

Affinity maturation of antibodies assisted by *in silico* modeling

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Rational engineering methods can be applied with reasonable success to optimize physicochemical characteristics of proteins, in particular, antibodies. Here, we describe a combined CDR3 walking randomization and rational design-based approach to enhance the affinity of the human anti-gastrin TA4 scFv. The application of this methodology to TA4 scFv, displaying only a weak overall affinity for gastrin17 ($K_D = 6 \mu\text{M}$), resulted in a set of nine affinity-matured scFv variants with near-nanomolar affinity ($K_D = 13.2 \text{ nM}$ for scFv TA4.112). First, CDR-H3 and CDR-L3 randomization resulted in three scFvs with an overall affinity improvement of 15- to 35-fold over the parental. Then, the modeling of two scFv constructs selected from the previous step (TA4.11 and TA4.13) was followed by a combination of manual and molecular dynamics-based docking of gastrin17 into the respective binding sites, analysis of apparent packing defects, and selection of residues for mutagenesis through phage display. Nine scFv mutants were obtained from the second maturation step. A final 454-fold improvement in affinity compared with TA4 was obtained. These scFvs showed an enhanced potency to inhibit gastrin-induced proliferation in Colo 320 WT and BxPc3 tumoral cells. In conclusion, we propose a structure-based rational method to accelerate the development of affinity-matured antibody constructs with enhanced potential for therapeutic use.

antibody engineering | gastrin | *in vitro* affinity maturation | pancreatic cancer

The hormone gastrin, which is produced by G cells in the antral mucosa, is considered to be an important growth factor for pancreatic cancer and other gastrointestinal malignancies (1–5). Gastrin is expressed early in the development of gastrointestinal carcinomas (6, 7), and it is being tested as a therapeutic target in pancreatic, gastric, and colorectal cancer (6, 8, 9). An anti-gastrin vaccine is currently in phase III clinical trials (9) for pancreatic cancer. The use of this vaccine has resulted in a significant increase in the survival time of patients.

In previous studies, we prepared a large repertoire of human and mouse antibodies against gastrin (10, 11). Among other neutralizing antibodies, we developed a fully human antibody scFv, TA4, with an overall affinity of $6 \mu\text{M}$ for gastrin, which was able to inhibit Colo 320 WT cell growth by 30% in a gastrin17-dependent proliferation assay.

To date, a variety of different mutagenesis strategies have proven useful to enhance the affinity of candidate therapeutic antibodies obtained by phage display. These strategies vary from substitution of specific selected residues within the complementarity determining region (CDR) loops to random mutagenesis of the entire variable fragment (Fv) sequence (12–15). One of the main problems associated with affinity maturation by phage display is that of library completeness: It is practically unfeasible to generate all possible (combinations of) CDR residue mutants. Therefore, various methods focused on selected CDR loops that are thought to encompass the paratope, i.e., the antibody region in contact with the antigen. CDR-L3 and, especially, CDR-H3 have been intensely studied, because they are usually responsible for most of the stabilizing contacts (16). However, the actual binding site usually

involves multiple CDRs and exact mapping of the paratope is a laborious task. Moreover, it is not guaranteed that substituting any of the contact residues will improve binding. For example, randomization and selection studies often yield substituted residues that are not in contact with the antigen (17). *In vitro* affinity maturation by somatic hypermutation yields, as a rule, mutations that are located in the periphery of the paratope (18). Hence, it is extremely complicated to determine which residues make up the binding site, which of them can be improved, and which peripheral residues should also be considered.

Application of *in silico* analysis and prediction methods to antibody Fv regions may be helpful in a number of ways. In the ideal case where high-resolution antibody structures or, preferably, antibody-antigen complex structures are available, determination of contact residues is straightforward and this information can be applied to guide the maturation process (19). If experimentally determined structures are not available but the paratope has been reliably mapped, a 3D model of the variable domains can be constructed (20) and the residues affecting affinity can be projected onto it (21), thereby facilitating the selection of candidate positions for maturation.

In the present study, 3D experimental structures were not available for gastrin-antibody binding. However, the epitope on gastrin17 had been experimentally determined (11). So, we obtained Fv sequence variation data from the first-round affinity maturation step (this study). Hence, we were confronted with a triple task, i.e., to (i) construct relevant models for the parental scFv TA4 and two first-round variants, (ii) dock at least the epitope fragment of the gastrin17 peptide into the binding sites, and (iii) combine theoretic and experimental information to make semirational proposals for affinity enhancement in a second-round maturation step. To our knowledge, this is the first time that a strategy like this has been successfully followed for the affinity maturation of an antibody. Thus, to increase the binding affinity of the parental TA4 scFv construct, we relied on phage display randomization of CDR-H3 and CDR-L3 and *in silico* procedures [supporting information (SI) Fig. S1A]. The latter included the challenging step of docking an intrinsically flexible peptide into the binding pocket of a modeled antibody Fv structure.

Results

Interactive Docking of gastrin17 to TA4. The binding epitope of gastrin17 to TA4, 4-WLEEEEE-10, was determined by alanine-scanning (11). In view of its strong affinity contribution and explicit

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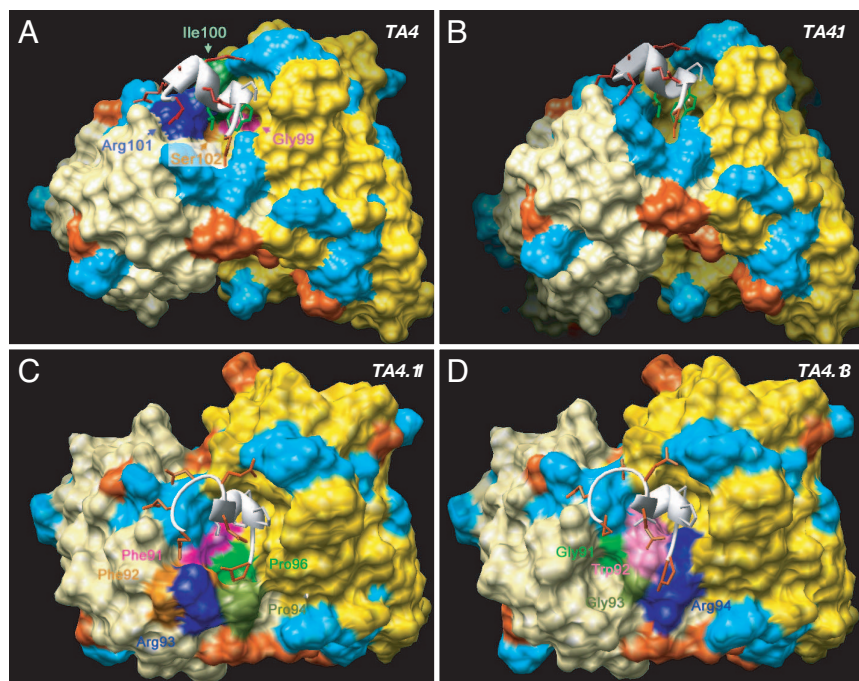


