Ribosome display: a potent display technology used for selecting and evolving specific binders with desired properties

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Abstract

Currently, a variety of display technologies have been developed in the life science field, such as phage display, ribosome display, and yeast display. Many studies have found that display technologies are powerful and universal methods when they are combined with large genetically encoded binder libraries, which results in the generation of high-performance binders against nearly any antigen interested. As a result, display technologies are widely applied to molecular biology, clinic and medicine. Ribosome display based on cell-free display systems has been established as a different type of technology for 23 years until now. Compared to other related methods, ribosome display possesses unique advantages and is successfully exploited to the selection for functional and specific binders *in vitro*, exhibiting potent development prospects. Here in this paper, we will review the theories and advantages of ribosome display, and will highlight how it is being used now to select and evolve functional proteins as well as applications in diagnostics and therapeutics.

Keywords: ribosome display, display technologies, selection, affinity maturation, stability optimization, evolution, diagnostics, therapeutics

Introduction

Over the past decades, researchers have established various display techniques with the rapid development of molecular biology and proteomics. Meanwhile, the development level of these disciplines and relatives allow the generation of antibody, polypeptide or nucleic acid libraries featuring large populations and broad diversity. Display techniques in combination with binder libraries have proven to provide a powerful means to isolate individual variants with characteristics of high potency (Sergeeva et al. 2006). So far, plenty of display tools have been developed. In general, these approaches are mainly divided into two categories: cell-based and cell-free techniques (Farajnia et al. 2014). The versatile principle of display technologies is that they create a linkage between phenotype (the binding molecule displayed) and genotype

(corresponding genetic information encoding that binding molecule) (McCafferty & Schofield 2015).

As the most diffusely used and maturest in-vitro antibody selection method, phage display belonging to cell-based techniques was first elucidated by Smith (Smith 1985) in 1985. Several evident characteristics of this technique facilitate its widespread application triumphantly, for example, operational flexibility and convenience, high concentration and small size of phage particles existing in solution, and low cost of propagation. Nowadays, phage display turns into a quite useful approach in multifarious scientific fields: developing diagnostic or therapeutic reagents in medicine (Imai et al. 2011), developing nanomaterials in material science (Petrenko 2008), and identifying receptor agonists or antagonists in cell biology. In terms of therapeutic reagents, phage-displayed antibody libraries are utilized to obtain effective reagents against multiple toxins, such as botulinum neurotoxin (Miethe et al. 2014), tetrodotoxin (Wang et al. 2014), and ricin (Pelat et al. 2009), which obtains neutralizing antibodies with affinities in the nanomolar or picomolar range.

Cell surface display methods involve bacterial display (Georgiou et al. 1997), yeast display (Boder & Wittrup 1997), and mammalian cell display (Higuchi et al. 1997), emerging as newer kinds of display methods in the 1990s, which have unique advantages because of the introduce of fluorescence-activated cell sorting (FACS) for quantitative selection compared to phage display. As a resultful alternative choice to phage display, bacterial display is exploited for displaying enzymes to offer improved production technologies for industrial applications that overcome limitations of traditional processes (Muñoz-Gutiérrez et al. 2012). Yeast as eukaryotic microorganism owns post-translational modifications as well as processing machinery and conditions homologous to the mammals' (BoderRaeeszadeh-Sarmazdeh & Price 2012). All of these efficiently facilitate production of correctly folded proteins characterized by pragmatic activities. To date, yeast display has been testified to be an indispensable and efficient way for affinity maturation in antibody engineering (van Rosmalen et al. 2017). A few achievements

have expanded application domains of yeast display, such as in the non-antibody protein engineering field (Mei et al. 2017). Another cell-based display platform is mammalian cell surface display. Noticeably, the mammalian expression system is inherently competent for processing and secreting eukaryotic proteins. Consequently, mammalian cell display offers vast potential to display full-length human antibodies. Recently, a method (Bowers et al. 2014) which incorporated mammalian cell display with *in vitro* somatic hypermutation (SHM) was introduced to identify or mature therapeutic antibodies. Nevertheless, an obvious challenge remains to be solved that relatively low transformation efficiency of mammalian cells diminishes the actual repertoire size in contrast with phage display, leading to unlikely straightforward isolation of antibodies with remarkable affinity. Moreover, the mammalian cell proliferation rate is slower, and such cells require pretty harsher culture conditions *in vitro* than microbial cells. These drawbacks necessitate great efforts to improve the mammalian cell display platform.

Ribosome display and mRNA display are the two most repeatedly used cell-free display systems, taking place entirely *in vitro*. The strategy of mRNA display was set up chiefly by Roberts and Szostak (Roberts & Szostak 1997) on the basis of ribosome display. The high-throughput screening capability of mRNA display extends its application areas in protein-protein interactions. As new intrabodies, RasIn1 and RasIn2 which were selected and affinity matured respectively via mRNA display not only exhibited outstanding selectivity for the GTP-bound Ras and little or no specific interaction with close Ras homologs, but also still ensured functionality under cytosol environment (Cetin et al. 2017).

In the past two decades, ribosome display has been gaining great attention from scientists from different fields because of its unique advantages compared to other display technologies. The broad application fields of this technology include antibody engineering, proteomics, diagnostics and therapeutics. It allows the selection for affinity, stability and enzymatic activity. The most powerful applications of ribosome display have been the rapid isolation and direct evolution of high-affinity functional proteins, particularly antibodies. It accelerates the

production process of novel, active and correctly folded antibodies. This review will discuss the theories and advantages of this technology, and survey recent advances in selecting and evolving functional proteins. Finally, we will provide a summary on its applications in diagnostics and therapeutics. Before this review, we have done some researches, and related papers were published (Li 2015; Li et al. 2015), another manuscript on ribosome display is in preparation.

Survey Methodology

We conducted a systematic literature search for relevant studies published between 1994 and 2018 using databases which included Web of Science, Google Scholar, Baidu Scholar, PubMed and Scopus. We used single or a combination of the following keywords: "ribosome display", "cell-free display system*", "diagnostics and therapeutics", "evolution", "evolv*", "antibody librar*". No language restrictions were applied. Furthermore, additional articles referring to these selected studies were also found by cross-referencing. We scanned the reference lists of the selected studies to identify any further studies, and discussed new developments and research directions related to ribosome display.

