

# Chapter 2

## Design and Construction of Synthetic Phage-Displayed Fab Libraries

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### Summary

Diversity—the variability carried by the amino acid sequences of a synthetic antibody library—can be generated by synthetic degenerate oligonucleotides. One can experiment with different diversity designs in the variable domains of light and heavy chains ( $V_H$  and  $V_L$ ) to generate antibody libraries with different properties. The ability to precisely define the final diversity of a library facilitates the process of isolating, characterizing, and optimizing an antibody lead. Here we describe detailed protocols for the design and construction of phage-displayed synthetic antibody libraries in which diversity is generated in the complementarity determining regions (CDRs) of the  $V_H$  of a single humanized bivalent Fab scaffold. The example used in the protocol provides a general methodology for generation of libraries with engineered CDR diversity that can be applied to a template antibody sequence of choice.

**Key words:** Diversification, Synthetic, Scaffold, Random, Degenerate

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### 1. Introduction

Synthetic antibody libraries have proven to be highly effective for the generation of functional, high-affinity antibodies against a wide variety of antigens (1–4). This chapter describes a method to design and construct such a synthetic antibody library in which diversity is created in the heavy chain variable domain. The method involves introducing diversity into an antibody scaffold by site-directed mutagenesis using synthetic oligonucleotides. In contrast to natural immune repertoires, the diversity of synthetic antibody libraries can generate well-defined repertoires.

There are numerous examples of different design strategies that have generated functional antibody libraries (2–5). The ability to precisely define the diversity of the final library facilitates the process of isolating, characterizing, and optimizing an antibody lead. Another advantage of synthetic libraries is that they allow control over parameters such as the subgroups of the light and heavy chain. The diversity of libraries generated in this protocol is restricted to 17 positions in the complementarity determining regions (CDRs) of the  $V_H$ . A single humanized bivalent Fab served as the scaffold. The libraries have proven to be efficient in generating antibodies with high affinity and specificity. This protocol will guide the reader through the process of defining the diversity to be achieved, using degenerate oligonucleotides to generate the library, and finally constructing the phage-displayed antibody library.

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## 2. Materials

### 2.1. Generation of Library DNA

1. Competent *Escherichia coli* CJ236 (New England Biolabs).
2. Carbenicillin stock (5 mg/mL). Sterile filter and store at 2–8°C.
3. LB agar plates: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 20 g/L agar. Mix all components, heat to dissolve, autoclave, and allow to cool. Store at room temperature. Melt in a microwave when required, cool to 60°C, and, when required, supplement with carbenicillin to a final concentration of 50 µg/mL before pouring plates.
4. 2YT broth: 10 g/L yeast extract, 16 g/L tryptone, and 5 g/L NaCl in deionized water. Mix all components, autoclave, and cool down. Store at room temperature.
5. M13KO7 helper phage (New England Biolabs).
6. Kanamycin stock (5 mg/mL). Sterile filter and store at 2–8°C.
7. Phage precipitation solution: 20% (w/v) polyethylene glycol (PEG 8000) and 2.5 M NaCl in deionized water.
8. Phosphate-buffered saline (PBS).
9. QIAprep Spin M13 Kit (QIAGEN).
10. Degenerate oligonucleotides diluted in water to 10 OD<sub>260</sub>/mL.
11. 10× Tris-Magnesium (TM) buffer: 0.5 M Tris-HCl pH 7.5, 0.1 M MgCl<sub>2</sub>.

12. 100 mM adenosine 5'-triphosphate (ATP, New England Biolabs).
13. 100 mM dithiothreitol (DTT).
14. T4 polynucleotide kinase (10,000 U/mL, New England Biolabs).
15. Deionized water.
16. 25 mM deoxynucleotide solution mix (dNTPs, New England Biolabs).
17. T4 DNA ligase (400,000 U/mL, New England Biolabs).
18. T7 DNA polymerase (10,000 U/mL, New England Biolabs).
19. Agarose.
20. Tris acetate EDTA (TAE) buffer: prepare a 50× stock containing 242.2 g/L Tris base, 18.6 g/L of Na<sub>2</sub>EDTA·2H<sub>2</sub>O (EDTA disodium dihydrate), and 57.47 mL/L of 17.4 M glacial acetic acid. Dilute 50-fold with deionized water before use.
21. Ethidium bromide (EtBr).
22. QIAquick Gel Extraction kit (QIAGEN).