Fig. 1. Modeling of the interaction between gastrin17 and scFvs. (A) TA4 parental anti-gastrin17 scFv. (B) TA4.1 scFv derived from the CDR-H3 round of maturation. (C and D) TA4.11 (C) and TA4.13 (D) scFvs derived from the maturation of the CDR-L3 of TA4.1. The docked gastrin fragment (residues 1–10) is shown by sticks plus a backbone ribbon. The scFv domain is surface-rendered in all images. The gastrin anchor residues 4-WL-5 are represented by green sticks. Red, acidic residues (Asp, Glu); blue, basic residues (Lys, Arg); unsaturated and saturated yellow, other V_L and V_H residues, respectively. Specific colors are used for residues of interest as indicated. The scFv models in C and D are tilted forward by $\approx 90^\circ$ relative to those in A and B.

hydrophobic character, the 4-WL-5 motif was expected to be somehow contained in a hydrophobic pocket formed by the CDR loops. Different 3D structural models were constructed, and a funnel-like pocket was observed, which suggested that the binding of gastrin17 would be largely driven by hydrophobic anchoring, a typical feature of peptidic antibody ligands (22). Moreover, the models showed the presence of an “apical crown” of solvent-exposed Arg residues, suggesting charge complementation with the highly acidic penta-Glu subfragment 6-EEEE-10. Consequently, the 4-WL-5 motif was interactively placed into the central pocket and the penta-Glu fragment was directed as much as possible toward the Arg-rich surface. The interactively docked complex was further refined by a combination of molecular mechanics and molecular dynamics steps. This resulted in a model wherein the gastrin epitope adopts an α -helical conformation.

We found that this predicted binding mode bears strong similarity to a described anti-HIV-1 Env Fab 4E10 (23) (Fig. S2 and Fig. 14). Similar features include the α -helical conformation and orientation, the deep anchoring by a WF motif in a pocket formed by CDR-L3 and CDRs H1-H3, and the anchor residues initiating the helical binding mode.

CDR-H3 and CDR-L3 Affinity Maturation by Random Mutagenesis. The CDR-H3 and CDR-L3 affinity maturation of TA4 scFv was performed by *in vitro* evolution. The use of degenerate oligonucleotides reduces the number of oligonucleotides and introduces a natural evolution in the process (24). In a first step, a phage scFv library of 1×10^6 transformants was constructed by randomizing the four amino acids between positions 99–102 in CDR-H3 (Fig. S1B). Three scFvs, TA4.1, TA4.2, and TA4.3, with improved recognition of gastrin17 by ELISA (data not shown) were obtained after four rounds of phage selection in solution. Analysis by surface plasmon resonance (SPR) showed that TA4.1 and TA4.2 had a 9.3 and 5.6-fold improvement with respect to the parental TA4 scFv affinity ($K_D = 6 \mu\text{M}$; Table 1), respectively. The third scFv, TA4.3, showed no enhanced affinity. The most improved variant (TA4.1) presented two amino acid mutations (V_H I100L and S102V, Table 2). For the TA4.2 scFv, we observed three different substitutions (V_H G99S, I100F, and S102L). Three positions were also changed in the

TA4.3 scFv (V_H G99N, I100K, and R101K); however, these did not lead to any enhanced affinity.

These results can be explained on basis of the 3D model (Fig. 1 A and B). At position V_H 99, a small side chain is required to permit placement of gastrin Trp 4. Ser and Asn, occurring in TA4.2 and TA4.3, fit well and can theoretically form a H-bond linkage to CDR-H1. Residues at position 100 face away from the ligand and various amino acid types should be possible (Ile, Leu, Phe, and Lys were observed). Position 101 is assumed to interact with the acidic part of gastrin (Arg and Lys were observed). Position 102 is an interface residue between V_L and V_H and does not form contact with gastrin in the model. The WT Ser at this position is fully buried and is suitably placed to H-bond to Asn 34, the C-terminal residue of CDR-L1. Substitution of the WT Ser into a hydrophobic residue (Val and Leu were observed) is expected to break the H-link with Asn 34 in V_L . The latter is indirectly supported by the observation of additional substitutions of Asn V_L 34 in the final maturation round (see below and Table 2). The model further suggests that disruption of the H-link permits conformational adaptation of CDR-H3. Thus, this is probably an example of affinity improvement through rearrangements caused by peripheral substitution.

TA4.1 scFv was selected to initiate the second round of matu-

Table 1. Affinity and binding kinetic parameters of wild-type and CDR-H3 and CDR-L3 matured scFvs

scFv	k_{onr} $10^4 \text{ M}^{-1} \text{ s}^{-1}$	k_{offr} 10^{-3} s^{-1}	K_D , nM	Relative improvement respect to	
				TA4	TA4.1
TA4	—	—	6,000*	—	—
TA4.1	5.12	32.9	643	9.3	—
TA4.2	—	—	1,080*	5.6	—
TA4.3	—	—	9,720*	0.7	—
TA4.11	11.1	37.6	339	17.7	1.9
TA4.12	41.6	71.4	172	34.9	3.7
TA4.13	8.37	33.6	402	14.9	1.6

*Binding parameters were fitted according to the Langmuir model, except TA4.2 and TA4.3 scFvs that were fitted according to the steady state model.