Ribosome display

The principle and advantages of ribosome display

Originally proposed as an alternative to selection for target-binding peptides by Mattheakis (MattheakisBhatt & Dower 1994a) and his coworkers, ribosome display shares an analogous concept with other display systems which is the physical association of phenotype and genotype. The complete ribosome display includes several standard steps (Douthwaite & Jackson 2012): preparation of large DNA libraries, *in vitro* transcription and translation, selection and recovery of selected mRNA, reverse transcription and PCR amplification for further selection rounds or analysis. The DNA libraries are typically a collection of various PCR fragments. A prominent feature in this technology is the generation of non-covalent ternary complexes (polypeptide–ribosome–mRNA). The elimination of translational stop codons on mRNA assures that the

116 newly synthesized peptide fails to be released from the ribosome as well as its encoding mRNA.
117 While the release of the polypeptide and mRNA are realized by the release factors (RFs) (Marr et
118 al. 2011) and ribosome recycling factors (RRFs) (Shang et al. 2014) respectively in normal
119 conditions. Generally speaking, the peptide gene of interest is integrated with a T7 promoter, a
120 stem loop, and a translation initiation sequence (the prokaryotic Shine-Dalgarno or eukaryotic
121 Kozak sequence) at its 5' site, and is fused to a spacer sequence and a stem loop on the 3' site
122 (**Fig. 1**). All of these elements constitute the DNA cassette necessary to ribosome display.
123 Studies (Hanes & Plückthun 1997; He & Taussig 1997; He & Taussig 2002) demonstrate that
124 gene III of filamentous phage M13, Cκ (constant region of Igκ chain), and the CH3 domain of
125 human IgM are all suitable to serve as spacers. It is the spacer length that affects display
126 efficiency. When establishing a human single chain variable fragment (scFv) gene library, some
127 authors (Zhao et al. 2009) amplified the coding gene of amino acids 211–299 of gene III of
128 filamentous phage M13mp19 as the spacer, and identified high-affinity scFvs binding to RVGp
129 specifically as well as rabies virus. Bencurova (Bencurova et al. 2015) *et al.* designed a distinct
130 and universal ribosome display construct for producing mRNA–ribosome–VHH complexes both
131 in diverse cell-free expression systems and in viable *Escherichia coli* (*E. coli*) cells (*in vivo*). In
132 addition to components essential for transcription and translation, the encoding gene of VHH
133 was also fused with the F2 fragment consisting of mCherry, Myc-tag, tether, SecM arrest
134 sequence, and 3' stem loop during the construction of the DNA expression cassette in which the
135 SecM stall sequence captured the translation in *E. coli* cells bringing about the formation of
136 stable ribosomal complexes. Thereby, the expression of ternary complexes became rapid and
137 cost-saving by this pipeline.

138 Like mRNA display, ribosome display circumvents transformation to microbial cells, thus a
139 tremendous encoded library incorporating 10^{12}-10^{14} independent sequences can be established
140 rapidly depending on PCR amplifications during a shorter date. In fact, the maximal amount of
141 practical ribosomes included in cell-free expression systems limits the upper size of ribosome-

displayed libraries. Furthermore, some special molecules, such as potent toxis (Pacheco et al. 2015), proteolytically sensitive proteins, and proteins possessing non-natural or chemically modified amino acids (Manandhar et al. 2017; Tada et al. 2013), can also be exhibited on the ribosomal surface, which would be futile ordinarily employing cell-surface display platforms. More importantly, due to PCR reactions involved in every selection cycle, additional DNA diversification is consecutively accomplished by PCR-based mutagenesis technologies, promoting affinity maturation and molecular evolution totally *in vitro*.

The choice of a cell-free translation system

Undoubtedly, transcription and translation call for cell machinery. Four eukaryotic (rabbit reticulocyte lysates, wheat germ extracts, insect cell extracts, and human cell extracts) and one prokaryotic (*E. coli* S30 extracts) cell-free translation systems already commercialized are commonly used and obtainable conveniently. Researchers choose the apposite expression system mainly according to the origin and succeeding applications of displayed proteins. In other words, eukaryotic proteins synthesis would be performed in eukaryotic translation systems. However, this is not absolute. There is no definite evidence that eukaryotic cell-free expression systems perform better with proteins from eukaryotic sources than with prokaryotic proteins (Douthwaite & Jackson 2012). Hanes (Hanes et al. 1999) and other authors prepared different DNA constructs of scFv fragments applicable for ribosome display utilizing an optimized *E. coli* S30 extracts and two optimized rabbit reticulocyte systems (coupled and uncoupled). After making a comparison, they concluded that the amount of full-length scFvs expressed by *E. coli* system were 30% more than those translated by the rabbit reticulocyte system in same reaction volumes; moreover, the uncoupled rabbit reticulocyte system whose efficiency was about 100-fold lower than that of *E. coli* S30 extracts showed no inherent advantage of better folding of scFvs over the *E. coli* system.

Ribosome-displayed library selection cycles *in vitro*

A complete selection cycle involves a few moves, starting with the preparation of the

ribosome display libraries and ending up with the retrieve of mRNA and reconstruction of the DNA cassette. Iterative selection cycles are able to be carried out under experimental conditions which permit extra human manipulation if necessary. At present, ribosome display is chiefly applied to obtain therapeutic or diagnostic antibodies characterized by high affinity and stability from naïve, immune or synthetic antibody repertoires. Due to not implicating biological cells, ribosome-displayed unbiased selection cycles aiming at any antigen can be achieved with naïve and synthetic libraries in reality. Some special antigens, such as self-antigens, haptens, and toxic molecules, possess no immunogenicity, low immunogenicity, and toxicity severally. As a result, *in vivo*-based display technologies can't accomplish the isolation of binders targeting these antigens.

Selection cycle steps

In simple terms, the biopanning (selection) procedure incorporates antigen presentation, incubation, wash and elution (**Fig. 2**). For the method of solid-phase selection, researchers immobilize the target antigen on certain solid surface (e.g., microtiter plates, sensor chips, and immune tubes) via direct adsorption using the coating buffer or indirect capture with an antigen-binding protein, streptavidin or others. Despite the simple operation, effectiveness and high throughput of direct adsorption, it is likely to generate issues that epitopes of interest on some antigens may be not consistent with their natural states, or this method may arouse partial denaturation of protein antigens (ZahndAmstutz & Pluckthun 2007). These problems signify that isolated antibodies will not recognize corresponding natural antigens. However, the indirect capture approach can overcome the drawbacks to some extent (Pardon et al. 2014). Binding of ternary complexes to the antigen presented can also happen in solution, followed by washing away non-specific and low-affinity binders inside libraries. In this method, researchers capture the antigen ordinarily realized by the biotin-streptavidin system and magnetic beads; alternatively, they adopt fixed antigen-binding or tag-specific antibodies to immunocapture the antigen, while the tag is present on the antigen. Biopanning in solution avoids changing the