**2.2. Generation  
of Electrocompetent  
SS320 for Phage  
Production**

1. *E. coli* SS320 (see **Note 1**).
2. 2YT top agar: 10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl, and 7.5 g/L agar. Mix components and heat to dissolve. Autoclave. Store at 2–8°C. Melt in a microwave when required then hold at 44°C until required.
3. Tetracycline stock (5 mg/mL): dissolve initially in ethanol then add deionized water to 50% (v/v). Store at –10°C in brown bottles to protect from light.
4. LB agar plates supplemented with tetracycline to a final concentration of 10 µg/mL.
5. Super broth. Solution A: 12 g tryptone, 5 mL glycerol, 24 g yeast extract, and deionized water to 900 mL. Solution B: 125 g/L K<sub>2</sub>HPO<sub>4</sub> and 38 g/L KH<sub>2</sub>PO<sub>4</sub>. Prepare solutions A and B and autoclave separately. Add 100 mL of Solution B to 900 mL of Solution A. Store at room temperature.
6. 1 mM HEPES, pH 7.2: 238 g HEPES (Cellgro) dissolved in 750 mL sterile deionized water. Adjust to pH 7.2 using 50% (w/v) NaOH. Add deionized water to 1 L. Store at room temperature. Dilute to 1 mM in sterile deionized water before use.
7. Glycerol.
8. Dry ice.

### 3. Methods

#### 3.1. *Selecting the Library Scaffold*

The performance of a synthetic library depends on the scaffold chosen as the foundation for the library, the design strategy for diversity, and the overall size of the final library. The first step in designing a synthetic phage-displayed antibody library is therefore to choose an appropriate scaffold for the library. Synthetic libraries have been reported that employ either single or multiple frameworks (1, 5–8). Libraries that are built on a single framework rely mainly on the CDR diversity that is introduced with synthetic degenerate oligonucleotides; while this is also true of libraries based upon multiple scaffolds, these resources also generate diversity through the provision of different frameworks.

A single framework facilitates the library construction and the downstream characterization and optimization of antibodies that are isolated from the library through their interaction with target. In the example described here, the library template is the Fab derived from the humanized antibody 4D5 (hu-4D5). The antibody binds to the extracellular domain of the human receptor tyrosine kinase ErbB2 with high affinity and is a validated therapeutic (9). This scaffold was chosen because it contains variable domain subgroups ( $V_H3$  and  $V_{kappa}1$ ) that are prevalent among naturally occurring human antibodies. It has been optimized for protein expression and it displays well on phage. The hu-4D5 antibody has been extensively characterized and structural information is available, which facilitated the strategy for library construction (9–11). These issues should be borne in mind when choosing a template for library production.

#### 3.2. *Choosing the Optimal Display Format*

In the example described here, Fabs are engineered to assemble as dimers on the phage particle, thereby presenting the antibody in a bivalent format. Protein assembly results from fusion of a dimerizing leucine zipper motif to the Fab heavy chain as described below. This enables bivalent display (Fig. 1). The bivalent format mimics natural IgG in that avidity effects can allow the recovery of antibodies from the library that possess moderate affinity to the target. Antibodies recovered can then be readily reformatted into IgG.

#### 3.3. *Construction of the Library Template*

A phagemid for displaying hu-4D5 as bivalent Fabs on phage particles is outlined in Fig. 2. The vector contains a bicistronic operon under the control of the alkaline phosphatase promoter. The first open-reading frame of the operon encodes a polypeptide consisting of the stII secretion signal (12), and the light chain variable ( $V_L$ ) and light chain constant domains ( $C_L$ ) of hu-4D5 followed by a gD epitope tag (sequence: MADPNRFRGKDLGG) (13). The second reading frame encodes the stII signal peptide followed by the heavy chain variable domain ( $V_H$ ) and the first

V<sub>L</sub> V<sub>H</sub> V<sub>H</sub> V<sub>L</sub>

C<sub>L</sub> C<sub>H1</sub> C<sub>H1</sub> C<sub>L</sub>

gD Tag

Leucine Zipper

cP3

Fig. 1. The phage in the synthetic libraries display Fabs in a bivalent format. The leucine zipper enables the bivalent display of Fabs on the phage.

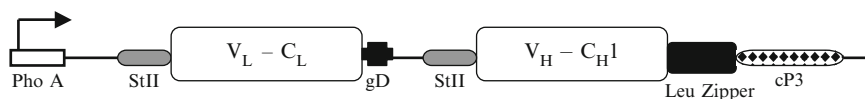


Fig. 2. Phagemid expression construct for bivalent display of Fabs on phage.

### 3.4. Library Diversity Design

As discussed previously, the productivity of a synthetic antibody library depends on the design strategy for introducing diversity and the final size of the repertoire. The size of phage libraries generated by the method described in this chapter is limited to  $10^9$ – $10^{10}$  by the efficiency with which library DNA can be electroporated into the *E. coli* host. Therefore, the design strategy for introducing diversity is key to a phage display resource that will be of general utility. One challenge is to select which residues to diversify (spatial diversity) and the range of amino acids (the degree of randomization) that will be incorporated at these positions (chemical diversity). Varying the length of the hypervariable loops present in the variable domain (length diversity) can further increase the diversity of the library. Different CDR-H3 lengths allow diverse structural conformations to form in this region.

