

Phage Display Vectors for the In Vitro Generation of Human Antibody Fragments

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Summary

A major source of human antibodies are phage display libraries, which are constructed from various genetic sources. Antibodies are expressed as scFV and Fab antibody fragments using various vector systems. This review offers a comprehensive overview of M13 phage display antibody vectors and discusses their applications.

Key Words: Antibody (Ab); fragment antigen binding (Fab); fragment variable (Fv); single-chain Fv (scFv); variable part of heavy chain; variable part of light chain; wild type.

1. Introduction

The production of polyclonal antibodies by the immunization of animals is a method that has been used for more than a century (1). Hybridoma technology was the next development, allowing the production of monoclonal antibodies (2). However, hybridoma technology has some limitations, some resulting from the instability of the aneuploid cell lines, but most of all, difficulties in producing human antibodies especially to toxic or highly conserved antigens (3).

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In the past decade, the *in vitro* selection of antibodies from recombinant antibody repertoires has proven to bypass these limitations. *In vivo* libraries offer the capability of generating human antibodies in transgenic mice carrying the human IgG loci. Here, antigens are injected into the humanized mouse, followed by conventional hybridoma technology for selection and production of antibodies (4–6).

To be able to raise human antibodies against toxic, highly pathogenic, or nonimmunogenic antigens, the method of choice is the use of libraries and an *in vitro* selection process. The first antibody repertoires were generated and screened in phage Lambda (7,8) with limited success. The phage display method most commonly used today is based on the groundbreaking work of Smith (9). Here, the genotype and phenotype of a polypeptide are linked by fusing short gene fragments to the minor coat protein III gene of the filamentous bacteriophage M13. This results in the expression of this fusion protein on the surface of phage and allows affinity purification of the gene by the peptide binding. Shortly after this breakthrough, another method was developed in which antibodies were presented on the surface of M13, fused to pIII (10–15). By uncoupling antibody gene replication and expression from the phage life cycle by locating them on a separate plasmid (phagemid), genetic stability, propagation, and screening of antibody libraries was greatly improved (11,12,14,15). To date, “single-pot” antibody libraries with a theoretical diversity of up to 10^{11} independent clones have been assembled (16) to serve as molecular repertoires for phage display selections. An overview of antibody libraries is given by Hust and Dübel (17).

The novel way of isolating antibodies by their binding activity *in vitro* is called “panning,” which refers to the method used by gold prospectors (18). The antigen is immobilized to a solid surface, such as nitrocellulose (19), magnetic beads (20), a column matrix (12) or, the most widely used, plastic surfaces as polystyrene tubes (21) or 96-well plates (11). The antibody phage are incubated with the

surface-bound antigen, followed by thorough washing to remove the excess nonbinders. The bound antibody phage can subsequently be eluted and amplified by infection of *Escherichia coli*. This method allows the detection of a single antibody phage by panning, and as it can be selected by its resistance marker, it can give rise to a bacterial colony after elution. This illustrates the tremendous sensitivity of the method. This selection cycle can be repeated, by infecting the resulting *E. coli* colonies with a helper phage to produce new antibody phage, which can then be used for a new round of panning, and so on. The number of antigen specific antibodies will increase with every panning round. Usually two to six panning rounds are necessary to get specifically binding antibodies.

Other display techniques have been developed and successfully applied for antibody selection. Techniques requiring intracellular antibody expression, such as the two-hybrid system (22), are of restricted use because of the limited folding of antibody fragments in the reducing milieu of the cytoplasm (23). Bacterial surface display techniques were developed, which rely on fusing antibodies to peptidoglycan-associated lipoprotein (24,25). Recently, fusions to lipoprotein NlpA have been used (26), but a universal method has not yet been developed. To display antibodies on yeast, antibodies were fused with the Aga2p mating adhesion receptor embedded in the cell wall (27). An extravagant technique completely avoiding the use of living cells in the process uses the linkage of messenger ribonucleic acid (mRNA) to the translation product (protein). In ribosomal display, mRNA and the related protein (antibody) complexed to the ribosome are selected by antigen binding of the attached antibody fragment. Then, the antibody mRNA is amplified by reverse transcription polymerase chain reaction and a further round of selection can be started (28,29). A related concept is the puromycin linked display ("profusion"). The 3' end of the mRNA is fused to puromycin, which serves as a peptidyl acceptor. After translation, the resultant polypeptide is covalently linked to mRNA by the puromycin (30), allowing selection as previously described.