antigen conformation and increases binding chances between the antigen and its specific molecules. Thence, this approach makes the isolation of low-copy and desirable binders successful. Whereas, anti-streptavidin binders or anti-antibodies (targeting the antigen or tag) binders could be identified simultaneously. Preincubation of ribosomal complexes with coated streptavidin, antibodies referred to just now or other molecules is inevitable to eliminate non-specific binding molecules (Zadravec et al. 2016; Zhao et al. 2016). Furthermore, when incubating ternary complexes with antigen analogues for pre-binding, independent individuals will be acquired which recognize that antigen specifically without cross-reactivity to antigen analogues (Pan et al. 2012). We have already been aware that ribosome-displayed libraries embody vast amount of dissimilar sequences, whereas the realistic effective library size can be decreased owing to the emergence of stop codons incorporated during PCR amplifications probably. Additionally, another reason is that the configuration of polysome complexes or DNA mutation improves the ratio of truncated nascent peptides or proteins generated by cell-free expression systems. To conquer these challenges, the preselection step is equally applicable to remove problematic sequences and enlarge the proportion of complete proteins or peptides, yielding libraries with better quality. Substractive panning is applied to isolate active antibodies targeting haptens while not binding to their carrier proteins such as BSA (Liu et al. 2012; SheedyRoger MacKenzie & Hall 2007). In a research (Rothe et al. 2007), the authors performed six rounds of subtractive panning against the non-correlative Fc-fusion protein CD22-Fc from a T-NHL related scFv library by ribosome display. Next, the resultant “subtracted’’ ribosome complex supernatant was panned on the target antigen CD28-Fc in every selection cycle. Binding analysis outcome revealed that both the selected hscFv cl83 and cl184 exhibited no specific binding to CD22-Fc. The power of subtractive panning turned out to be markedly formidable.

Except for solid-phase selection and selection in solution, other selection methods include direct selection targeting integral cells or tissue slices, in-vivo selection, and automated screening

based on the high-throughput screening technology.

After washing away unbound complexes repeatedly, the attached mRNA inside complexes which bind specifically to the antigen needs to be recovered and then undergoes reverse transcription and PCR (RT-PCR) reactions to rescue the genotype for the next selection cycle or for analysis by cloning and sequencing. A powerful recovery procedure is crucial to obtain rare binders that aim at the antigen of interest and carry extraordinary features. Presently, two major recovery methods have been explored successfully. The method referred to as prokaryotic ribosome disruption (Hanes & Plückthun 1997) (**Fig. 2A**) disconnects ternary complexes by treatment of the elution buffer incorporating a certain concentration of EDTA (AhangarzadehBandehpour & Kazemi 2017), in which the released mRNA must be purified for subsequent RT-PCR amplifications. While the distinct eukaryotic in situ RT-PCR (Gan & Jewett 2016; He & Taussig 1997) (or "direct RT-PCR") (**Fig. 2B**) approach allows DNA recovery directly from selected ribosome complexes with no procedure of above-mentioned complexes dissociation and mRNA purification. The in situ RT-PCR method supplies relatively simplified operation, greatly lowers the probability of mRNA loss because of degradation, and is regarded as a way to accelerate realizing automation of ribosome display. Even so, there may be certain selection scenarios, where selection rounds are performed on immobilized target antigens or cell monolayers, making in situ RT-PCR impossible. Experiments have suggested that prokaryotic ribosome disruption conditions are less efficient for DNA recovery from eukaryotic ribosome-bound mRNA than in situ RT-PCR. Pan (Pan et al. 2012) *et al.* dissociated eukaryotic ribosomal complexes with EDTA after washing thrice and observed that only one of seven clones showed higher specific-binding activity against the antigen protein. They attributed this result to a significant proportion of undissociated mRNA which still existed in the form of ribosomal complexes after EDTA treatment. From these unsatisfactory facts, we deduce that the prokaryotic elution conditions can not be directly transferable to eukaryotic ribosome display systems. In other words, it is obligatory to optimize some conditions of prokaryotic ribosome

disruption to enable it effective for the disintegration of eukaryotic complexes. In an example (Douthwaite et al. 2006), the comparison of prokaryotic and eukaryotic ribosome display systems proved that EDTA-based ribosome disruption functioned quite efficiently for recovering mRNA sequences from prokaryotic complexes. Further, authors verified that reverse transcription conditions could be applied to the eukaryotic ribosome complex disruption. The elution conditions may be temperature-dependent as the reverse transcription buffer alone failed to release mRNA sequences. Eventually, they offered an improved step-by-step protocol advisable for ribosome display based on eukaryotic in-vitro translation systems. In this protocol, the ribosome disruption step was implemented with PBS containing 100mM EDTA and 10 μg/ml *S.cerevisae* RNA, reacting at 50℃ for 5 min.

In brief, whether we choose prokaryotic ribosome disruption or eukaryotic in situ RT-PCR as the recovery method, it will not destroy the combination of the target antigen and its specific binding protein. This is no doubt conducive to the selection for high-affinity binders. On the contrary, competitive elution using an excess of free antigen as the competitor disrupts antigen-antibody complexes. The concrete amount of the competitor rests with the relative affinity of binders to the antigen, while a 10- to 100-fold molar excess is generally adding. This kind of elution strategy shows a tendency to obtain specific binders recognizing precise epitopes on the antigen surface (Veggiani et al. 2011). Unfortunately, the competitive elution approach is inclined to acquire low-affinity binders.

Key factors to successful selection

The successful selection is closely related to the proper folding of displayed binders to active structures. Molecular chaperones (Douthwaite & Jackson 2012; Murray & Baliga 2013), such as heat shock proteins, Dna K, and Dna J, accompany with the protein synthesis process in the translation mixture, serving as one of significant factors for protein folding. Researches have indicated that the ribosome itself also plays an important role in insuring correct structures of binders (Blondel et al. 2016). Besides, several scaffold based libraries especially antibody

libraries demand to be exogenously supplemented with protein disulfide isomerase when translation *in vitro* to form disulfide bonds for stability. Adding right amount of oxidized or reduced glutathione (ForsterCornish & Blacklow 2004) into the cell-free translation mix is another instance of facilitating the production of functional binders. In a word, divergent in-vitro expression systems are flexible tools on the grounds of allowing addition of favorable reagents to meet our requirements for further optimization and improvement (Chong 2014).

Clearly, the stability of ternary complexes directly affects display efficiency, as well as the successful selection for ligand-binding molecules. A few measures have been adopted to enhance and maintain ternary complexes stability: high concentration of magnesium ions contained in the selection buffer, low temperature (4 °C) during the whole selection process, and addition of an antisense RNA complementary to tmRNA existing in the *E. coli* extract which grants the freedom of nascent polypeptides. Kim (Kim et al. 2007) *et al.* paid their attention on how to stabilize mRNA -ribosome-protein complexes. They introduced a pseudoknot derived from the genomic RNA of infectious bronchitis virus between VH/K and spacer sequence to establish the ribosome display construct. This kind of mRNA secondary structure gave rise to ribosomal pausing validly while translation *in vitro*. The study observed that the pseudoknot-including DNA cassette showed higher affinity selection efficiency than that without a pseudoknot, which implied that this pseudoknot conformation certainly stabilized ternary complexes.