Table 2. Sequences of anti-gastrin17 clones derived from the random CDR-H3 and CDR-L3 and the punctual maturation libraries

scFv	CDR-H3							CDR-L1											CDR-L3								
	99	100	101	102	103	104	105	24	25	26	27	28	29	30	31	32	33	34	89	90	91	92	93	94	95	96	97
TA4	G	T	R	S	F	D	Y	R	A	S	Q	S	I	S	S	Y	L	N	Q	Q	H	G	R	R	P	V	T
TA4.1		L		V																							
TA4.2	S	F		L																							
TA4.3	N	K	K	S																							
TA4.11		L		V																	F	F		P		P	
TA4.12		L		V																	F	F	L	P		P	
TA4.13		L		V																	G	W	G			A	
TA4.111		L		V																	F	F		P		T	
TA4.112		L		V																	F	F		P		A	
TA4.113		L		V																	F	F		P		V	
TA4.114		L		V																	F	F		P		I	
TA4.115		L		V														A			F	F		P		P	
TA4.131		L		V														A			G	W	G			A	
TA4.132		L		V														M			G	W	G			A	
TA4.133		L		V																	A	W	G			A	
TA4.134		L		V														G			G	W	G			A	

In grey, amino acid positions where no mutation was observed. In color are shown the amino acid positions that were mutated during the maturation process.

ration based on CDR-L3 mutagenesis. A new library was created by randomization of the five amino acids located at positions 91–94 and 96 of CDR-L3. Pro 95 was kept invariable in the library. The resulting phage library contained 5×10^6 different transformants (Fig. S1B). Three scFvs, TA4.11, TA4.12, and TA4.13, were obtained after four rounds of selection. They showed an improved recognition of gastrin17 by ELISA (data not shown). The scFvs were purified to homogeneity and tested for affinity by SPR. They presented 1.6- to 3.7-fold higher affinity than TA4.1 (Table 1). These mutants displayed a high hydrophobic content, with occurrence of Phe and Trp at positions 91 and 92 and Leu and Gly at position 93 in CDR-L3 (Table 2). The occurrence of Pro at positions 94 and 96 produced a motif of three consecutive prolines in two of three scFv sequences (Table 2).

The 3D model of the TA4-gastrin complex showed an obvious packing defect in the contact region between CDR-L3 and the primary gastrin anchor residues 4-WL-5. Thus, it was encouraging to observe that high-affinity mutants of residues His 91 and Gln 92 showed general preference for bulky side-chains (Tables 1 and 2). The matured sequences provide two different solutions to the packing problem. First, TA4.11 and TA4.12 contained the substitution H91F in combination with R94P. This is structurally explainable (Fig. 1C) because the fully buried His 91 points toward the hydrophobic gastrin anchor residues but is too small for making optimal van der Waals contacts. At the same time, Arg 94 interacts with the gastrin17 pyroGlu residue and partially holds CDR-H3 in an open (and probably hydrated) state. Cosubstitution of H91F and R93P solves the problem of loose packing by allowing CDR-H3 to close up around the anchors. Residues at position 93 point straight into solvent and were expected to be less contributive (Arg and Leu observed). Position 92 side chains are covered by CDR-L1, which explains the preference for the nonpolar residue Phe. Finally, position 96 is deeply buried in the complex and the model suggests that many hydrophobic side chains can optimally interact with Trp 4 of gastrin. Indeed, apart from WT Val, we observed the presence of Pro in this maturation round (and Thr, Ala, Val, and Ile in the final round, as described below).

The other mutant, TA4.13, partially conflicted with the previous reasoning. Residues 91–93 were found to have the striking sequence GWG, followed by WT Arg. This mutant provides strong evidence

that the packing problem observed for the WT complex can also be resolved in an alternative way, i.e., by rotating the bulky Trp at position 92 inward into the binding pocket (Fig. 1D). This requires major conformational changes in the flanking residues, which is probably why the latter were identified as Gly. Given the bulky nature of a Trp side chain, we also speculated that the CDR-H3 loop would in this case not need to close up on gastrin, which explains why WT Arg was conserved at position 94.

In Silico-Guided Affinity Maturation of TA4.11 and TA4.13 scFvs.

TA4.11 and TA4.13 mutants (Fig. 1 C and D, respectively) were selected for further maturation. Although TA4.12 possessed the highest affinity, it was discarded because of its strong hydrophobicity. To locate potential remaining deficiencies in local interactions, a systematic, interactive survey was conducted on all CDR residues in both models. All residues that were presumed suboptimal were given a priority rank 1 (“high”). To include some controls and because molecular modeling can be error-sensitive, a comparable number of residues showing no obvious defects but still

Table 3. Proposed and observed amino acid mutations for *in silico*-guided maturation

scFv	Location	Priority*	Amino acid	Suggested mutations	Observed mutations
TA4.11	CDR-L1	1	N 34	H, E, A, T, F	A
		1	Q 89	L, M, V, H, A, S	—
	CDR-L3	2	F 91	Y, W	—
		1	P 96	F, Y, L, M	T, A, V, I
		2	T 50	M, V, I, Q	—
		2	I 59	T, V, L, M, Q, E	—
	CDR-H2	2	L 100	Polar	—
	CDR-H3	2	L 100	Polar	—
	CDR-L1	1	N 34	H, L, A, T, F, E	A, M, Q
		2	Q 89	L, M, V, H, A, S	—
TA4.13	CDR-L3	1	G 91	A, S	A
		2	I 59	T, V, L, M, Q, E	—
	CDR-H2	2	I 59	T, V, L, M, Q, E	—
	CDR-H3	2	L 100	Polar	—
		2	L 100	Polar	—

*All residues that were presumed suboptimal were given a priority rank 1 or 2. Priority 1, high priority, i.e., those residues showing clear packing deficiencies; priority 2, low priority or uncertain, i.e., those residues showing no obvious defects but considered as potentially critical for binding.

Table 4. Affinity and binding kinetic parameters for punctual scFv mutants

scFv	k_{on} , $10^6 \text{ M}^{-1}\text{s}^{-1}$	k_{off} , 10^{-3}s^{-1}	K_D , nM	Relative improvement respect to	
				TA4 (WT)	Parental scFv
TA4.111	0.54	11.8	21.8	275.2	15.6*
TA4.112	1.78	23.6	13.2	454.5	25.7*
TA4.113	1.69	34.7	20.6	291.3	16.5*
TA4.114	0.39	24.1	61.7	97.2	5.5*
TA4.115	0.42	17.1	60.8	98.7	5.6*
TA4.131	0.64	29.1	45	133.3	8.9†
TA4.132	0.58	29.9	51.2	117.2	7.9†
TA4.133	0.69	45.9	66.5	90.2	6.1†
TA4.134	0.72	42.9	69.8	85.9	5.8†

*Parental scFv TA4.11.
†Parental scFv TA4.13.