2. Phage Display

Because of its robustness and ease of use, phage display has been the method most widely used in the past decade. This review therefore focuses on libraries using this selection principle. For an overview of available strategies and protocols, *see* McCafferty et al. (31) and Kontermann and Dübel (32). Display systems that require the insertion of antibody genes into the phage genome have been developed for phage T7 (33), phage Lambda (7,34,35), and the Ff class (genus *inovirus*) of the filamentous phage f1, fd, and M13 (10). Being well established for peptide display, the phage T7 is not well suited for antibody phage display because protein assembly takes place in the reducing milieu of the cytoplasm, thus leaving most antibodies unfolded (33). In contrast, the oxidizing milieu of the bacterial periplasm allows antigen binding fragments of antibody heavy and light chain to be folded and assembled properly (36). The Ff class nonlytic bacteriophage are assembled in this cell compartment and allow the production of phage without killing the host cell. This is a major advantage compared with the lytic Lambda phage (7). In addition, filamentous phage allow the production of soluble proteins by introducing an amber stop codon between the antibody gene and gene III. In an *E. coli* supE suppressor strain, the fusion proteins will be produced, whereas soluble antibodies are made in a nonsuppressor strain (37,38). As a result the members of the Ff class are the phage of choice for antibody phage display.

To achieve surface display, five of the M13 coat proteins have been used in fusion to foreign protein fragments. In the most widespread system the antibody is coupled to the N-terminus or second domain of the minor coat protein pIII (11,12,14). The naive function of the three to five copies of the pIII, in particular their N-terminal domain, is to provide binding of the phage to the f-pili of *E. coli* to initiate infection (39). The major coat protein (pVIII) has been used as an alternative fusion partner, with only very few successes reported in the past decade (40). This fusion technique is more useful for the display of short peptides (41,42). Fusions to pVI have been tried, but not yet with antibodies (43). pVII and pIX were

used in combination by using the variable part of light chain domain to pIX and the variable part of heavy chain domain to pVII, which allowed the expression of a fragment variable (Fv) on the phage surface. This particular method offers the potential for heterodimeric display (44). However, the fusion with pIII remains the most widely used system for phage display and is the only system of practical relevance so far.

Two different systems have been developed for the expression of the antibody/pIII fusion proteins. First, the fusion gene can be inserted directly into the phage genome substituting the wildtype pIII (10). Second, the fusion gene can be provided on a separate plasmid with an autonomous replication signal, promoter, resistance marker and phage morphogenetic signal, allowing this “phagemid” to be packaged into assembled phage particles. A helper phage, usually M13K07, is necessary for the production of the antibody phage to complement the phage genes not encoded on the plasmid. Because of its mutant origin, the M13K07 helper phage genome is not efficiently packaged during antibody phage assembly when compared with the phagemid (45), thus increasing the selection of the phagemid of interest during panning.

What are the practical differences of the vectors described so far? In the system using direct insertion into the phage genome, every pIII protein on a phage carries an antibody fragment. This is of a particular advantage in the first round of panning, where the desired binder is diluted by millions of phage with unwanted specificity. The oligovalency of these phage improves the chances of a specific binder being enriched because of the improved binding provided by the avidity effect. This advantage, however, has to be weighed against a number of disadvantages. The transformation efficiency of phagemids is two to three orders of magnitude better than phage vectors, thus facilitating the generation of large libraries in phagemids. Second, the additional protein domains fused to pIII may reduce the function of pIII during reinfection. In a phagemid system, the vast majority of the pIII assembled into phage are wild-type (wt) proteins, thus providing normal pilus binding. This may explain why only two “single-pot” antibody libraries (38,46) have