In-vitro protein directed evolution by ribosome display

An intrinsic prominent advantage of ribosome display can be described as permitting Darwinian protein evolution to occur *in vitro* via alternating cycles of mutagenesis and selection. The rapid and sustainable development of biomedical science and protein engineering puts forward higher demands for the functional characteristics of proteins against various antigens. Great efforts have been made to improve either affinity or stability of protein binders, thence currently establishing a number of evolutionary strategies (**Table 1**).

Affinity maturation by in-vitro diversification strategies

Affinity optimization of proteins means improving binding characteristics. This process is significant for medical and biotechnological applications of selective binders. Nowadays, in-vitro diversification and modified selection strategies, which can be carried out separately or simultaneously, contribute to yielding higher-affinity binding proteins. In-vitro diversification producing approaches can be classified into two wide categories: targeted methods and random mutagenesis (non-targeted) methods (Harel & Benhar 2012). Random mutagenesis more closely imitates the in-vivo process of somatic hypermutation events, and as a consequence, it brings sequence diversity into the pre-existing DNA libraries. Strategies like hotspot mutagenesis, parsimonious mutagenesis, and CDR walking pertain to targeted methods, which empower randomness to occur in given positions (e.g. specific CDRs or framework regions) supposed to be advantageous to antigen recognition in accordance with relative structural information and molecular modelling data of proteins. Hotspot mutagenesis and parsimonious mutagenesis are included in site-directed mutagenesis. Mutational hotspots (Chowdhury & Pastan 1999; Jiao et al. 2017) can be interpreted as short nucleotide sequence motifs inside DNA fragments with a characteristic of being innately liable to hypermutations during the process of in-vivo affinity maturation. Some concrete information with respect to mutational hotspots was reported previously. The identified hotspots comprise direct and inverted repeats, palindromes, secondary structures, and certain consensus sequences that can be utilized as targets for mutation. PTH22 (Yau et al. 2005), a peptide targeting VHH selected from the llama-derived naïve phage display library, was chosen as the lead binder to construct a mutant library relying on an AGY/RGYW mutational hotspot mutagenesis approach coupled with ribosome display. In this case, CDR2 and CDR3 codons that incorporated mutational hotspots AGY and RGYW were randomized selectively, leading to a library with 5.12×10^{11} variants in theory. The highest affinity-improvement mutant was isolated after three rounds of selection with up to an over 30-fold increase in contradistinction to the progenitor sequence determined by surface plasmon resonance (SPR). However, the enhancement in affinity was moderate, the reason could be that

some of the AGY/RGYW mutational hotspots played a role in other aspects of VHH evolution except affinity maturation. Parsimonious mutagenesis (PM) is another protein evolution technique where mutagenic oligodeoxynucleotides are designed to minimize the redundancy of the coding gene and restrict the number of mutations. To restrict mutation numbers, scientists aim at blocks of around six successive residues generally per library (Thom et al. 2006) using degenerate oligonucleotides, or they utilize biased nucleotide mixtures biasing for the parent amino acids, which can prevent mutagenesis of all positions in a designated region from emerging and reduce the quantity of variants no longer recognizing the antigen to enhance the functional library size. PM has been developed to mature certain antibodies, such as a humanized rat anti-RAGE monoclonal antibody (Finlay et al. 2009). The mutant library created by PM holds a high proportion of well-folded and potentially active binding molecules, since the oligos used mutate only about half of targeted amino acid residues and keep the remaining residues indispensable to neutralize antigens conserved.

According to the currently existing literatures, it appears that non-targeted mutagenesis strategies are also frequently proposed to improve protein potency *in vitro*. These strategies consist of error-prone PCR, error-prone Qβ RNA replicase, *E. coli* mutator strains, and shuffling, introducing diversifications into random positions throughout the whole pre-existing DNA sequences before or in-between selection cycles. The principle of error-prone PCR is utilizing the low-fidelity polymerase to give rise to point mutations randomly across the gene sequences when PCR amplifications proceed. Experiments have illustrated that combining error-prone PCR with ribosome display can be a robust approach to evolve protein biophysical characteristics containing affinity and specificity. In a previous study, highly Her2 ECD-specific designed ankyrin repeat proteins (DARPins) with low nanomolar affinities were obtained from large synthetic ribosome-displayed libraries. To make the selected binders become qualified candidates for therapeutic and diagnostic applications, Zahnd (Zahnd et al. 2007) *et al.* extended randomization to frameworks of the selected pools and matured their affinity by error-prone PCR

combined with subsequent three rounds of selection under very stringent conditions. The usage of dNTP analogs increased the error rate, and the sequencing data of mutant pools indicated that the average mutation rate was 1.3–5.9 mutations at framework positions per gene. The equilibrium dissociation constants (K_D) of the highest-affinity DARPin (H10-2-G3) evaluated by kinetic SPR measurements was up to 90 pM, a 3000-fold affinity improvement in contrast with the consensus framework variant. Cross-reactivity tests of some mutants showed that specificity was retained after affinity maturation. At the level of structure, authors interpreted that amino acid substitutions in frameworks resulted in subtle conformational changes of DARPins. These changes adapted the binding site to the target surface, which was thought to be the grounds for affinity maturation. *E. coli* mutator strains (CoiaHudson & Irving 2001; Ponsel et al. 2011), such as mutD5-FIT and XL1-RED available commercially, are conditional mutants which can lead to random mutations of antibody genes and be beneficial to rapidly select novel antibodies with a notable affinity enhancement or expression level increase. Single base transversion or transition events take place at a higher rate in such *E. coli* mutator cells than that in corresponding normal cells. When achieving antibody affinity refinement with mutator cells, the protocol executes iterative steps of mutation, selection, and amplification. As effective recombination-based ways to exploit molecular diversity, shuffling technologies are universally applied to rapid evolution of proteins. Normally, three approaches can be taken: DNA shuffling, chain shuffling, and staggered extension process (StEP, for short). In DNA shuffling, library DNA members are digested by DNase I into different gene fragments. Whereafter, these homologous segments serve as primers for each other and are randomly reassembled into full-length mutant sequences as a result of PCR cycles. Initially exploited by Stemmer (Stemmer 1994), the aforementioned principle of DNA shuffling is similar to the in-vivo molecular evolution mechanism of somatic hypermutation. Three rounds of mutagenic DNA shuffling and two backcrossing cycles were performed on parental DNA of β-lactamase for evolution. The published results of panning on increasing cefotaxime dosage suggested that a 32000-fold improvement in minimum inhibitory