considered as potentially critical were selected as well; these were assigned priority rank 2 (“uncertain”). For each position, a list of suggested mutations was elaborated (Table 3).
The site-specific amino acid substitutions were performed by constructing two different phage libraries based on the respective TA4.11 and TA4.13 sequences. For a faster procedure, we decided to randomly mutate selected positions by using degenerated oligonucleotides with the NNS motif, which allowed us to incorporate all natural amino acids at each position, select by phage display the best anti-gastrin17 scFv binders, and compare the experimental results with the theoretical approach. The library sizes were 1.2×10^7 and 2.3×10^7 for TA4.11 and TA4.13, respectively (Fig. S1B). Equal

amounts of those libraries were mixed and four rounds of selection were carried out in solution, using streptavidin-coated magnetic beads. Nine different scFv mutants were obtained from the fourth round of selection (Table 4). Five distinct scFvs were obtained from the TA4.11 library and four were obtained from the TA4.13-based library. Interestingly, we only observed amino acid substitutions on those positions that were previously assigned priority one, at positions 34 and 96 of CDR-L1 and CDR-L3, respectively (Table 3). The substitution of Pro 96 in CDR-L3 was found in all mutants except one. Mutations P96T, P96A, and P96V in CDR-L3 increased the affinity by a factor of 15.6–25.7 with respect to the parental TA4.11, whereas P96I in CDR-L3 and N34A in CDR-L1 increased the affinity 5.5 and 5.6-fold, respectively. For the TA4.13 scFv-derived mutants, the increase in affinity ranged between 5.8 and 8.9-fold (Table 4). The most improved scFv was TA4.131 with the substitution CDR-L3 N34A (Tables 2 and 4). In all cases, the k_{off} of the punctual mutants derived from the TA4.11 and TA4.13 remained relatively constant, while the best improvements were obtained for the association rate constants.

gastrin Neutralization by Mutant scFvs. All of the scFv variants were produced in the HB2151 *Escherichia coli* strain and the monomers were purified by two consecutive chromatographic steps, using IMAC and size-exclusion (Fig. S3A). All of the scFvs showed a significant and progressive enhancement in the binding to gastrin17 along the maturation process (Fig. S3B). Then, we tested the neutralizing activity of the affinity-improved scFvs (Fig. 2). Although parental TA4 showed a 30% inhibition of gastrin-derived proliferation in Colo 320 WT cells and almost no inhibition in BxPc3 cells, the optimized scFvs were able to block gastrin-induced proliferation up to 60% in Colo 320 WT (Fig. 2A) and 45% in BxPc3 (Fig. 2B). For the pancreatic tumoral cell line BxPc3, the best neutralizing scFvs were TA4.112 and TA4.131, which showed the

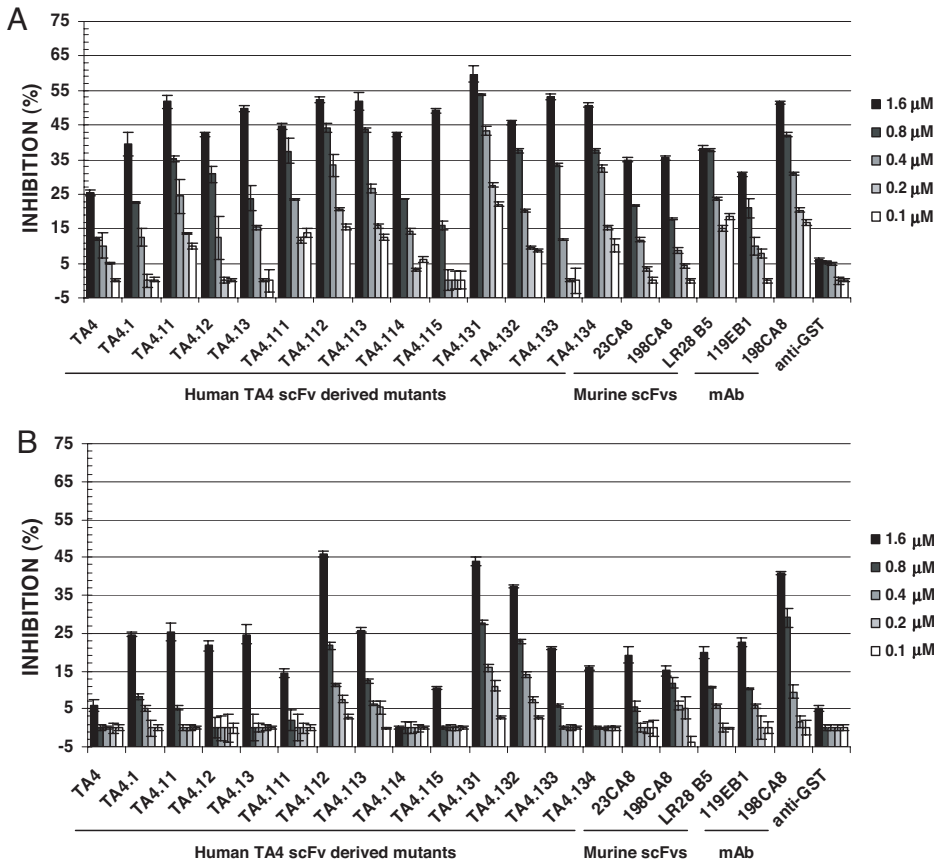


Fig. 2. *In vitro* gastrin-dependent cell proliferation assay. (A) Colo 320 WT colorectal tumor cells were incubated with 0.5 nM gastrin17 and twofold serial dilutions of human TA4-derived scFvs in serum-free RPMI, antibiotics and G418. (B) BxPc3 pancreatic adenocarcinoma cells were incubated in serum-free DMEM plus antibiotics with 10 nM gastrin17 in the same conditions as in A. In both cases, after 72 h, the cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 570 nm and represented as inhibition (%). Absorbance of the untreated control cells was taken as 100% of cellular growth and the inhibition of the cellular growth calculated according to the following formula: (relative growth of untreated cells – relative growth of treated cells)/relative growth of untreated cells $\times$ 100. Each column is the average of three independent cell proliferation experiments (each concentration tested in duplicate). Error bars indicate SD. The human scFvs were tested in comparison with anti-gastrin17 murine scFvs obtained from *in vivo* immunization (23CA8, 198CA8, and LR28 B5) or with anti-gastrin17 mAbs (119EB1 and 198CA8).

highest affinities in their series and were suggested by the *in silico* modeling. At equal concentrations, the neutralization capacity of TA4.112, TA4.131, or TA4.132 scFvs were similar to or higher than those obtained with the best anti-gastrin17 murine scFvs and mAbs obtained by *in vivo* immunization (11).