ever been made using phage vectors. In phagemid systems, by contrast, both replication and foreign fusion protein expression are independent from the phage genome. Because the propagation of the phagemid occurs in the absence of helper phage, there is no selection pressure from this end. The fusion protein can be produced in adjustable quantities by the use of the amber stop/suppressor system for switching expression to antibody (Ab) fragments without a pIII domain. Finally, despite not usually being derived from highest copy plasmids, the dsDNA of phagemids is more easy to handle than phage DNA, facilitating both cloning and analysis. Therefore, most pIII display systems use the phagemid approach. There is, however, a disadvantage that originates from the two independent sources for the pIII during phage packaging. During assembly, the wt pIII of the phage are inserted into the phage particles with much higher rate than the pIII fusion protein. As a result, the vast majority of resulting phage particles carry no antibody fragments at all. The few antibody phages in these mixtures are therefore mainly monovalent, with phage carrying two or more antibodies being extremely rare. This allows the selection of antibodies with high monovalent affinity, since avidity effects which would decrease the dissociation rate from the panning antigen can be avoided. In the first panning round, however, when a few binders have to be fished out of a huge excess of unwanted phage, the fact that only a few percent of the phage carry antibodies hampers the efficiency of the system (11,12,14,46–48). Recently, this problem has been solved by using a helper phage (Hyperphage), which avoids the need to introduce wt pIII into the packaging process, thus leaving the phagemid as the sole source of pIII and therefore offering multivalent display for phagemid vectors as well. This method improves Ab display by two orders of magnitude and vastly improves panning efficiency (49). Multivalent display has however been achieved by integrating two amber stop codons into the gIII gene of the helper phage genome. This allows the production of a functional helper phage (ex-phage) in an *E. coli* suppressor strain. In the associated phagemid pIGT3, the Ab/pIII fusion occurs without an amber stop codon and the Ab phage is produced in an *E. coli* nonsuppressor strain (50). However,

the deletion of the amber stop codon in the phagemid makes it imperative that the antibody gene is subcloned or that a protease is used to produce soluble antibodies. This is in contrast to the Hyperphage system where the amber stop/suppressor system can be used for switching expression to Ab fragments without a pIII domain.

An elegant application of phage display is selective infective phage technology. Here, antibodies are fused to the C-terminal domain of pIII by cloning into the phage genome; therefore, every pIII carries an antibody and deletion of the pIII aminoterminal region makes the phage noninfective. Antigen is then fused to the C-terminal end of separately produced soluble pIII N-terminal domain. The functional, f-pili binding pIII is reconstituted when the antibody phage binds to the antigen, allowing only the correct antibody phage to infect *E. coli* and be propagated (51). However, because of the fast kinetics of pIII/pilin interactions and very low concentrations of the three reaction partners if not co-expressed in the same cell, this method does not lend itself to the rapid panning of larger libraries.

3. Phage Display Vectors

A large number of different phage display vectors have been constructed. With pretending to be complete, **Table 1** lists a selection of phage display vectors. Some of them have not been used for the construction of a library up to now but have been included because they offer possible alternatives. For example, one of the systems allows the success of antibody gene cloning to be monitored by the expression of green fluorescent protein (52).

A variety of different promoters have been used for the expression of antibody genes. Widely used is the lacZ promoter (lacZ) derived from the lactose operon (53). The gIII promoter (gIII) from the bacteriophage M13 (9), the tetracycline promoter (1X tet^{o/p}; ref. 54) and the phoA promoter of the *E. coli* alkaline phosphatase (47) also have been used successfully. It appears that very strong promoters, for example, the synthetic promoter PAI/04/03 (55), are

Table 1
Phage Display Vectors for Cloning of Antibody Genes in Alphabetical Order