#### 3.4.1. Analyzing the Natural Antibody Diversity to Guide Library Design

The diversity of natural antibodies has been successfully used to guide the design of antibody libraries (1, 2, 16–19). In constructing a functional repertoire of high-affinity antibodies, it is important that the degeneracy of the DNA does not greatly exceed the actual library size. Naturally occurring antibodies provide guidance on how to limit synthetic diversity.

The diversity of the libraries described here (“V<sub>H</sub>-Fab” libraries) is designed to mimic the natural antibody repertoire (2). Heavy chain variable domains of natural human antibodies are more diverse than light chain variable domains, in both amino acid sequence and structural conformations. The heavy chain is the main direct contributor to interaction with antigen (mainly through CDR-H3) (20–23). Based on these observations, the diversity of the V<sub>H</sub>-Fab library is focused to the three CDRs of the V<sub>H</sub> domain of the Fab, while the V<sub>L</sub> domain is of fixed sequence.

#### 3.4.2. Determining the Spatial Diversity

Analysis of the sequences of natural human V<sub>H</sub> domains in the Kabat database (24, 25) reveals that the degree of amino acid variation at individual positions in the CDRs is itself variable. This kind of analysis identifies positions with the greatest natural variability. In the absence of structural information on the template chosen for library construction, one approach to diversification is to randomize amino acids at these naturally variable positions. In the case of hu-4D5, detailed structural information is available (11), and can be used to further guide library design by identifying solvent-exposed residues in the CDRs. These residues have the greatest potential to directly impact upon interaction with antigen. Overall, then, residues in the CDRs that are both highly variable in the natural V<sub>H</sub> repertoire and solvent exposed

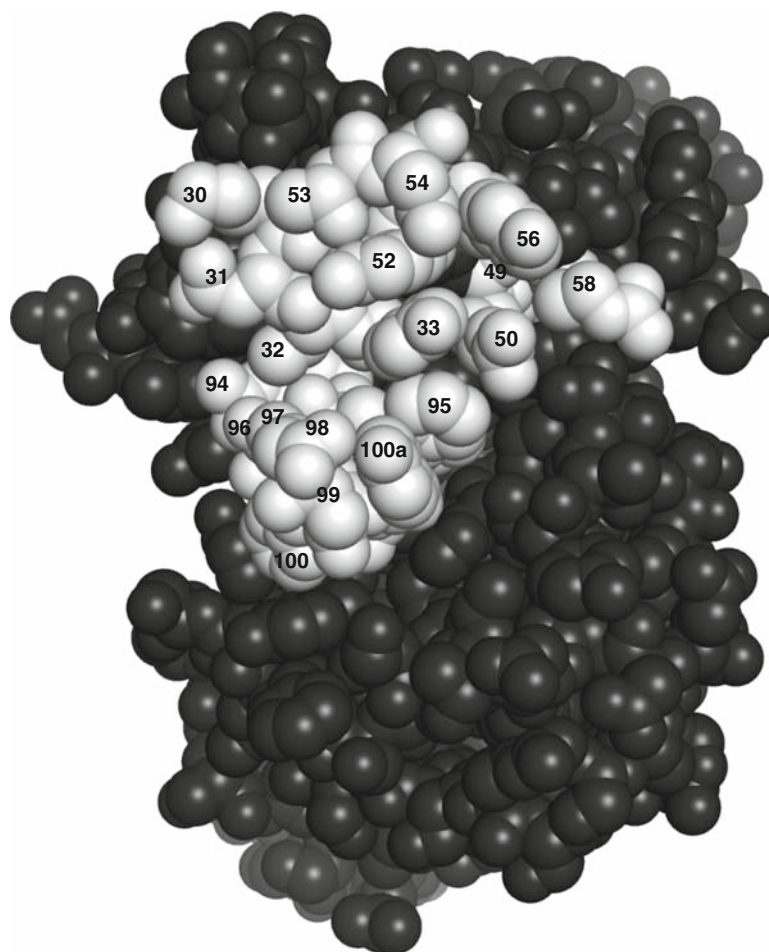


Fig. 3. Space-filling model of hu-4D5 (PDB reference number 1FVC). The heavy chain residues that were randomized in the library are highlighted in *white* and *numbered* according to Kabat (24).

in the hu-4D5 structure were selected for randomization (**Fig. 3**, **Tables 1** and **2**). These comprised residues 30–33 in CDR-H1 and 50, 52–54, 56, and 58 of CDR-H2. The framework residue at position 49 was also included, as this position has a degree of variation in the natural repertoire (**Table 1**). For CDR-H3, residues 95–101 were randomized. In contrast to CDR-H1 and CDR-H2, natural CDR-H3 loops are highly diverse in sequence and length. To mimic CDR-H3 length diversity, oligonucleotides were designed that insert residues between positions 98 and 100 (**Table 2**). The CDR-H3 length thus varies from 7 to 19 residues.