Discussion

Antibodies are increasingly being used in cancer therapy (25). A growing number of antibodies are being applied for the treatment of neoplasias with high success (26). The application of technologies such as phage display for the preparation of human antibodies has paved the way for this success. However, the initial affinities of these antibodies are typically too low for therapeutic application. High affinity and selectivity are critical issues for antibody therapeutic capacity. Many different approaches have been reported for improving the affinity of scFvs derived from phage antibody libraries, including error-prone PCR (27), CDR walking (28), hot-spot mutagenesis (29), parsimonious mutagenesis (30), etc. None of them is based on a rational approach to accelerate and direct the maturation process.

For this study, we investigated the combination of two approaches: CDR-H3 and CDR-L3 consecutive walking mutagenesis (24) followed by modeling-guided specific mutagenesis of interacting residues. The saturation mutagenesis was limited to CDR-H3 and CDR-L3, because most of the binding energy is usually contributed by these two CDR loops (31). This stepwise strategy was carried out by using phage selection in solution followed by capture on streptavidin-coated magnetic beads. This was demonstrated (31) to result in the isolation of higher affinity monomeric scFv. Also, the use of lower antigen concentration in the selection phase elicits the largest enrichment of high-affinity binders.

Part of our strategy relied on the knowledge of the epitope sequence of the gastrin17 antigen. It was possible to model the structure of various complexes and to identify a number of suboptimal interactions. Knowledge of the epitope should not be a limiting step in this process for there exist several validated epitope mapping methods such as Pepscan (32), Ala-scan (33), phage display libraries (34), etc. Given this information, one can attempt to find a structural match between antigen and antibody by using an appropriate docking method (20). In the present study, this task was particularly complicated because the antigen consisted of a soluble flexible peptide. However, the mapping profile (11) suggested that binding would be dominated by the hydrophobic 4-WL-5 motif in combination with electrostatic complementation of the penta-Glu segment. Interestingly, the TA4 scFv model showed a “natural” complementarity, i.e., a wide central pocket and a crown of positively charged residues. This led to the hypothesis that the 4-WLEEEEE-10 epitope fragment assumes a helical conformation in the complex, and the peptide was docked accordingly. The model was supported by the ability to find a structure-based rationalization for the initial maturation results. Further confidence arose from the *a posteriori* observation that the antibody 4E10 recognizes a helical conformation of a viral membrane-proximal HIV-1 gp41 fragment, where the epitope is similarly anchored through a WF motif (23). All of the improved mutants from the final maturation round were located at positions marked formerly with the highest substitution priority, suggesting that the structure is essentially correct. The conservation of residue Q89 in CDR-L3, the only priority-1 position for which no mutations were observed (Table 3), can be explained by the (underestimated) importance of a double intrachain H-bond. We also generally overestimated the available space for residue packing in the complex, because most observed mutants were smaller in size than the predicted ones.

If reliable complex structures are available, molecular modeling-assisted maturation has a number of advantages over “blind” random mutagenesis. A first advantage is that variation can be confined to a few selected positions only. This should increase the probability to identify “gain-of-function” mutants, where “func-

tion” can involve affinity but also stability and solubility. With random mutagenesis, error-prone PCR or ribosome display, mutations will be scattered over different CDR loops, some of which can be irrelevant for binding, yet introducing undesired properties, i.e., antigenicity and immunogenicity (35). Second, when targeting specific structurally distant regions (in our case, the consecutive variations in CDR-H3, CDR-L3, and selected buried and surface positions), a fair degree of additivity can be achieved, as shown in this work. Third, the mutagenesis process can be sped up by parallel testing of small libraries. The low complexity of these libraries, compared with larger random libraries, can also reduce the risk of false negatives, i.e., the failure to retrieve improved variants because of incompleteness of the library or a suboptimal display/selection procedure. A nice example of this is that various improved V_L P96 mutants were identified from the structure-based maturation round, despite the fact that these should theoretically have emerged from the random maturation of CDR-L3 (TA4.1x mutants).

The overall improvement in affinity for the best optimized scFvs was found to be 454-fold compared with the parental scFv, going from micromolar to near-nanomolar affinity, which is the habitual affinity range for therapeutic antibodies (36, 37). We were able to increase the affinity in three consecutive rounds by 5.6 to 9.3 (V_H), 1.6 to 3.7 (V_L), and 5.8- to 25.7-fold (structure-based). Thus, the *in silico* maturation step yielded the most efficient affinity improvement, especially in view of the fact that the parental molecules had already been significantly enhanced. We observed that substitutions in the V_H chain provoked higher affinity binders when compared with V_L in the CDR walking mutagenesis and just the opposite in the *in silico*-guided maturation.

The gastrin-neutralizing capacity of several mutants and, especially, the highest affinity variants TA4.112 and TA4.131, increased significantly, as reflected in the capacity to inhibit the proliferation of the tumoral cell lines Colo 320 WT or BxPc3 cells. The matured scFvs compared favorably with the neutralization ability of hybridomas produced from mice that were immunized with gastrin coupled to diphtheria toxin, thereby confirming the potential of this approach. In summary, we have described a stepwise affinity maturation method that substantially improved the affinity, from the micromolar to the low-nanomolar range, of a potential therapeutic antibody for the treatment of gastrointestinal malignancies susceptible to the trophic effect of gastrin molecules. Further studies are needed to test the effect of these scFv variants on *in vivo* gastrin neutralization.

Materials and Methods

Peptides and Antibodies. Gastrin17 (pEGPWLEEEEEAYGWMDF-NH₂, in which pE is pyroglutamic acid); cysteine-extended gastrin17 (pEGPWLEEEEEAYGWMDFC-NH₂ and Ac-QGPWLEEEEEAYGWMDF-NH₂); and various forms of biotinylated gastrin, alone and/or conjugated to diphtheria toxin (DT) were provided by Pepscan. Mouse anti-c-myc mAb (clone 9E10) was purchased from Sigma. Horseradish peroxidase-conjugated anti-c-myc (clone 9E10) was purchased from Roche.