Phage display vector	Promoter	Secretion	Antibody format used by reference	C-domains in vector	Sites heavy chain	Sites light chain	Tags	gIII	Expression of soluble Ab	Reference
pAALFab	1x lacZ, 2x RBS	2x pelB	Fab	no	<i>EcoRI</i> – <i>BstPI</i>	<i>SpeI</i> – <i>XhoI</i>	-	truncated	subcloning	77
pAALFv	1x lacZ, 2x RBS	2x pelB	Fv	no	<i>EcoRI</i> – <i>BstPI</i>	<i>SpeI</i> – <i>XhoI</i>	-	truncated	subcloning	
pAALSC	1x lacZ, 1x RBS	1x pelB	scFv	no	<i>EcoRI</i> – <i>BstPI</i>	<i>SpeI</i> – <i>XhoI</i>	-	truncated	subcloning	
pAK100	1x lacZ, 1x RBS	1x pelB	scFv	no	<i>SfiI</i> (tat resistance will be removed)	<i>SpeI</i> – <i>XhoI</i>	FLAG, myc	truncated	amber, supE strain	78
pAPIII, scFv	1x phoA, 1x RBS	1x ompA	scFv	no	<i>HindIII</i> – <i>SalI</i>		FLAG, His	truncated	<i>SalI</i> – <i>KI</i> digest, deletion of gIII	79
pCANTAB3his	1x lacZ, 1x RBS	1x g3p	scFv	no	<i>NcoI/SfiI</i> – <i>NotI</i>		His, myc	full	amber, supE strain	59
pCANTAB5his/ pCANTAB 6	1x lacZ, 1x RBS	1x cat	scFv	no	<i>NcoI/SfiI</i> – <i>NotI</i>		His, myc	full	amber, supE strain	
pCANTAB 5 E	1x lacZ, 1x RBS	1x g3p	scFv	no	<i>SfiI</i> – <i>NotI</i>		E tag	full	amber, supE strain	
pCES1	1x lacZ, 2x RBS	1x gIII (L) 1x pelB (H)	Fab	yes	<i>SfiI</i> – <i>PstI/BstEII</i> (VH)	<i>ApaI</i> 1 – <i>AscI</i> (L chain), <i>ApaI</i> 1 – <i>XhoI</i> (VL)	His, myc	full	amber, supE strain	73
pComb3	2x lacZ, 2x RBS	2x pelB	Fab	no	<i>XhoI</i> – <i>SpeI</i>	<i>SacI</i> – <i>XbaI</i>	-	truncated	<i>NheI</i> – <i>SpeI</i> digest, deletion of gIII	11
pComb3H	1x lacZ, 2 x RBS	ompA (LC) pelB (HC)	Fab, scFv	yes	<i>XhoI</i> – <i>SpeI</i>	<i>SacI</i> – <i>XbaI</i>	-	truncated	<i>NheI</i> – <i>SpeI</i> digest, deletion of gIII	80
pComb3X	1x lacZ, 2 x RBS	ompA (LC) pelB (HC)	Fab, scFv	yes	<i>XhoI</i> – <i>SpeI</i>	<i>SacI</i> – <i>XbaI</i>	His, HA	truncated	amber, supE strain	81
pCW93/H, pCW99/L ¹	1x lacZ, 1x RBS	1x pelB	scFv	no	<i>NcoI</i> – <i>NheI</i>	<i>SacI</i> – <i>BglII</i>	myc	truncated	amber, supE strain	
pDAN5	1x lacZ, 1x RBS	undiscribed leader	scFv	no	<i>XhoI</i> – <i>NheI</i>	<i>BstPVI</i> – <i>SalI</i>	SV5, his	full	amber, supE strain	
pDH188	2x phoA, 2x RBS	2x stII	Fab	no	n.d.	n.d.	-	truncated	subcloning	47
pDN322	1x lacZ, 1x RBS	1x pelB	scFv	no	<i>NcoI</i> – <i>NotI</i>		FLAG, His	full	amber, supE strain	82
pDNEK	1x lacZ, 1x RBS	1x pelB	scFv	no	<i>NcoI</i> – <i>NotI</i>		FLAG, His	full	amber, supE strain	83
pEXmide3	1x lacZ, 2x RBS	2x pelB	Fab	yes	<i>SfiI/NcoI</i> – <i>KpnI/ApaI</i>	<i>EagI/NotI</i> – <i>NheI/SpeI</i>	-	full	amber, supE strain	8 4
pEXmide4	1x lacZ, 1x RBS	1x pelB	scFv	CH1	<i>NcoI</i> – <i>SalI</i>		-	full	amber, supE strain	85
pEXmide5	1x lacZ, 1x RBS	1x pelB	scFv	?	<i>NcoI</i> – <i>SalI</i>		-	full	amber, supE strain	86
pFAB4	2x lacZ, 2x RBS	2x pelB	Fab	no	<i>SfiI</i> – <i>NotI</i>		-	truncated	amber, supE strain	87
pFAB4H	1x lacZ, 2x RBS	2x pelB	Fab	CH1	<i>SfiI</i> – <i>NotI</i>		-	truncated	subcloning	88