concentration could be noticed. To build up quite highly diverse ribosome-displayed pools of variants, DNA shuffling (Dreier & PlÃ¼ckthun 2011) is employed to recombine mutations after randomization by error-prone PCR in selection procedures, or collaborate with adequate selection strategies such as off-rate selection, both having the great capacity to produce highly potent proteins. Chain shuffling (Klarenbeek et al. 2016; Marks 2004) is likewise believed to successfully facilitate evolution of antibodies in affinity at a faster rate than that *in vivo*. This approach consists in recombining V_H and V_L repertoires of antibodies with each other at random. Specifically speaking, the technique keeps one variable region fixed and shuffles the other followed by pairing with the constant chain. Then, the resulting secondary library is expected to seek for promising antibodies with superior properties. As one more PCR-based shuffling strategy, the in-vitro DNA recombination efficiency of StEP is comparable to that of DNA shuffling, which has been validated by the published literature (Zhao & Zha 2006). The distinction between the two methods lies in the missing manipulation of DNA fragmentation by the enzyme and the feasibility of operation in a single PCR tube in StEP. The modified PCR technique StEP brings about template switching when DNA fragments anneal to templates due to extremely shortened polymerase-catalyzed extension time. A mutant pool full of chimeric progeny clones is constructed after repetitive rounds of denaturation and abbreviated annealing/extension. Note that functional progeny sequences in the library account for a large percentage. So far, scientists have taken full advantage of StEP to engineer multiple antibodies, enzymes, and viruses to endow them with reinforced or new functions, and ultimately achieved satisfactory and inspiring results. The highest-affinity single-domain antibody CSF2A (Sheedy et al. 2006) targeting IAA was derived from a naïve llama library and evolved in affinity by StEP PCR which shuffled CDRs of CSF2A with those of four different IAA-specific sdAbs. SPR determination revealed that the affinity of two discovered shuffled antibodies was similar to that of CSF2A. Even so, cross-reactivity experiments offered no proof that such shuffled clones interacted with any of auxinic herbicides. In addition, the StEP method generated an L-

asparaginase mutant which carried higher half-inactivation temperature contrasted with the wild-type enzyme. To sum up, recombination-based mutagenesis methods favor round-by-round accumulation of beneficial mutations rapidly in variant pools as selection cycles are conducted.

Nevertheless, non-targeted mutagenesis strategies have a disadvantage that they apparently heighten the ratio of invalid or nonfunctional clones appearing in mutant pools, which implies that we must build up large libraries incorporating adequate active species to satisfy the needs of biopanning. Some studies (Groves & Osbourn 2005; Thom et al. 2006), searching for high-affinity specific binders, concentrate on integrating non-targeted mutagenesis strategies with targeted methods to evolve antibodies. Ribosome display is confirmed as a suited medium to combine these strategies. Such integration allows for simultaneous optimization of non-targeted regions, accompanied by targeted region evolution. For instance, affinity maturation was performed on the IL-13-neutralizing antibody BAK1——a therapeutic candidate for the treatment of asthma——by in-vitro protein evolution approaches along with phage and ribosome display (Thom et al. 2006) . In this paper, authors randomized BAK1 targeting its amino acids within H-chain CDR3, followed by introducing error-prone PCR amplifications in between rounds of ribosome display. The selected BAK1.1 scFv was optimized relying on CDR walking mutagenesis in VH CDR1 and VH CDR2 to achieve further improvement in potency and affinity. Finally, the collaboration of targeted and random mutagenesis strategies yielded a BAK1.45 variant, exhibiting 345-fold improvement in IC50 and 167-fold enhancement in affinity over the parental antibody BAK1 determined by the TF-1 proliferation assay and kinetic analysis respectively. Certainly, the aforesaid combination approach is prone to achieve more favorable potency gains than targeted mutagenesis methods alone. It is very important that reliable affinity optimization methods are capable of sampling broad regions of protein sequence space and selecting for mutants with excellent performance.

Affinity maturation by modified selection strategies

Several well-established modified selection strategies can also add tremendous value to

protein affinity maturation. One of them is off-rate selection (Huang et al. 2015; Zahnd et al. 2004) which has been defined as a valuable tool to obtain high-affinity binders from libraries by researches. An off-rate selection procedure in concert with ribosome display can be implemented on the antigen in the immobilized form or in solution. Nonetheless, the biotin-labeled (or otherwise tagged) antigen in solution is preferred, as there is a great chance that immobilizing the target antigen on a solid surface could alter its premier conformation. Here, we take the biotinylated antigen as an example to describe the selection process. In this selection strategy, the ribosomal complexes are first exposed to the biotin-tagged antigen; after equilibration, a large excess of unbiotinylated antigen is adding to the selection reaction together with an optimum incubation time, trapping each complex dissociating fast from the biotinylated antigen. The library members with lower off rates still retain bound to the biotinylated antigen, rescued subsequently by the streptavidin-coated magnetic beads. The published literature (ZahndSarkar & PlÃ¼ckthun 2010) has evidenced that the most vital parameters for searching for the lowest off-rate binders are the incubation time of the complexes with unlabeled competitor antigen and the ratio of competitive antigen to labeled antigen. Extending the exposure time to the competitor means enhancing the selection pressure, causing a stronger possibility that the ribosomal complexes dissociate from the labeled ligand. Starting from a single PrP binder, scientists constructed a scFv antibody library with DNA-shuffling and error-prone PCR using dNTP analogs (LuginbÃ¼hl et al. 2006). To mature the P scFv, they applied five repeated cycles of off-rate selection in the entire course of ribosome display. As a critical factor in the experimental set-up, the competitive incubation time was gradually prolonged from 12 h up to ten days. This selection strategy managed to isolate the affinity-improved scFv named C1 against BoPrP (90–105), which was the tightest peptide-binding antibody at that time with a K_D value of 1.2 pM heightened by about 13-fold compared to the original P scFv.

Other useful selection strategies taken by many published articles (Chin et al. 2015; Douthwaite & Jackson 2012; GrimmSalahshour & Nygren 2012) are reducing the target antigen

concentration and increasing the frequency and stringency of washing steps in a stepwise manner during sequential selection rounds, which can increase the likelihood of obtaining high-affinity binding molecules.

Stability optimization strategies

Other than accelerating affinity maturation of antigen-binding molecules, ribosome display technology has been developed to evolve binders in stability. An approach for stability optimization depends on altering redox potential of the cell-free translation system in ribosome display, which is trustworthy and implemented in many experiments. For example, DTT can be added during the ribosome complex production step to generate antibodies that still fold correctly under the reducing environment (Buchanan et al. 2012; Dreier & PlÃ£Â¼Ckthun 2012). Now we know that disulfide bonds are crucial factors for maintaining stability and activity, a common feature of antibodies. It could be speculated that considerable or even total activity of antibodies could lose attributing to lacking disulfide bridges. While, such stable antibodies acquired by this rapid method act as "intrabodies" (Chames et al. 2009) showing functional expression in the cytoplasm to inactive target antigens intracellularly. In a scenario (Jermutus et al. 2001), an anti-hag scFv was evolved for stability with ribosome display by choosing decreasing redox potentials as selection pressure. During every translation process within the five selection rounds, the concentration of DTT was gradually heightened from 0.5mM to 10 mM, which impaired the formation of disulfide bonds. The selected mutant scFvs still folded stably in the existence of DTT and maintained the ability to bind antigen specifically. What's more, urea denaturation experiments gave delightful results that these mutants manifested a higher stability compared with the wild type under the circumstance where disulfide linkages were able to form again.

