Camel (VHH)	H7N2 virus	Avian influenza virus subtype H7N2	[61]
Single-chain fragment antibody (scFv)	Mice	*Bacillus anthracis* spores (exosporium)	Anthrax	[60]
	–	Domoic acid	Amnesic shellfish poisoning (ASP)	[102]
	Mouse	*Clostridium difficile* toxin B	*C. difficile* infection, cause of nosocomial diarrhoea	[103]
	Mice	Prostate-specific membrane antigen (PSMA)	Prostate cancer	[104]
Fragment antibody binding (Fab)	Human	Anti-carcinoembryonic antigen (CEA)	Colorectal carcinoma	[105]
	Mouse	prostate-specific antigen (PSA)	Prostate cancer	[106]
	Human	Hepatitis B surface antigen (HBsAg)	Hepatitis B	[54]

Table 4. Application of antibody fragment as diagnostic probe.

5. Application of recombinant antibodies in rapid diagnostics

Recombinant antibodies are used in diagnostics due to its binding specificity and affinity. There are many platforms, such as lateral-flow assay (LFA), ELISA and cell imaging available in the market today, which are rapid and accurate in identifying the target antigens found in sample. Most of the platforms make use of either the antigen-capture assay or the antibody-capture assay to diagnose the presence of certain diseases [75].

5.1. Lateral-flow dipstick assay

Lateral-flow assay or immunochromatography assays are normally found as a test strips with the most common being the pregnancy test strip. The theory behind lateral-flow assay is based on the capillary action that occurs in the nitrocellulose membrane to migrate molecules along the membrane to cause a reaction and detect target antigen [75, 76]. This is because the presence of antigens in the sample matrix against a specific antibody reflects the onset of certain disease and treatment should be carried out immediately. (**Figure 4**) shows the actual design, polyclona antibodies against the target antigen were conjugated with gold nanoparticles and are deposited on the membrane. The migration of the sample when mixed with the antibody-coated particles will allow the particles to flow along the membrane until it is captured by a secondary antibody that is permanently fixed along the membrane as a line. Therefore, the presence of the antigen will be reported by the appearance of a band on the dipstick that represents the concentration of the gold nanoparticles on the target line.

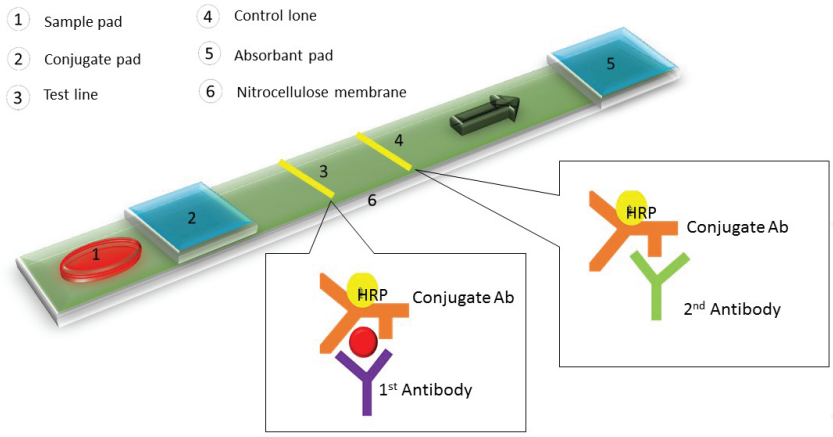

Figure 4. Lateral-flow dipstick assay design.

The conventional design of the lateral-flow strips will show a single control line and another test line. The control line indicates the control assay to show that the lateral-flow system is in order. The working assay requires buffer to improve the performance as well as the compatibility with other components used in assay. There are two formats used in LFAs, sandwich

and competitive format [77]. In short, dipstick assay is sensitive enough to detect the antigen constituent within 10 min besides the simple usage that does not require professional personnel to carry out the test. The conjugation of recombinant antibodies to gold nanoparticles is essential for the generation of lateral-flow assays. The ability to produce recombinant antibodies easily using bacterial expression systems would facilitate rapid kit production at a lower cost.

5.2. Microfluidic platform

Microfluidics makes use of the movement of small amounts of fluid along a small diameter channel. Microfluidics was vastly applied due to the small sample volume needed for fast and precise result. The microfluidic platform is highly sensitive, efficient and portable [78].