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pFAB5c	2x lac Z, 2x RBS	2x pelB	Fab	no	SfiI – NotI	-	truncated	amber, supE strain	87
pFAB5c-His	1x lac Z, 2x RBS	2x pelB	scFv	no	SfiI – NotI	his	truncated	amber, supE strain	89
pFAB60	1x lac Z, 1x RBS	2x pelB	Fab	CH1	SfiI – SspII (VH) SfiI – NotI (Fd)	his	truncated	EagI digest, deletion of gIII	90
pFAB73H	1x lac Z, 1x RBS	2x pelB	Fab	CH1	NheI – ApaI (VH) NheI – ApaI (Fd)	his	truncated	EagI digest, deletion of gIII	91
pGP-F100	1x tdk ⁺ , 1x RBS	1x pelB	scFv	no	SfiI (GFPuv will be removed)	myc	truncated	TEV protease site	52
pGZ1	1x tdk ⁺ , 1x RBS	1x pelB	scFv	no	SfiI – NotI	myc	full	amber, supE strain	54
pHEN1	1x lac Z, 1x RBS	1x pelB	scFv, Fab, Fd, LC	no	SfiI – NotI	myc	full	amber, supE strain	14
pHEN1-V3	1x lac Z, 1x RBS	1x pelB	scFv	no	NcoI – XhoI	myc	full	amber, supE strain	92
pHEN2	1x lac Z, 1x RBS	1x pelB	scFv	no	NcoI – XhoI	his, myc	full	amber, supE strain	93
pHENIX	1x lac Z, 1x RBS	1x pelB	scFv	no	SfiI/NcoI – Sall/XhoI	myc	full	amber, supE strain	94
pHG-Im/A27Jk1	1x lac Z, 1x RBS	1x pelB	scFv	no	ApaLI – SfiI	his, myc	full	amber, supE strain	95
phi3mu-y1	2x lac Z, 2x RBS	2x pelB	Fab bidirectional	yes	XhoI – EcoRI	-	truncated	subcloning	96
pIG10	1x lac Z, 1x RBS	1x OmpA	scFv	no	EcoRV – EcoRI	myc	full	amber, supE strain	50
pIGT2 (vector)	1x lac Z, 1x RBS	1x G3p	scFv	no	SfiI – NotI	myc	full	amber, supE strain	97
pIGT3 (vector)	1x lac Z, 1x RBS	1x G3p	scFv	no	SfiI – SfiI	His, myc	full	subcloning	98
pIT2	1x lac Z, 1x RBS	1x pelB	scFv	no	SfiI/NcoI – XhoI	-	truncated	subcloning	99
pLG18	1x phoA, 2x RBS	2x attL	Fab	yes	BssHII – NcoI (CDR2-3)	-	full	subcloning	100
pM834, pM827	2x lac Z, 2x RBS	2x pelB	Fab	no	XhoI – SspII	-	full	subcloning	101
pMorph series	1x lac Z ?	1x phoA	scFv	no	XbaI – EcoRI	FLAG ?	?	subcloning	12
pScUAGvcp3	1x lac Z, 1x RBS	1x pelB	scFv with Cx	Cx	XhoI – NheI	-	truncated	amber, supE strain	75
pSEX	1x PA1/04/03 1x RBS	1x pelB	scFv	no	-	Yoi1/34	full	subcloning	74
pSEX20	1x PA1/04/03 1x RBS	1x pelB	scFv	no	-	Yoi1/34	full	subcloning	74
pSEX81	1x lac Z, 1x RBS	1x pelB	scFv	no	NcoI – HindIII	Yoi1/34	full	subcloning	74

¹Cre/lox recombination. ² λ recombination. ³Construction of the HuCAL library is described, but the pMorph vectorsystem is unpublished.

rather a disadvantage. To our knowledge, a systematic comparison of the different promoters has not been conducted to date.

The targeting of antibodies to the periplasm requires the use of signal peptides. The *pelB* leader of the pectate lyase gene of *Erwinia carotovora* (56) is commonly used. The *gIII* leader (9), the *phoA* leader of the *E. coli* alkaline phosphatase, and the *ompA* leader of *E. coli* outer membrane protein OmpA have also been used, being common to many protein expression vectors (57,58). Further examples are the heat-stable enterotoxin II (stII) signal sequence (47) and the bacterial chloramphenicol acetyltransferase (*cat*) leader (59).