Anti-gastrin17 human scFv TA4 was selected from the tomlinson I+J libraries (10). It contained a TAG stop codon in the CDR-H2 of the heavy chain and an overall affinity with K_D = 6 μM. Before maturation, the amber TAG stop codon was mutated to a CAG codon by PCR, using the primers MutTA4 sense, Mut TA4 antisense, LMB3, and pHENseq (Table S1).

Semiautomated Antibody Fv Model Construction. Structural models for the antibody Fv domains of the scFvs TA4, TA4.1, TA4.11, and TA4.13 were generated by a proprietary, semiautomated tool for antibody construction (ABC). The ABC method is primarily based on standard homology modeling techniques and consists of two main parts: (i) the selection of suitable framework region (FWR) and CDR template fragments from the Protein Data Bank (PDB), and (ii) the actual model building and energetic optimization. The selection of suitable template structures is based on scores that take into account fragment sequence similarity, FWR-CDR compatibility, and crystallographic resolution. Candidate template structures for V_L and V_H are scored independently, and the best-ranked V_L and V_H templates are selected for assembly by default. Optionally, V_L and V_H templates can be constrained to the same antibody structure from the PDB. For the CDRs, all

templates with the highest sequence identity and different sequence are selected, with a maximum of 5. If no candidate CDR templates of the correct length are available, the user has to select manually one or more template structures from a list of proposals (not applicable in this work). Optionally, every FwR and CDR fragment can be selected manually, but we used the default settings here: We selected one and the same template structure for V_L and V_H , the single best-ranked template for CDRs L1, L2, H1, and H2 and the five best-ranked templates for CDRs L3 and H3. Finally, an overview table of selected PDB templates, sequences, and highlighted substitutions to be performed are output, together with the commands required for 3D structure building.

The actual modeling steps have been performed with the Brugel modeling package (38). In brief, template structures are superimposed and recombined after retrieval from a local standardized antibody structural database. Different combination models are constructed if different CDR templates had been selected (in this work, $5 \times 5 = 25$ different CDR-L3 and -H3 combinations in the context of a single template structure for the FwR plus other CDRs). The amino acid substitutions required to obtain the correct target sequence are initially introduced with standard side-chain conformations. In the final step, all models are energy-optimized, first by global side-chain conformation optimization, using the FASTER algorithm (39), then by 200 steps of conjugate gradient energy minimization.

Docking of gastrin to Constructed scFv Models. Molecular docking of gastrin onto selected scFv models was accomplished by an iterative process of manual predocking of hypothetical anchor residues (gastrin17 residues 4-WL-5), followed by chain extension under control of standard energy refinement methods (conjugate gradient minimization and short molecular dynamics simulations). This combination method was chosen because interactive manual docking allows gaining structural insights and generates reasonable starting structures, whereas energy optimization methods, in particular MD simulations, can efficiently explore a local search space (SI Text).

Targeted TA4 scFv Libraries. Two libraries were constructed for sequential randomization of CDR-H3 and CDR-L3, using degenerated oligonucleotides with the NNS motif (N, any nucleotide; S, guanine and cytosine) (40). The first library was constructed by mutating the four residues -GIRS- at CDR-H3

located at positions 99–102 (numbered according to Kabat) (41). The second library was prepared to randomize CDR-L3 residues 91–94 and 96 of the TA4.1 mutant. The oligonucleotides were designed to permit any amino acid at each position while decreasing the presence of stop codons and cysteines. The flow chart of the maturation process and the PCRs performed to construct the libraries are summarized in Fig. S1 A and B, respectively. PCRs were performed by using the KOD polymerase (Novagen).

The *in silico*-guided maturation process was carried out by building two small libraries designed for punctual amino acid mutagenesis. This maturation step was performed on the scFv TA4.11 at positions Asn 34 (CDR-L1), Gln 89, Phe 91 and Pro 96 (CDR-L3), Thr 50 and Ile 59 (CDR-H2), and Leu 100 (CDR-H3) and on the scFv TA4.13 at positions Asn 34 (CDR-L1) and Gln 89 and Gly 91 (CDR-L3), Ile 59 (CDR-H2), and Leu 100 (CDR-H3). The oligonucleotides used for building the libraries are shown in Table S1.

Phage Display Selections. Phage selections were performed in solution, using streptavidin-coated magnetic beads (Invitrogen) and C-terminal biotinylated gastrin17 as described in ref. 11. For the CDR-H3 library, the gastrin17 concentration used varied from 1 μ M to 1 nM and for the CDR-L3 library from 100 nM to 100 pM. In the case of the punctual mutation libraries, we used 10 nM to 1 pM. The washes in the fourth round of selection were performed 10 times during 20 min in PBS containing 0.1% Tween 20. Elution, infection and production of phages for other rounds of panning were performed as described in ref. 42. The human scFvs were produced and isolated according to protocols established in refs. 42 and 43. The binding affinities of human anti-gastrin17 monoclonal recombinant antibodies were determined by surface plasmon resonance (SPR), using a Biacore X (11).

gastrin-Dependent Colo 320 WT and BxPc3 Cell Proliferation Assays. Proliferation assays to determine the inhibitory activity of the matured scFv mutants on Colo 320 WT and BxPc3 cells were carried out as described in ref. 11.

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Supporting Information

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SI Text: Docking of gastrin to Constructed scFv Models

Interactive predocking of the 4-WL-5 motif into the central pocket observed in the TA4 model was initially complicated by the wide-open space between the CDRs. However, we could exploit in this case the experimental finding that the entire penta-Glu fragment of gastrin forms part of the epitope. It was observed that five Arg residues are organized in a crown-like fashion at the apex of the TA4 model and we hypothesized that a helical conformation would ideally fit in between. Besides providing an explanation for the large central pocket in TA4 and its highly basic character, the polyGlu segment of gastrin17 also has a high helical propensity and the preceding Pro is a good helix initiator [Chou PY, Fasman GD (1974) Conformational parameters for amino acids in helical, beta-sheet, and random coil regions calculated from proteins. *Biochemistry* 13:211–222]. Therefore, the gastrin epitope sequence 4-WLEEEEE-10 was manually docked in an idealized helical conformation. This brought 4 of the 5 Glu residues (positions 7–10) in close proximity of the apical Arg residues and permitted full burial of

the WL-motif in a strainless conformation. This binding mode was considered well suited for further chain extension in the next step.