Recent developments in microfluidic technology including on-chip detection and imaging, on-chip flow cytometry, on-chip immunoassay and nanosensor for point of care (POC) diagnostic application [79] help to overcome conventional ELISA's limitation for immunoassay-based diagnosis; micro-ELISA systems have been proposed for sensitive and rapid diagnostics using a fluidic chamber. This modified ELISA platform uses a microfluidic platform that reduces the amount of sample required by 10 times (<10 μL), 20 times faster analysis (<20 min) with a higher sensitivity range (Um–pM) as compared to conventional ELISA [79, 80]. Rapid diagnosis is the most important selling point for biomarkers such as protein, carbohydrate, lipid, metabolites, genomic DNA and RNA by using immunoassay due to the obvious advantage in disease management.

To improve the quantitative result, fluid-handling components and data acquisition software were used. In addition, microfluidic kits are integrated with electrical, optical and mechanical transducer to improve the platform [79, 80]. Other than that, the development of on-chip diffusion assay allows the measurement of small molecules within the microfluidic channels. This is done by detection of the labelled probe by antibodies against the probe itself. This study also proves that microfluidic diffusion is suitable for blood sample analysis [81]. There have been other versions of assays utilizing recombinant antibodies in fluidic platforms such as malaria detection with on-card dry reagent storage of microfluidic immunoassay from blood samples [79].

5.3. Enzyme-linked immunosorbent assay

The ELISA platform takes advantage of the specific interaction between antibody and antigens to allow a capture base for detection. The method requires a series of incubation and wash steps, which can be time consuming and tedious. Thus, antigens are first coated on a microtitre plate and then blocked overnight before incubation with antibodies. To detect binding between antigen and antibody, bio-conjugate and chemical-conjugate proteins coupled with reporter enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) are used. Lastly, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) is used as a colour indicator for successful binding between antigen and antibody. However, the conventional ELISA approach has setbacks such as long assay time, large amount of expensive antibodies,

chemicals, plastic ware and liquid-handling platforms. This process although straightforward may still require professional training in order to minimize any false-positive result and ensure reproducibility [82].

5.4. Nanoparticles, silica, polystyrene and latex agglutination test (LAT)

The advent of nanotechnology has brought about the introduction of nanosized particles and with it a series of new diagnostic platforms. There are many nanoparticles that have been used for diagnostic platforms. This includes gold nanoparticles (AuNPs) [83–86], carbon nanotubes and carbon nanoparticles (CNPs) [87]. The chemical property of these nanoparticles has allowed the expansion of the diagnostic platforms from simple colorimetric assays to the introduction of electrical signal, fluorescence and even phase transition readouts [83–85].

Although the use of AuNPs is associated with its application in lateral-flow dipstick assay as the immobilization surface for DNA, antibodies and proteins for the line development, it has also been used independent of the lateral flow. An assay was developed using AuNPs with influenza-specific antibodies acting as labels to detect influenza virus. This will result in the AuNPs probe aggregating and causing a readout in dynamic light-scattering (DLS) spectroscopy. Thus, the detection of AuNP aggregation was analysed using the DLS to determine the concentration of virus present. This method monitors the size change of aggregated nanoparticles and not change colour. However, the DLS approach is more suitable in detecting larger viruses with multiple epitopes [88].

The latex antigen detection or latex immunoagglutination test established in 1959 makes use of protein-conjugated latex microspheres to magnify the antigen-antibody interaction [89]. This LAT assay is fast and simple to use for the diagnosis of circulating antigens in patients with systemic infection because latex is sensitized with the serum of an immune donor [90]. The latex complexes will agglutinate if the target antigen is present [91]. The LAT platform has been used for the diagnosis of systemic candidiasis [90, 91], visceral leishmaniasis [89], invasive pulmonary aspergillosis [92], *Helicobacter pylori* infection [93] and *Meningococcal meningitis* [94]. The diagnostic test can be done using blood, cerebrospinal fluid or other body fluids depending on the design of the agglutination test.

5.5. Fusion with DNA technology—DNAzyme probe system, immuno-PCR (IPCR), immuno-quadruplex priming amplification (IQPA), immuno-rolling circle amplification (IRCA)

Enzyme-linked immunosorbent assay is an assay that uses the enzymatic reaction as a basis of reporting. However, such assays sometimes suffer from a lack of sensitivity to detect low-dose drugs or biomarkers [95]. To overcome this issue, the fusion of DNA technology with protein engineering has brought about newer reporter systems that can increase the sensitivity of assays. This includes modified hybrid methods such as antigen-DNAzyme, immuno-PCR, immuno-QPA and immuno-RCA.