Because of the general inability of *E. coli* to assemble complete IgG, with only one exception documented (60), smaller antibody fragments are used for display. In particular, fragment antigen binding (Fabs) and single-chain Fvs (scFvs) have been shown to be the antibody fragments of choice. In Fabs, the *fd* fragment and light chain are connected by a disulfide bond. In scFvs, the V_H and V_L are connected by a 15–25 amino acid linker (61–63). Soluble scFvs tends to form dimers, in particular when the linker is reduced to 3 to 12 amino acid residues. Without or with only a few amino acid linker, diabodies or tetrabodies can be found, with high variability in the behaviour of different V regions (refs. 64–66, Schmiedl and Dübel, unpublished). The dimerization can cause problems with binding because of possible avidity effects of the antibody complex (67). Furthermore, some scFvs have a reduced affinity of up to one order of magnitude compared with Fabs (62), only in rare cases, scFvs with a higher affinity than the associated Fab have been found (68). Small antibody fragments like Fv and scFv can easily be produced in *E. coli*. The yield of functional Fvs expressed in *E. coli* is higher than the yield of the corresponding Fabs, because of a lower folding rate of the Fabs (69,70). In one example, the stability in long-term storage was much higher for Fabs than for scFvs. After 6 mo, the functionality of scFvs stored at 4°C was reduced by 50%; Fabs, however, showed no significant loss of functionality after 1 yr (71). The overall yield of Fvs expressed in *E. coli* vary from 0.5 to 10 mg/L culture compared with 2 to 5 mg Fabs/L culture (72).

Therefore, the choice of the antibody fragment, scFv or Fab, depends on the desired application.

For the expression of Fabs in *E. coli*, two polypeptide chains have to be made. To achieve this, there are two strategies. In the monocistronic systems, for example, pComb3, the antibody genes are under control of two promoters and each has its own leader peptide (**11**). In plasmids like pCES1 with a bicistronic Fab operon, both chains are under control of a single promoter, leading to mRNA with two ribosomal binding sites (**73**).

Two variants of pIII-fusions to antibody fragments have been made. Either full-size pIII or a truncated version of pIII have been used. The truncated version is made by deleting the pIII N-terminal domain. This domain mediates the binding to the f-pili of *E. coli*. Infection is provided by wt pIII because only a small percentage of phage in phagemid-based systems are carrying an antibody. These truncated vectors are therefore not compatible for use with either Hyperphage or Ex-phage, as the full-size pIII is necessary to provide infections (**49,50**). Some vectors, for example, pSEX81 (**74**), allow an elution during panning by protease digestion instead of pH shift. This is possible because of a protease cleavage sequence between pIII and scFv and allows a complete recovery of bound phage for infection, even in case of very strong antigen binding.

Most of the described phagemids have an amber stop codon between the antibody gene and gIII. This allows the production of soluble antibodies after transformation of the phagemid to a non suppressor bacterial strain like HB2151 (**38**). For phagemids like pComb, it is necessary to delete the gIII by digestion followed by religation of the vector before transformation into *E. coli* (**11**). In the case of vectors like pSEX81, the selected antibody genes have to be subcloned into a separate expression vector like the pOPE series (**12,75,76**).

4. Outlook

The vast variety of available methodologies and constructs have not been systematically compared for their efficiency. This is mainly

Table 2
Human Single-Pot Phage Display Libraries

Library vector	Library type	Antibody type	Library cloning strategy	Library size	Reference
fdDOG-2lox,pUC19-2lox	Semisynthetic	Fab	PCR with random CDR3 Primers, Cre-lox	6.5×10^{10}	38
fdTet	Naive	scFv	Recloning of a naive library ¹	5×10^8	46
pAALFab	Semisynthetic (anti-hen egg white lysozyme Ab framework)	Fab	PCR with random CDR Primers, assembly PCR	2×10^8	77
pAP-III ₆ scFv	Naive	scFv	Assembly PCR	n.d.	79
pCANTAB 6	Naive	scFv	Assembly PCR	1.4×10^{10}	102
pCES1	Naive	Fab	Three-step cloning (Lchain, VH)	3.7×10^{10}	73
pComb3	Semisynthetic (anti-tetanus Ab framework ²)	Fab	PCR with random CDR H3 Primers	5×10^7	103
pComb3	Semisynthetic (anti-tetanus Ab framework ²)	Fab	PCR with random CDR H3 Primers	$>10^8$	104
pDAN5	Naive	scFv	Cre-lox	3×10^{11}	16
pDN322	Semisynthetic (VH DP47 and VL DPK22 V-genes)	scFv	Random CDR3 Primer, assembly PCR	3×10^8	82