The selected binding modes from the manual docking experiments on TA4 were completed by the aid of short (20 ps), partially restrained MD runs (applying soft force constants to the scFv main-chain, side-chain and gastrin anchor main-chain atoms). The purpose of these simulations was not to reach energetic equilibrium but to observe spontaneous conformational drifts. Having added the missing flanking segments (1-pEGP-3 for TA4), their conformation was gradually adjusted by performing another MD simulation, followed by interactive inspection of the simulation path, picking one or more snapshot structures, energy minimizing them, analyzing different aspects of packing quality (with special focus on H-bond saturation), and restarting the process from a selected structure. Only two such cycles were judged necessary to obtain the final model for the TA4 complex. The main adjustments during TA4 simulation were the optimization of H-bond networks for all gastrin glutamic acids.



B		PCR1		PCR2		Overlapping extension		Library size
Library	DNA	oligonucleotides	DNA	oligonucleotides	DNA	oligonucleotides		
CDR3 V _H	TA4	LMB3 anti CDR3 _{VH}	TA4	pHENseq NNS CDR3 _{VH}	PCR1 PCR2	LMB3 pHENseq		1.0x10 ⁶
CDR3 V _L	TA4.1	LMB3 NNS CDR3 _{VL}	PCR1	LMB3 RE CDR3 _{VL}	No	No		5x10 ⁶
TA4.11 punctual mutation library	TA4.11	pHENseq 5H N34 sense	TA4.11	LMB3 5H N34 antisense	No	No	1.2x10 ⁷	
	TA4.11	LMB3 5H Q89 antisense	PCR1	LMB3 5H Q89 reantisense	No	No		
	TA4.11	LMB3 5H F91 reantisense	PCR1	LMB3 5H F91 reantisense	No	No		
	TA4.11	LMB3 5H P96 antisense	PCR1	LMB3 5H P96 reantisense	No	No		
	TA4.11	LMB3 5H T50 antisense	TA4.11	pHENseq 5H T50 sense	PCR1 PCR2	LMB3 pHENseq		
	TA4.11	LMB3 5H I59 antisense	TA4.11	pHENseq 5H I59 sense	PCR1 PCR2	LMB3 pHENseq		
	TA4.11	LMB3 5H L100 antisense	TA4.11	pHENseq 5H L100 sense	PCR1 PCR2	LMB3 pHENseq		
TA4.13 punctual mutation library	TA4.13	LMB3 9G N34 antisense	TA4.13	pHENseq 9G N34 sense	PCR1 PCR2	LMB3 pHENseq	2.3x10 ⁷	
	TA4.13	LMB3 9G Q89 antisense	PCR1	LMB3 9G Q89 reantisense	No	No		
	TA4.13	LMB3 9G G91 antisense	PCR1	LMB3 9G G91 reantisense	No	No		
	TA4.13	LMB3 9G I59 antisense	TA4.13	pHENseq 9G I59 sense	PCR1 PCR2	LMB3 pHENseq		
	TA4.13	LMB3 9G L100 antisense	TA4.13	pHENseq 9G L100 sense	PCR1 PCR2	LMB3 pHENseq		

Fig. S1. Scheme of the scFv TA4 maturation process. (A) Flowchart of the process to get improved TA4-derived anti-gastrin17 recombinant antibodies. (B) Summary of the PCRs required to build the libraries, with indication of the final size.

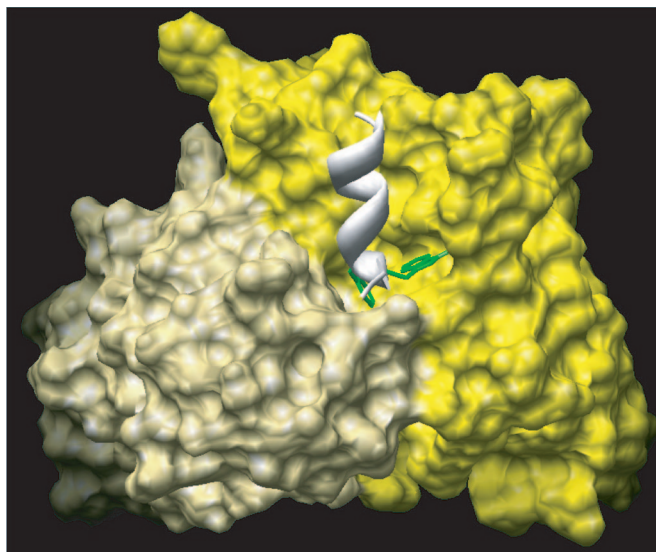


Fig. S2. Binding mode of the HIV-1 gp41 epitope (residues 669–680) in complex with the HIV-1 neutralizing human Fab 4E10. The variable domains V_L and V_H are surface rendered in unsaturated and saturated yellow, respectively. The epitope backbone is depicted as a gray ribbon. The anchor residues 672-WF-673 are represented by green sticks.