The fact that the addition of DTT into the translation system indeed achieves a stability improvement of the evolved proteins enlightens us that other selection pressures, such as the presence of proteases, heat or organic solvents (Hussack et al. 2011), can be employed in

cooperation with ribosome display to find out binders equipped with corresponding better stability.

Applications of ribosome display in diagnostics and therapeutics

So far, ribosome display has been exploited in developing therapeutics and diagnostics in several scientific fields (AhangarzadehBandehpour & Kazemi 2017; Heyduk & Heyduk 2014; Qi et al. 2009), such as cancer treatment, allergic disorders, infectious diseases, autoimmune and metabolic diseases. The prominent virtue of this *in vitro* display system is that it enables toxic substrates to be displayed, which not only broadens the range of displayed molecules, but also provides an increased probability to discover diagnostic and therapeutic candidates carrying the characteristic of desired specificity and potency. Analysis to large amounts of data created by human genome sequencing (Consortium 2004) has contributed to the discovery of disease-related genes serving as drug targets. Ribosome display, an ideal means adopted in drug discovery, is gradually playing a more prominent role in the process of drug discovery. The technology has applications in obtaining recombinant antibodies, peptides, enzymes, and ligand-binding proteins. In the past few years, various antibodies have been authorized for diagnostic and therapeutic applications (Galan et al. 2016), and the number of antibodies accepted for clinical use has been increasing. In addition, scientists recently make further advances in improving the efficacy of antibodies for therapeutic applications by which people are able to treat some kinds of cancers, arthritis, immune and infectious diseases (Mahmuda et al. 2017). Nonetheless, all of the therapeutic reagents as well as antibodies have side effects and limitations. Therefore, in view of these shortcomings such as immunogenicity, some researchers direct their work towards developing novel non-immunoglobulin affinity proteins considered as an alternative choice to current antibodies used in the fields of diagnostics and therapeutics.

In some examples, ribosome display is conducted to select DARPins from synthetic libraries with affinities in picomolar range against different targets of interest (Binz et al. 2004; Dreier et al. 2011; Stefan et al. 2011; Zahnd et al. 2007). DARPins have significant success in acting as

diagnostic or therapeutic reagents, which attributes to their advantages, for instance, DARPins can fold well in cell free translation systems reliant on ribosome display, have one-tenth of the molecular weight of a full IgG on the average and possess better specificity and higher thermodynamic stability. The smaller structural size means a great power on capillary extravasation, tissue penetration and diffusion. Selected DARPins targeting human EGFR-2 provide the ability to inhibit the proliferation of breast cancer cells. Furthermore, due to efficient pharmacokinetics and favorable tumor penetration, EGFR-2-specific DARPins can be promising drug carriers which convey a high concentration of drugs to the tumor sites precisely. Zellweger (Zellweger et al. 2017) and other scientists conducted recursive rounds of selection to obtain anti-huFcγRII DARPins with ribosome display. Detailed analysis on the selected binding protein D11 involving SPR and flow cytometry indicated its high affinity and specificity. They also generated a bispecific molecule D11_E53 which could recognize FcγRIIB and FcεRI-bound IgE simultaneously. In the cellular degranulation assays, they observed that D11_E53 not only inhibited allergen-induced basophil degranulation remarkably in a dose-dependent manner, but also performed quite better compared to the therapeutic antibody. Furthermore, D11_E53 exerted great in-vivo functionality of alleviating antigen-induced systemic anaphylaxis. Thus, D11_E53 had broad application prospects in efficient allergy therapeutics. Another non-immunoglobulin alternative to antibodies is designed albumin binding domains (ABD) (Zadravec et al. 2016). The investigators found that the selected Shiga toxin 1 B subunit binders based on the ABD scaffold held great promise for coping with a severe medical issue stemming from Shiga toxin-generating bacteria. Ribosome display is also proposed to develop novel early diagnostic reagents or therapeutic drugs against Hepatitis C virus (HCV) infection. In an endeavor, 12-mer peptides specifically binding to the HCV E2 envelope glycoprotein were produced from a distinct random 12-mer peptide library through a 13-cycle selection by ribosome display (Chen et al. 2010). The affinity data of the four obtained peptides measured by the SPR technique unveiled that PE2D with a K_D of 19 nM had the highest binding affinity. Not only that, the following virus capture

assays on PE2D also demonstrated its validity for targeting HCV particles and diagnosing HCV infection at the early stage. For further characterization, the authors investigated the inhibitory effects of PE2D on HCV infection of Huh-7.5.1 target cells. It was encouraging that PE2D exhibited extremely strong effects on blocking the hepatocyte binding ability of E2 and inhibiting HCVcc from entering into hepatocytes. In summary, there were ample reasons to consider that the selected peptide PE2D was well suited to serve as an early-diagnostic agent or a therapeutic drug for HCV infection.

Overall, these promising results provide strong evidence that ribosome display can discover diagnostic or therapeutic candidates aiming to multiple diseases. These candidates which are superior to existing drugs are worthy of further clinical research. And from another perspective, these examples extend the application area of this technology.

Concluding remarks

Since Mattheakis (MattheakisBhatt & Dower 1994b) *et al.* first reported ribosome display in 1994, this technology has been diffusely employed to selection of functional proteins, polypeptides or others, and molecular directed evolution entirely *in vitro*. Moreover, the enormous display power of ribosome display permits it to be combined with high-throughput protein microarrays to assess protein–protein interactions, which opens doorways into the proteomics field. However, there still exist limitations to conventional ribosome display, such as low stability of ternary complexes and nuclease-susceptible mRNA, making it less popular than phage display. In addition, nucleases, proteases and other inhibitory factors inevitably appear in the cell-extract-based cell-free translation systems. To overcome these shortcomings, scientists reconstituted the PURE (protein synthesis using recombinant elements) system (Douthwaite & Jackson 2012; KanamoriFujino & Ueda 2014; Ohashi et al. 2007) for protein synthesis *in vitro*. This system contains individually prepared and purified components necessary for gene expression in *E. coli*, and has few nucleases and proteases, thereby increasing mRNA recovery rate and producing stable ternary complexes. All in all, ribosome display as a potent tool is

558 demonstrating its value in many domains. We anticipate that more significant advances will be
559 made to perfect this technique so that it may yield versatile molecules for various applications.