The antigen-DNAzyme-based probe reporter system is a simple and rapid immuno-based assay that depends on the peroxidase activity as a reporter signal and the affinity of antigen-

antibody for binding. DNAzymes also known as deoxyribozyme, founded by Ronald Breaker and Gerald Joyce in the year 1994, are catalytically active DNA molecules, which are able to function mimicking enzymatic reactions [96]. G-quadruplex (G-quad) structures are formed by guanine-rich nucleic acids. The guanine-rich sequence will form a guanine tetrad structure with intra-Hoogsteen hydrogen bonding. This will then allow two or more guanine tetrads bind to form a G-quad. The G-quad structure is further stabilized by the presence of cations. The complexation of G-quad structures with a hemin will form a peroxidase mimicking DNAzyme that catalyses the peroxidase-mediated oxidation of ABTS [97, 98]. The main difference of this antibody-antigen detection assay is the use of G-quad DNA structures in association with hemin as a reporter system. The addition of hemin to a G-quad structure will allow the transfer of electrons from the guanine to hemin in the presence of peroxide to oxidize the ABTS to form a green complex that is visible to the eye [98].

This assay design allows the fusion of the G-quad to function as a DNAzyme to generate a colorimetric readout as a reporter system for the rapid detection of small haptens such as hormones or drug molecules [99]. Thus, DNAzyme can be conjugated with antibodies for signal enhancement with the help of hemin and is suitable to be used as an immunoassay in biodiagnostic platforms [98]. As the G-quad sequence is made up of oligonucleotides, the sensitivity of the assay can be greatly improved via external DNA amplification processes to generate more G-quad sequences for reaction with hemin.

Immuno-quadruplex priming amplification is another hybrid method that couples both the calorimetric DNAzyme detection with an effective DNA amplification for improved sensitivity. IQPA is an immunoassay platform that generates G-quad reporter molecules via an isothermal quadruplex-priming amplification process. The reporter G-quad forming sequences, which were amplified from isothermal QPA, allow QPA to fuse with an immunoassay platform. In other words, we can avoid reporter conjugate enzyme in the immunoassay platform. QPA employs a set of primer and enzyme to maintain the stringent condition for target amplification to generate multiple copies of the G-quad sequence at an isothermal condition. Streptavidin can be sandwiched between the antigen and antibody DNA to form the binding for IQPA to work. The hemin molecules are then used complex with the amplified G-quad structures to catalyse the colour change of ABTS in the presence of hydrogen peroxide [100].

Immuno-PCR is another hybrid immuno-based assay that combines ELISA-type ligand-binding assay (LBA) technologies with PCR amplification signal without the use of antibody-enzyme conjugates. As a replacement, antibody-DNA conjugates were used whereby the DNA marker is physically linked to the capture antibody and a polymerase chain reaction step is introduced to generate copies of the DNA sequence. This allows improvements of 100–10,000-fold in limit of detection (LOD) as compared to conventional ELISA [95, 101]. Although the LOD of IPCR is almost in line with the ligand-binding assay, IPCR assay has been considered as challenging. Thus, various modifications are required to increase its sensitivity. This includes technical issues such as the availability of thermostable enzymes, high protein-binding capacity microplates and minimizing cross-contamination by avoiding plate trans-

fer steps. IPCR has been reported in detecting human interleukin 6 (IL-6) for neurological disease [95].

A further enhancement includes the immuno-RCA method that is another diagnostic platform, which utilizes DNA amplification steps to enhance the signal of immunoassay. This method employs similar concepts to IPCR but the method of DNA amplification varies. As IPCR utilizes the standard PCR amplification cycles, IRCA uses an isothermal amplification method, which does not require a thermal cycler. This makes it more attractive as there is a reduction of dependency on high-end instrumentation. IRCA was used to develop assays in detecting ovalbumin (OVA) allergens [102] and foot-and-mouth disease virus (FMDV) [103]. In IRCA, oligonucleotide primers are attached covalently to the antibody. However, DNA circularization is required to allow binding of the DNA primer to work with DNA polymerase and nucleotides for amplification to start [104]. The detection sensitivity of IRCA exceeds the conventional ELISA and microparticle formats. Thus, IRCA is a system that can allow detection of specific antigens using antibodies at high sensitivity with a wide dynamic range to detect a single molecule [104]. In short, DNA-fusion technologies with recombinant antibodies could potentially aid in the development of newer assays with improved sensitivities.

6. Conclusion

Phage display technology is commonly used for recombinant antibody production. The ability to produce antibodies via recombinant methods can help to improve the speed at which newer antibodies are produced at a fraction of the conventional cost. The freedom associated with recombinant antibodies would also allow the customization of antibodies for various downstream applications in diagnostics. This is particularly important as the development of newer technologies in sensing and reporting mechanisms, the flexibility of recombinant antibodies towards modifications would allow the antibodies to evolve with the advancement of sensing technologies. Therefore, phage display-derived recombinant antibodies provide an important platform for antibody generation for current and future diagnostic applications.

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