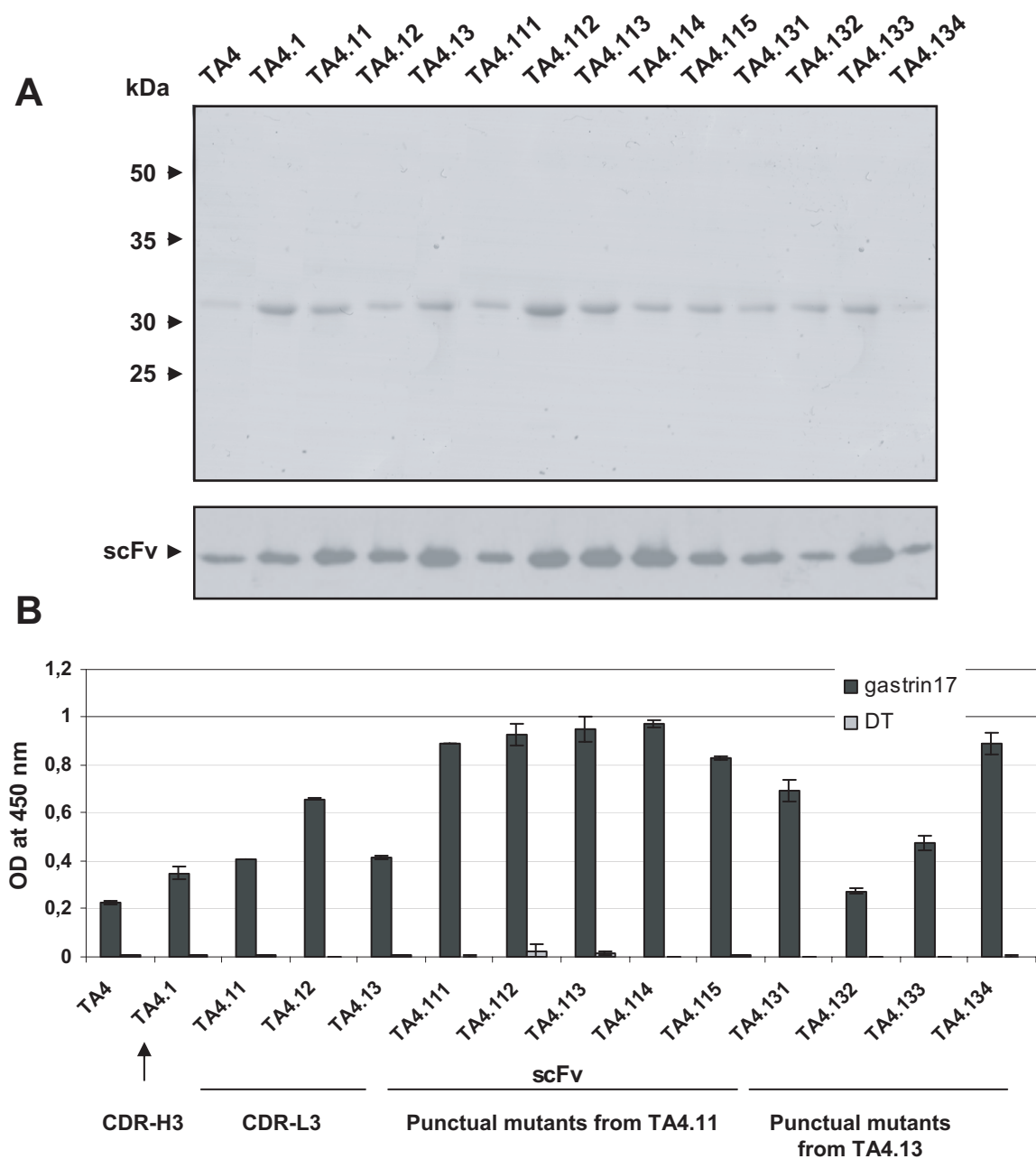


Fig. S3. Characterization of human anti-gastrin17 mutants. (A) Human scFv mutants were expressed in *E. coli*, purified by IMAC and size-exclusion chromatography and then analyzed by Coomassie blue staining (Upper) and Western Blot (Lower), using an anti-c-myc peroxidase-labeled antibody. (B) ELISA values obtained for 100 ng of purified scFv, using gastrin17 as antigen. The color was developed using TMB and stopped with 1M H₂SO₄. The Optical Density was measured at 450 nm.

Library	Primer	Oligonucleotide sequence
CDR-H3	NNS_CDR3.VH anti_CDR3.VH	GAC ACG GCC GTA TAT TAC TGT GCG AAA NNS NNS NNS NNS TTT GAC TAC TGG GGC CGC ACA GTA ATA TAC GGC CGT GTC
CDR-L3	NNS_CDR3.VL	TTT GAT TTC CAC CTT GGT CCC TTG GCC GAA CGT SNN AGG SNN SNN SNN SNN CTG TTG ACA GTA GTA
TA4.11 punctual mutation library	Rean_CDR3.VL	ATG ATG ATG TGC GGC CGC CCG TTT GAT TTC CAC CTT
	5H_N34_sense	ATT AGC AGC TAT TTA NNS TGG TAT CAG CAG AAA
	5H_N34_antisense	TAA ATA GCT GCT AAT
	5H_Q89_antisense	GGT CCC TTG GCC GAA CGT GGG AGG GGG GCG GAA GAA CTG SNN ACA GTA GTA AGT TGC
	5H_Q89_reantisense	ATG ATG ATG TGC GGC CGC CCG TTT GAT TTC CAC CTT
	5H_F91_antisense	GGT CCC TTG GCC GAA CGT GGG AGG GGG GCG GAA SNN CTG TTG ACA GTA GTA
	5H_F91_reantisense	ATG ATG ATG TGC GGC CGC CCG TTT GAT TTC CAC CTT
	5H_P96_antisense	GGT CCC TTG GCC GAA CGT SNN AGG GGG GCG GAA GAA
	5H_P96_reantisense	ATG ATG ATG TGC GGC CGC CCG TTT GAT TTC CAC CTT
	5H_T50_sense	CTG GAG TGG GTC TCA NNS ATT GGG AGG CGT GGT
	5H_T50_antisense	TGA GAC CCA CTC CAG
	5H_I59_sense	CGT GGT CAT CAG ACA NNS TAC GCA GAC TCC GTG
	5H_I59_antisense	TGT CTG ATG ACC ACG
	5H_L100_antisense	CCC TTT CGC ACA GTA
TA4.13 punctual mutation library	5H_L100_sense	TAC TGT GCG AAA GGG NNS CGG GTG TTT GAC TAC
	9G_N34_sense	ATT AGC AGC TAT TTA NNS TGG TAT CAG CAG AAA
	9G_N34_antisense	TAA ATA GCT GCT AAT
	9G_Q89_antisense	GGT CCC TTG GCC GAA CGT CGC AGG CCG CCC CCA CCC CTG SNN ACA GTA GTA AGT TGC
	9G_Q89_reantisense	ATG ATG ATG TGC GGC CGC CCG TTT GAT TTC CAC CTT
	9G_G91_antisense	GGT CCC TTG GCC GAA CGT CGC AGG CCG CCC CCA SNN CTG TTG ACA GTA GTA
	9G_G91_reantisense	ATG ATG ATG TGC GGC CGC CCG TTT GAT TTC CAC CTT
	9G_I59_sense	CGT GGT CAT CAG ACA NNS TAC GCA GAC TCC GTG
	9G_I59_antisense	TGT CTG ATG ACC ACG
	9G_L100_antisense	CCC TTT CGC ACA GTA
Common primers	9G_L100_sense	TAC TGT GCG AAA GGG NNS CGG GTG TTT GAC TAC
	pHENseq	CTA TGC GGC CCC ATT CA
	LMB3	CAG GAA ACA GCT ATG AC
Mutagenesis human TA4 scFv	MutTA4 sense	GGG AGG CGT GGT CAT CAG ACA ATT TAC GCA GAC
	Mut TA4 antisense	AGA ACC ACG CCT CCC AAT CGT TGA GAC