**Table 1**  
**Residues of hu-4D5 that are randomized in the libraries**

hu-4D5 residue	Diversity design				Target diversity			
	Codon	Encoded residues	Coverage (%)	Oligonucleotide	Pool	Amino acids in natural human antibodies (%)		
H1-28 N	ACC	T	54	H1	H1	T	S	
						54	36	
H2-30 K	AVT	STN	90	H1	a, b	S	T	N R D G
						68	18	4 3 2 2
H1-31 D	RRT	SNGD	82	H1	a, b	S	N	G T D R A
H1-32 T	WMY	YSN	80	H1	a, b	Y	S	N G F A
		T				64	9	7 4 3 3
H1-33 Y	KMT	AYS D	57	H1	a	A	Y	W G S D T N V
	KGG	WG	31	H1	b	22	20	17 14 12 3 3 2 2
H2-49 A	GST	GA	77	H2	a-c	H2	G S A	
						58	22	19
H2-50 R	DGG	RWG	35	H2	a	R	Y	W V G I E A S N L
	DHT	YVIASN	45	H2	b	17	10	9 9 9 8 8 6 6 4
	GAA	E	8	H2	c			
H2-52 Y	DMT	SYNDT	74	H2	a-c	S	Y	N K I R D T
		A				26	25	17 8 5 3 3 3



H2-53 T	DMT	SDYNT	A	66	H2	a-c	S	D	Y	G	H	N	I	T	W
							24	20	11	10	9	8	5	3	2
H2-54 N	RRC	GSDN		81	H2	a-c	G	S	D	N	K	F	T		
							37	26	11	7	6	5	4		
H2-56 Y	DMT	STNDYA		81	H2	a-c	S	T	N	D	Y	E	G	A	
							28	16	15	10	10	5	5	2	
H2-58 R	DAC	YND		69	H2	a-c	Y	N	D	R	S	I	T	H	
							32	25	12	7	4	4	3	2	

The target diversity was defined as the amino acids that cover 90% of the ~3,500 human amino acid sequences that were analyzed in the Kabat database (24). The occurrence of each amino acid is listed as a percentage. The diversity design depicts the amino acids that are encoded by the degenerate codons listed. The codons were designed to maximize the coverage (shown as percentages) while minimizing the occurrence of residues that are not part of the target diversity (shown in **bold**). Several oligonucleotides were mixed in the pools shown here in the Kunkel mutagenesis reaction, which is further described in the text

Table 2  
Designed diversity of CDR-H3

CDR-H3 positions									
Oligonucleotide	Length	93	94	95	96	97	101		102 Pools
H3-7a	7	A	R/K	NNK	NNK	NNK	NNK	F D Y	Y
H3-7b	7	A	R/K	XYZ	XYZ	XYZ	XYZ	F D/A Y	Y
H3-7c	7	A	R/H/ S/N	XYZ	XYZ	XYZ	XYZ	F/L G/V/ D/A	Y H3 1
H3-8a	8	A	R/K	NNK	NNK	NNK	NNK	F D Y	Y
H3-8b	8	A	R/H/ S/N	XYZ	XYZ	XYZ	XYZ	F/L G/V/ D/A	Y
H3-8c	8	A	R/H/ S/N	XYZ	XYZ	XYZ	XYZ	F/L D/A	Y
H3-9a	9	A	R	NNS	NNS	NNS	Y A/G/V	M D Y	H3 2
H3-9b	9	A	R	NNS	NNS	NNS	W/S/ A/G	M D Y	Y
H3-9c	9	A	R/K	NNK	NNK	NNK	NNK	F D Y	Y
H3-9d	9	A	R/K	XYZ	XYZ	XYZ	XYZ	F D/A Y	Y
H3-9e	9	A	R/K	XYZ	XYZ	XYZ	Y A	M D Y	Y
H3-9f	9	A	R/K	XYZ	XYZ	XYZ	W/S/ A/G	M D Y	Y
H3-10a	10	A	R	NNS	NNS	NNS	Y A/G/V	M D Y	H3 3
H3-10b	10	A	R	NNS	NNS	NNS	W/S/ A/G	M D Y	Y
H3-10c	10	A	R/K	NNK	NNK	NNK	NNK	F D Y	Y
H3-10d	10	A	R/K	XYZ	XYZ	XYZ	XYZ	F D Y	Y