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Figure 1

The DNA cassette used for ribosome display.

The gene of interest is integrated with a T7 promoter, a stem loop, and a translation initiation sequence (the prokaryotic Shine-Dalgarno or eukaryotic Kozak sequence) at its 5’ site, and is fused to a spacer sequence and a stem loop on the 3’ site. MCS stands for multiple cloning sites. Adapted from ref. (Douthwaite & Jackson 2012).

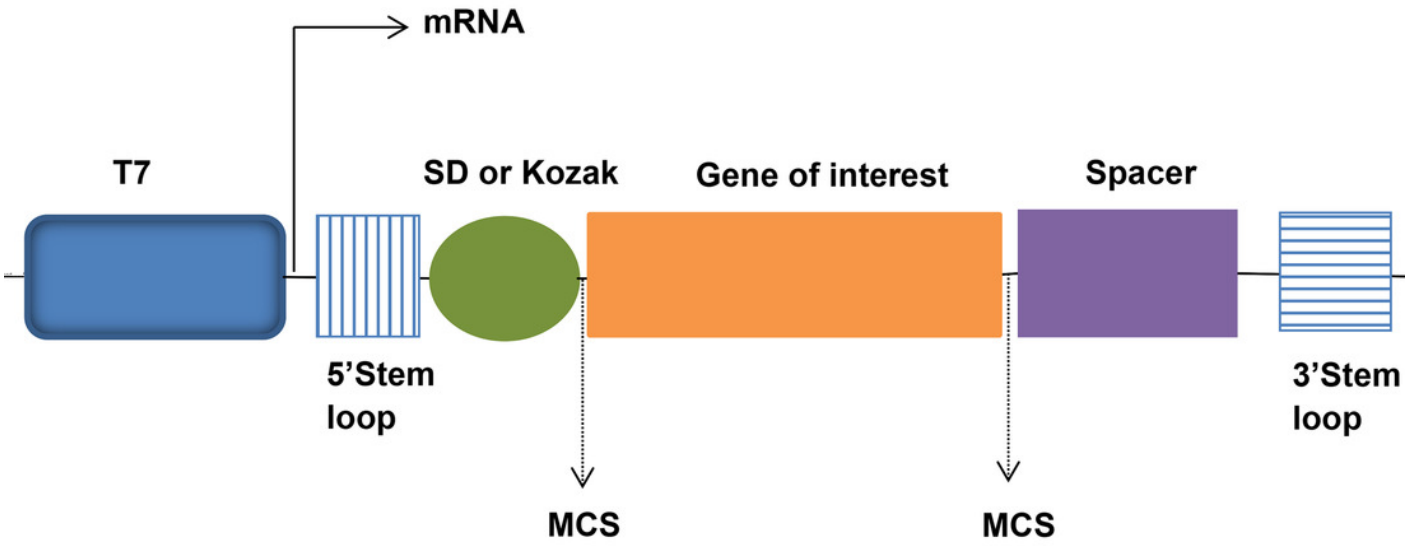
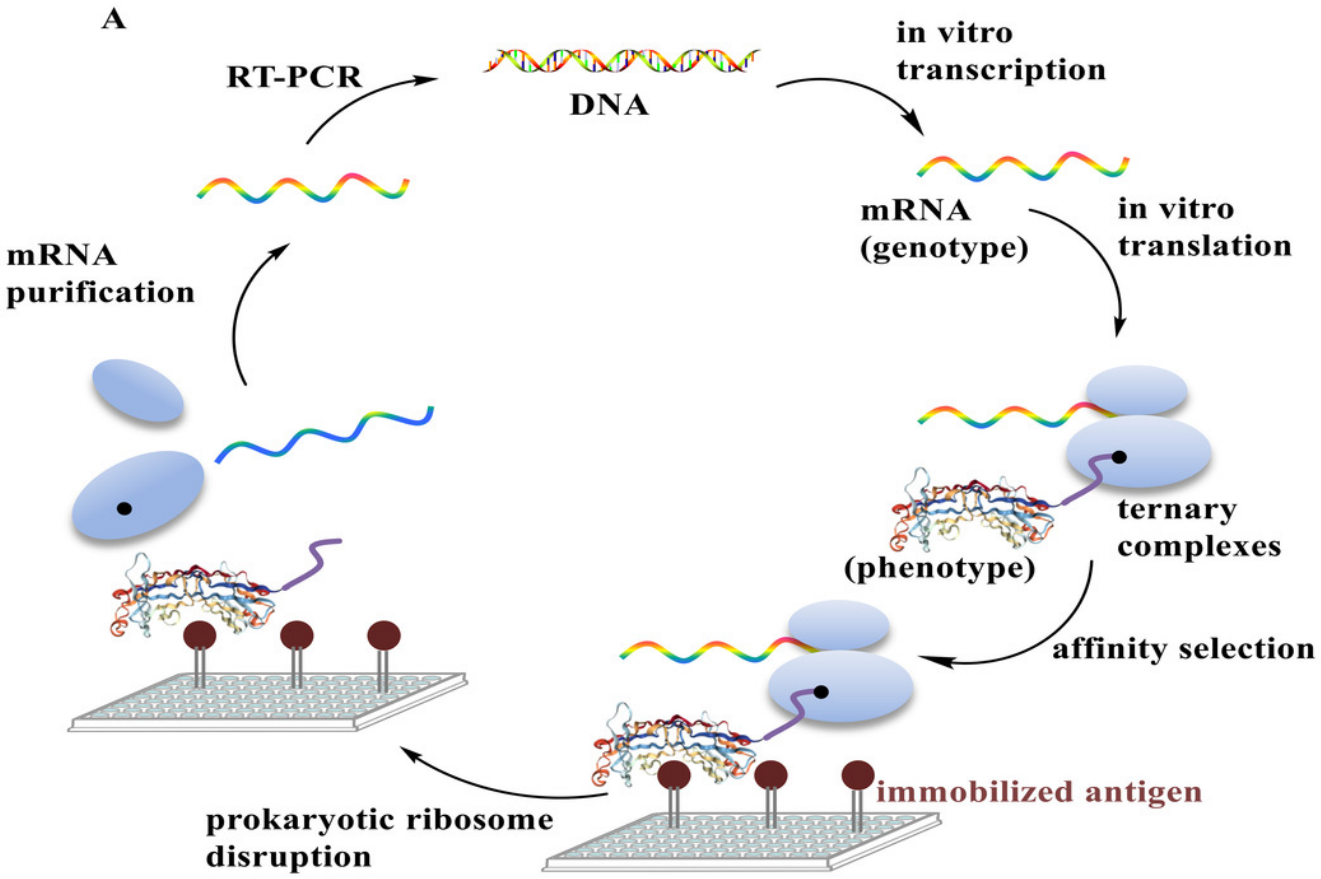


Figure 2

Schematic representation of the ribosome display selection cycle.