pDNEK (ETH2 library)	Semisynthetic (VH DP47, VI DPL16 and Vκ DPK 22 V-genes)	scFv	Random CDR3 Primer, assembly PCR	5×10^8	83
Semisynthetic (germline)					
pEXmide5	VH-DP47 and VL-DPL3 framework)	scFv	Assembly PCR, CDR shuffling	9×10^6	86
pFAB5c-His (n-CoDeR library)	Semisynthetic (germline VH-DP47 and VL-DPL3 framework)	scFv	Assembly PCR, CDR shuffling	2×10^9	89
pHEN1	Naive	scFv	Assembly PCR	$10^7 \times 10^8$	15
pHEN1	Naive	scFv	Assembly PCR	$2 \times 10^5/2 \times 10^6$	37
pHEN1	Naive	scFv	Assembly PCR	6.7×10^9	106
pHEN1-Vλ3	Semisynthetic (Vλ3 anti-BSA Ab light chain)	scFv	PCR with random CDR H3 Primers	10^7	92
pHEN1-Vλ3	Semisynthetic (Vλ3 anti-BSA Ab light chain)	scFv	PCR with random CDR H3 Primers	$>10^8$	107
			Three-step cloning, PCR with random CDR H3 Primers		
pHEN1-Vκ3	Semisynthetic (VH)/naive (VL)	scFv	Recloning of the lox library in scFv format ³	3.6×10^8	108
pHEN2 (Griffin 1 library)	Semisynthetic	scFv		1.2×10^9	109

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(continued)

Table 2 (*Continued*)
Human Single-Pot Phage Display Libraries

Library vector	Library type	Antibody type	Library cloning strategy	Library size	Reference
pIT2 (Tom I/ J library)	Semisynthetic (3x VH and 4x VK V-genes)	scFv	PCR with random CDR2 and CDR3 Primers	1.47×10^8 $/1.37 \times 10^8$	97
pLG18	Semisynthetic (anti-HER2 Ab framework)	Fab	PCR with random CDR Primers, 2 step cloning	$2-3 \times 10^8$	98
pMorph series (HuCAL library)	Synthetic	scFv	Two-step cloning + 2 step CDR3 replacement	2×10^9	100
pScUAGDep3	Semisynthetic	scFv	Three-step cloning with random CDR3 Primers	1.7×10^7	101
pSEX81	Naive	connected to Ck scFv (with N-terminus of CH1 and CL)	Two-step cloning	4×10^7	109
pSEX81	Naive	scFv (with N-terminus of CH1 and CL)	Two-step cloning	4×10^9	110
pSEX81	Naive	scFv (with N-terminus of CH1 and CL)	Four-step cloning	$1.6 \times 10^7/1.8 \times 10^7/4 \times 10^7$	111

¹(106), ²(8), ³(38).

owing to the fact that experimental strategies allowing evaluation of the performance of any given vector other than using it for library construction and panning, is hard to formulate. The proof of the pudding is in the eating so, we have to analyze the resulting libraries produced by the various methods. However, this is not an easy task because different methods are used for each type of panning. When comparing successfully used human “single-pot” libraries (**Table 2**), it is evident that most of them use scFv fragments, but this is because the construction of scFv phage display vectors is less complicated compared to Fab phage display libraries. More fine tuning is necessary when expressing two chains in the case of Fab fragments, because the ratio of light chain to heavy chain must be harmonized, as in the case of scFv fragments.

Various leader sequences, promoters, and cloning strategies have been successfully used, with none of them providing a perfect construct. In conclusion, there are many different ways to success with many factors that can be altered depending on individual applications. Two factors are probably more important than the others and these are the careful control of phage biology during the panning process and secondly the use of an initial antibody library as large as possible.

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