H3-10e	10	A	R/K	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	F/L	G/V// D/A	Y	
H3-11a	11	A	R	NNS	NNS	NNS	NNS	NNS	NNS	Y	A/G/V				M	D	Y	H3 4
H3-11b	11	A	R	NNS	NNS	NNS	NNS	NNS	NNS	W/S/ A/G	A/G/V				M	D	Y	
H3-11c	11	A	R/K	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	F	D	Y	
H3-11d	11	A	R/H/ S/N	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	W/S/ A/G	G/V// D/A				M	D	Y	
H3-11e	11	A	R/H/ S/N	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	Y	G/V// D/A				M	D	Y	
H3-12a	12	A	R	NNS	NNS	NNS	NNS	NNS	NNS	W/S/ A/G	A/G/V				M	D	Y	H3 5
H3-12b	12	A	R	NNS	NNS	NNS	NNS	NNS	NNS	Y	A/G/V				M	D	Y	
H3-12c	12	A	R	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	F/L	D	Y	
H3-12d	12	A	R/K	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	W/S/ A/G	A				M	D	Y	
H3-12e	12	A	R/H/ S/N	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	W/S/ A/G	G/V// D/A				M	D	Y	
H3-12f	12	A	R/H/ S/N	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	Y	G/V// D/A				M	D	Y	
H3-13a	13	A	R	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	F/L	D	Y	H3 6
H3-13b	13	A	R	NNS	NNS	NNS	NNS	NNS	NNS	NNS	Y	A/G/V			M	D	Y	
H3-13c	13	A	R	NNS	NNS	NNS	NNS	NNS	NNS	NNS	W/S/ A/G	A/G/V			M	D	Y	
H3-13d	13	A	R/K	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	X1Y2	Y	A			M	D	Y	

(continued)

**Table 2**  
(continued)

CDR-H3 positions												
Oligonu- cleotide	Length	93	94	95	96	97					101	102 Pools
H3-13e	13	A	R/K	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	W/S/ A A/G	M D	Y
H3-13f	13	A	R/K	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	Y A/G/V	M D	Y
H3-13g	13	A	R/K	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	W/S/ A/G/V A/G	M D	Y
H3-14a	14	A	R/K	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	Y A	M D	Y H3 7
H3-14b	14	A	R/K	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	W/S/ A/G/V A/G	M D	Y
H3-15a	15	A	R/K	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	Y A/G/V	M D	Y H3 8
H3-15b	15	A	R/K	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	W/S/ A/G/V A/G	M D	Y
H3-16a	16	A	R	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	W/S/ A/G/V A/G	M D	Y H3 9
H3-16b	16	A	R	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	Y A/G/V	M D	Y
H3-17a	17	A	R	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	W/S/ A/G/V A/G	M D	Y H3 10
H3-17b	17	A	R	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	Y A/G/V	M D	Y
H3-18a	18	A	R	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	W/S/ A/G/V A/G	M D	Y H3 11
H3-18b	18	A	R	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	Y A/G/V	M D	Y
H3-19	19	A	R	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	X1YZ	Y A/G/V M D	Y H3 12	

The table shows the oligonucleotide design encoding different diversity and length of CDR-H3. The designed diversity is shown as degenerate codons (***bold italics***) or amino acids (plain text). The oligonucleotides were mixed in different pools as indicated and used in the Kunkel mutagenesis reaction as described in the text. For specifications of the XYZ codon, *see* **Note 3**

### 3.4.3. Defining the Target Amino Acid Diversity of the Library

Analysis of natural human antibody sequences enables the calculation of amino acid occurrence at each variable position. This often reveals a bias toward certain residues. By aligning thousands of sequences (~3,500) in the Kabat database, the frequency with which each amino acid occurs at each position can be determined (**Table 1**). Where variation is observed, a limited number of residues may occur in more than 90% of all sequences in the database. For example, at position 49, glycine is the most common amino acid (58% of sequences in the database), followed by serine (22%) and alanine (19%). Together, these three amino acids account for the diversity at position 49 in 99% of natural sequences. These three amino acids are therefore taken forward into the strategy for diversification at position 49.

This analysis works well for CDR-H1 and CDR-H2 but is more difficult for CDR-H3 because this region is highly variable in naturally occurring antibodies. Due to the substantial diversity in the length and sequence of CDR-H3, the occurrences of amino acids at positions 94 to the position preceding 101 are totaled and analyzed as a group to determine the target diversity for all positions in this CDR (**Fig. 4**).