The DNA library is transcribed into mRNA. When the resultant mRNA is translated using a cell-free expression system in vitro, ternary complexes are generated due to the elimination of translational stop codons on mRNA. These complexes are directly applied to affinity selection on the immobilized antigen or the biotinylated antigen in solution which can be captured with streptavidin using magnetic beads. After washing away unbound ribosomal complexes, antigen-specific complexes are dissociated by EDTA to release mRNA. The purified mRNA is subjected to RT-PCR to recovery DNA for the next selection cycle or for analysis by cloning and sequencing. Alternatively, the DNA recovery is achieved by in situ RT-PCR from selected complexes without complexes dissociation and mRNA purification. (A): The panning cycle of prokaryotic ribosome display. (B): the panning cycle of eukaryotic ribosome display. Adapted from ref. (Douthwaite et al. 2006).



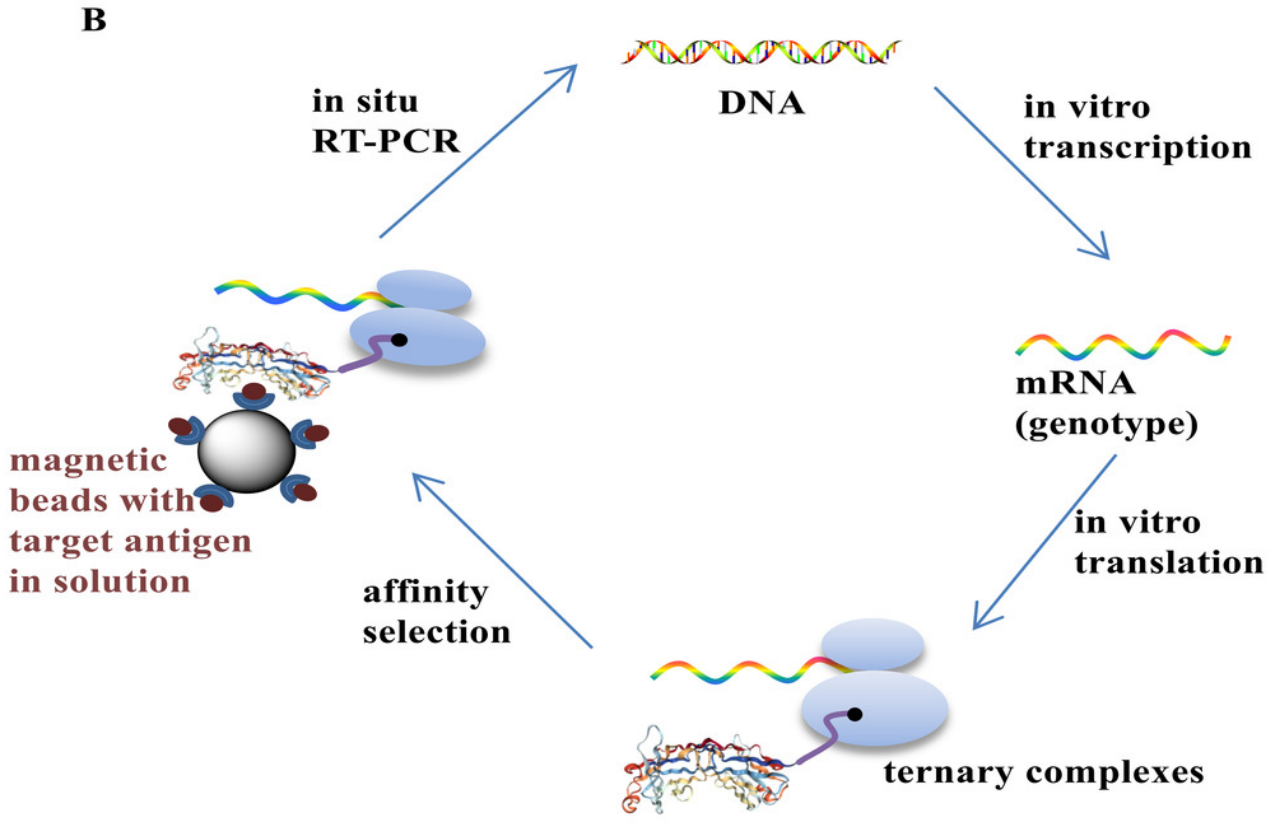


Table 1(on next page)

Different protein directed evolution strategies in vitro.

1 **Table 1** Different protein directed evolution strategies in vitro

Aspects of evolution	Directed evolution strategies in vitro		Characteristics	Relative application	Ref.
Affinity	In-vitro diversification strategies	Targeted strategies			
		Hotspot mutagenesis	Hotspots are innately prone to hypermutations	PTH22	(Yau et al. 2005)
		Parsimonious mutagenesis	Mutagenic oligodeoxynucleotides are designed to minimize the redundancy of the coding gene and restrict the number of mutations.	XT-M4	(Finlay et al. 2009)
		CDR walking	Mutations are only introduced in short (4–6) amino acid sequences related to a single CDR.	SWA11	(Shapira et al. 2015)
		Non targeted strategies			
		Error-prone PCR	It gives rise to point mutations randomly across the gene sequences.	Her2 ECD-specific DARPins	(Zahnd et al. 2007)
		E. coli mutator strains	Single base transversion or transition events take place at a higher rate in such *E. coli* mutator cells than that in corresponding normal cells.	Anti-Ag antibodies	(Irving et al. 2002)
		DNA shuffling	It is based on repeated cycles of point mutagenesis, recombination and selection.	CTX-M-15 β-lactamase	(Po et al. 2017)
		Chain shuffling	It can generate high-affinity antibodies from immunized animals quite rapidly.	Scfvs	(Lou & Marks 2010)
		Staggered extension process	The feasibility of operation in a single PCR tube; there is no need to digest DNA by the enzyme.	CSF2A	(Sheedy et al. 2006)

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Table 1 Continued

Aspects of evolution	Directed evolution strategies in vitro		Characteristics	Relative application	Ref.
Stability	Modified selection strategies	Off-rate selection	It uses the free or unlabeled antigen as the competitor.	LoopDARPins	(Schilling et al. 2014)
		Substractive panning	This method increases the likelihood of isolating high-affinity binders	anti-CD28 scFvs	(Rothe et al. 2007)
		Reducing antigen concentration and Increasing the frequency and stringency of washing steps gradually	The ease of operation	anti-hRaf-1 affibody molecules	(Grimm et al. 2012)
	Optimization strategies	Altering redox potential of the cell-free translation system	It can isolate stable binding antibodies under a reducing environment	G-CSF and EPO	(Buchanan et al. 2012)
		Adding proteases into the cell-free translation system	It can obtain protein binders resistant to proteases	VHHs	(Harmsen et al. 2006)

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