### 3.5. Designing Tailored Degenerate Oligonucleotides

Having assessed where diversity is best generated in the template and the degree/length of diversification that should be introduced, the goal moves to designing degenerate oligonucleotides with the capacity to encode the desired range of amino acids

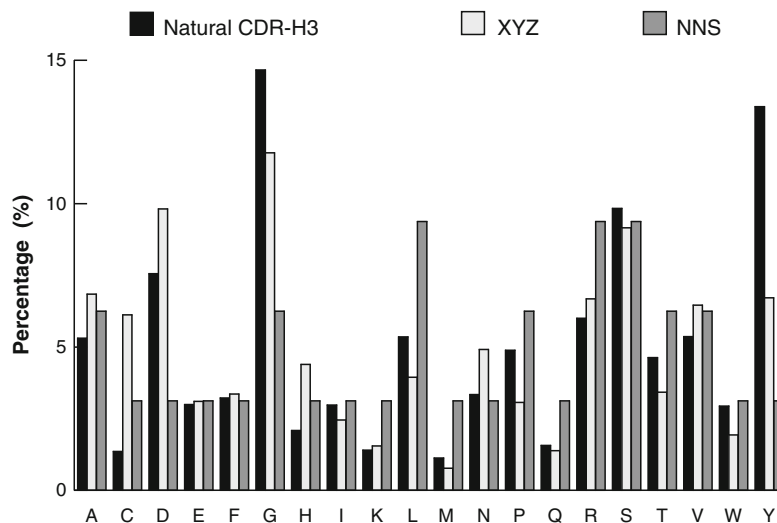


Fig. 4. The amino acid composition of natural human CDR-H3 sequences are calculated for position 94–101. The graphs depict the frequency at which each amino acid occurs in this region. The results are compared with the amino acid composition encoded by the degenerate codons NNS and XYZ. The latter was designed to closely mimic the natural CDR-H3 diversity. A standard single-letter amino acid code is used here and in all other figures. The text gives a detailed description of the XYZ codon.

for each selected position. Degenerate codons were designed to prioritize the most frequently observed amino acid residues, while encoding a minimum number of nontarget residues and excluding stop codons and cysteines (Tables 1 and 2, Fig. 4). DNA degeneracies are denoted by the IUB code (see Note 2). In creating these sequences, the goal should be to use an equimolar mix of the required nucleotides during synthesis. The codon XYZ (Table 2) is designed to represent the amino acid diversity of CDR-H3 sequences observed in natural antibodies. This is created by synthesizing the XYZ codon from different proportions of the nucleotides at each of the three positions in the codon (see Note 3). In some cases it is difficult to generate the diversity required using a single nucleotide mix. When this arises, several oligonucleotides are included that carry different codons at a single position. This is the case for positions 31, 33, and 50 of CDR-H1 and CDR-H2 (Tables 1 and 2).

### 3.6. Generation of Library DNA

The library is generated by Kunkel mutagenesis (26). Single-stranded template DNA is generated and used for mutagenesis together with the pools of degenerate oligonucleotides.

#### 3.6.1. Generation of Single-Stranded DNA Carrying the Antibody Library for Library Construction

1. Take phagemid vector DNA carrying the antibody sequence to be diversified and transformed into competent *E. coli* CJ236. Recall that, in our methods, stop codons are incorporated into the CDRs in a preliminary stage (Subheading 3.3).
2. Plate onto LB agar containing an antibiotic suitable for selection of the vector (carbenicillin in the case described here) and grow overnight at 37°C.
3. Pick a single colony of *E. coli* CJ236 harboring the phagemid vector and transfer to 5 mL of 2YT broth supplemented with 50 µg/mL carbenicillin. Grow at 37°C until log-phase (an OD<sub>600</sub> in the range 0.3–0.6). Infect the cells with M13KO7 helper phage ( $4 \times 10^{10}$  pfu/mL) for 60 min at 37°C.
4. Add 25 mL 2YT supplemented with 50 µg/mL carbenicillin and 50 µg/mL kanamycin and grow at 37°C for 20 h.
5. Centrifuge the culture at  $24,000 \times g$  for 10 min. Harvest the supernatant and add 1/5 volume of phage precipitation solution. Incubate on ice 5 min.
6. Centrifuge at  $24,000 \times g$  for 10 min. Decant the supernatant and resuspend the phage pellet in 5 mL of PBS. Centrifuge again at  $24,000 \times g$  for 5 min to remove any remaining bacterial debris.
7. Transfer the supernatant to a new tube and purify single-stranded DNA from the phage particles using two spin columns from a QIAprep Spin M13 kit. Elute the DNA from the columns with deionized water.

8. Determine the yield of single-stranded DNA by spectrophotometry ( $1.0 A_{260}$  is equivalent to 33 ng/ $\mu$ L of single-stranded DNA).

### 3.6.2. Generating Oligonucleotide Pools

Mutations are introduced simultaneously in all heavy chain CDRs to repair the engineered stop codons and introduce sequence diversity using the tailored degenerate codons. Oligonucleotide pools of defined composition are generated (**Table 1**).

For CDR-H1, oligonucleotides H1a and H1b are mixed 2:1 (Pool H1).

For CDR-H2, oligonucleotides H2a, H2b, and H2c are mixed 1:2:0.1 (Pool H2).

For CDR-H3, the oligonucleotides encoding sequences of the same length (**Table 2**) are each pooled at equimolar ratios, except for the two shortest lengths that are pooled together. This generates 12 separate pools (Pools H3 1–12) (*see Note 4*). A separate library is constructed for each length of CDR-H3 that is created from these pools (Libraries 1–12). This entails 12 mutagenesis reactions in which pools H3 1, H3 2, H3 3, etc. are individually mixed with Pool H1 and Pool H2 at a 1:1:1 ratio as detailed in **Table 3**.

**Table 3**  
**Individual pools of oligonucleotides for Libraries 1–12**

Library	Oligonucleotide pools			Molar ratio
1	H1	H2	H3 1	1:1:1
2	H1	H2	H3 2	1:1:1
3	H1	H2	H3 3	1:1:1
4	H1	H2	H3 4	1:1:1
5	H1	H2	H3 5	1:1:1
6	H1	H2	H3 6	1:1:1
7	H1	H2	H3 7	1:1:1
8	H1	H2	H3 8	1:1:1
9	H1	H2	H3 9	1:1:1
10	H1	H2	H3 10	1:1:1
11	H1	H2	H3 11	1:1:1
12	H1	H2	H3 12	1:1:1

The oligonucleotide pools H1, H2, and H3 1–12 are mixed according to the table and used for library DNA generation by Kunkel mutagenesis

**3.6.3. Kunkel Mutagenesis**

Standard Kunkel mutagenesis (26) is used to generate each synthetic antibody library (Libraries 1–12).

1. A kinase reaction is set up, preparatory to mutagenesis. Mix 1.5  $\mu$ L aliquots from pool H3 1, H3 2, H3 3, etc. with 1.5  $\mu$ L from pool H1 and 1.5  $\mu$ L from pool H2 (1:1:1 molar ratio).
2. Add 4  $\mu$ L of 10 $\times$  TM buffer, 0.5  $\mu$ L of 100 mM ATP, 2  $\mu$ L of 100 mM DTT, and 2  $\mu$ L T4 polynucleotide kinase (10 U/ $\mu$ L) to each mixture. Add deionized water to a final volume of 40  $\mu$ L. Mix and incubate at 37°C for 45 min. Place on ice.
3. In the next step, an “annealing reaction,” the oligonucleotides are allowed to anneal to the single-stranded library template (**Subheading 3.6.1, step 7**). Add 20  $\mu$ g single-stranded template to the kinase reaction mixture, and 25  $\mu$ L of 10 $\times$  TM buffer. Add deionized water up to 250  $\mu$ L. Incubate at 95°C for 2 min, then at 50°C for 5 min. Place the samples on ice.
4. The last step is a “filling-in reaction” that uses T7 polymerase and T4 ligase. To the 250  $\mu$ L annealing reaction, add 1  $\mu$ L of 100 mM ATP, 15  $\mu$ L of 100 nM DTT, 10  $\mu$ L of 25 mM dNTPs, 6  $\mu$ L T4 DNA ligase (400 U/ $\mu$ L), and 3  $\mu$ L T7 DNA polymerase. Mix and incubate at room temperature for at least 3 h.
5. Prepare a 1% agarose gel in 1 $\times$  TAE buffer. Add EtBr to a final concentration of 0.5  $\mu$ g/mL. Take 0.5  $\mu$ L aliquots of the mutagenesis products and load onto the agarose gel adjacent to the single-stranded template DNA. Run at 110 V for 20 min. Visualize the DNA bands under ultraviolet illumination. The mutagenesis products should appear higher on the gel than the single-stranded template and, after the reaction, three bands are normally visible on the gel, with the bottom band representing the correct, mutated product. The two upper bands are undesired products of the Kunkel mutagenesis reaction, which may lead to the presence of un-mutated template sequences in the final library.
6. Excise the mutagenesis product and purify using a QIAquick Gel Extraction kit using two spin columns per mutagenesis reaction. To elute the purified DNA from the spin columns, add 40  $\mu$ L deionized water and incubate the spin columns for 30 min at room temperature before centrifuging. Collect the eluate and measure the  $A_{260}$ . The total recovery should be at least 20  $\mu$ g.

**3.7. Generation  
of Electrocompetent  
SS320 for Phage  
Production**

1. Plate out *E. coli* SS320, pick a single colony, and grow into exponential phase (**Subheading 3.6.1, step 3**).
2. Set up infections of the bacteria with M13KO7 starting at  $6 \times 10^8$  pfu/mL (*see Note 5*) and, after allowing the infection to take place, mix the infected bacteria with just-molten 2YT top agar and pour to a dry LB agar plate supplemented with



10  $\mu\text{g}/\text{mL}$  tetracycline. Shake to distribute the top layer then incubate overnight for growth at  $37^\circ\text{C}$ .

3. Pick a single plaque and inoculate into 1 mL 2YT supplemented with 5  $\mu\text{g}/\text{mL}$  tetracycline and 25  $\mu\text{g}/\text{mL}$  kanamycin at  $37^\circ\text{C}$  for 4 h. Transfer to 50 mL of medium and grow overnight at  $37^\circ\text{C}$ .
4. Sample 5 mL of the overnight culture and inoculate into two flasks, each containing 900 mL Super broth with 5  $\mu\text{g}/\text{mL}$  tetracycline and 25  $\mu\text{g}/\text{mL}$  kanamycin. Incubate at  $37^\circ\text{C}$ .
5. When the  $\text{OD}_{600}$  reaches approximately 0.6, centrifuge at  $700 \times g$  at  $4^\circ\text{C}$ . Discard the supernatant.
6. All further steps require the cells to be kept on ice. Wash salt from the cells by resuspending the pellet in cold 1 mM HEPES, pH 7.5.
7. Pellet the cells as described previously and repeat the wash step with more cold 1 mM HEPES, pH 7.5.
8. Pellet the cells as described previously and resuspend in cold 1 mM HEPES, pH 7.5, containing 10% (v/v) glycerol.
9. Finally, resuspend the cells in 1 mL 10% (v/v) cold glycerol, aliquot, and snap freeze on dry ice. Store the cells at  $-80^\circ\text{C}$ .

### **3.8. Phage Production and Purification**

1. The 12 mutagenesis reactions are electroporated individually into the electrocompetent *E. coli* SS320 cells harboring M13KO7 helper phage (12). Each reaction should use 10  $\mu\text{g}$  of the mutated DNA template and approximately  $10^{11}$  electrocompetent *E. coli* cells. Approximately  $1\text{--}5 \times 10^9$  transformed clones should be expected from each transformation.
2. Recover all transformed cells from each reaction and grow overnight at  $30^\circ\text{C}$  in 2YT broth supplemented with 50  $\mu\text{g}/\text{mL}$  carbenicillin and 50  $\mu\text{g}/\text{mL}$  kanamycin.
3. Centrifuge the cultures for 10 min at  $24,000 \times g$  and transfer the supernatants to fresh tubes.
4. Harvest phage from the culture supernatants by the addition of 1/5 volume of phage precipitation solution followed incubation on ice for 5 min.
5. Pellet the precipitated phage by centrifugation at  $24,000 \times g$  and resuspend the pellets in 1/20 volume of PBS.
6. Remove insoluble matter by centrifugation at  $24,000 \times g$  and repeat twice to ensure purity.
7. The yields of phage can be estimated by measuring the optical density at 268 nm. 1 OD/mL corresponds to a phage concentration of approximately  $0.5 \times 10^{12}$  particles/mL.
8. Add glycerol to a final concentration of 50% (v/v). The phage libraries should be stored at  $-20^\circ\text{C}$  until they are used for selection (see **Note 6** and **7**).

## 4. Notes

1. For high-efficiency DNA transformation, the *E. coli* strain SS320 was constructed. The strain is generated by mating MC1061 (Sigma-Aldrich) and XL1-blue (Stratagene) and selection on agar containing tetracycline and streptomycin. *E. coli* SS320 retains the high-efficiency transformation qualities of MC1061, while acquiring the F' episome from XL1-blue that is required for bacteriophage infection and propagation (12).
2. IUB code for DNA degeneracy:

N = A/T/G/C	D = A/G/T	V = A/C/G	B = C/G/T
H = A/C/T	K = G/T	M = A/C	R = A/G
S = G/C	W = A/T	Y = C/T	

3. For the XYZ codon, the proportion of nucleotides at each codon triplet is as follows:  
X: 38% G, 19% A, 26% T, 17% C  
Y: 31% G, 34% A, 17% T, 18% C  
Z: 24% G, 76% C (2).
4. The generation of pools facilitates the precise mixing of the different oligonucleotides at specified ratios.
5. The generation of competent *E. coli* SS320 cells harboring M13KO7 helper phage facilitates library production, as there is no need for helper phage superinfection after the electroporation of DNA. In this way, each cell is guaranteed to harbor M13KO7 helper phage.
6. After purification and storage, the phage-displayed Fab libraries are stable at  $-20^{\circ}\text{C}$  for at least a year.
7. Selection can use the 12 libraries individually or as a pooled stock according to preference.

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