Supplemental Material to:

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Deep mutational scanning of an antibody against epidermal growth factor receptor using mammalian cell display and massively parallel pyrosequencing

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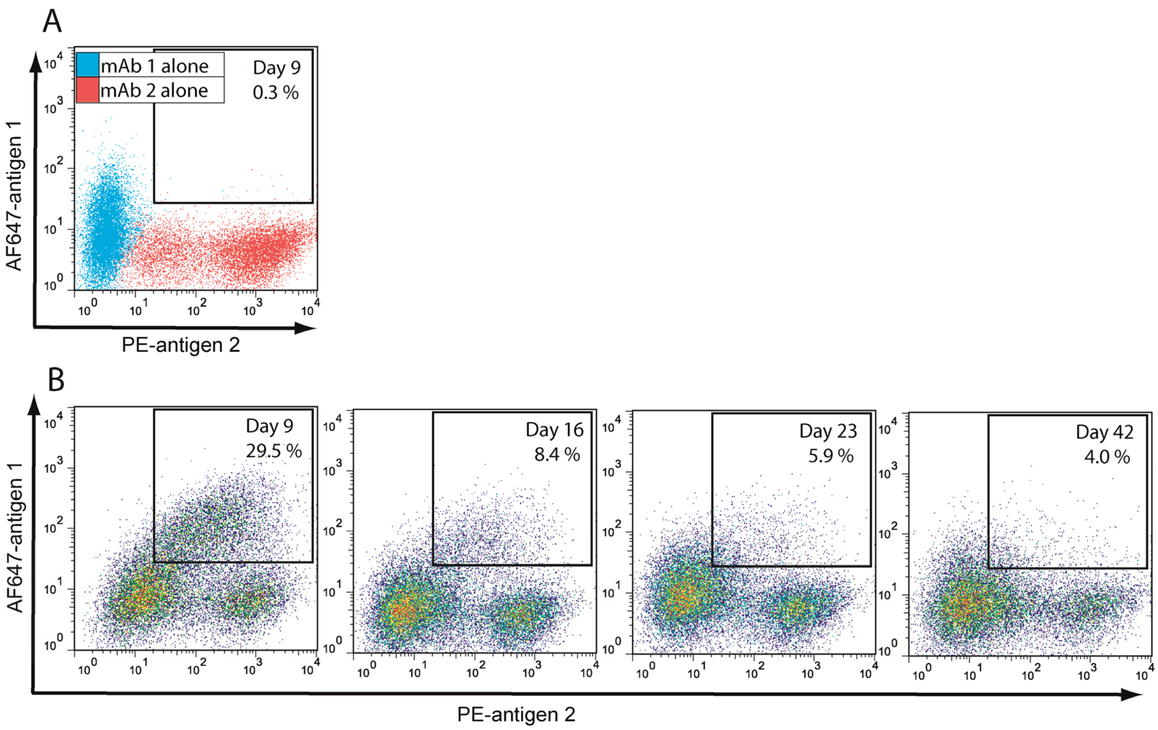
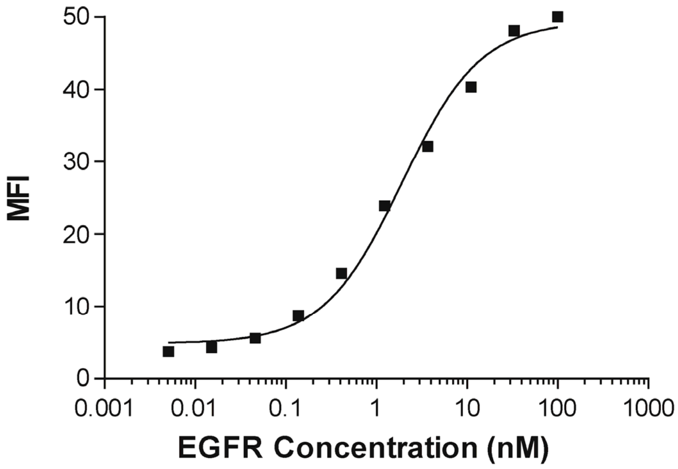
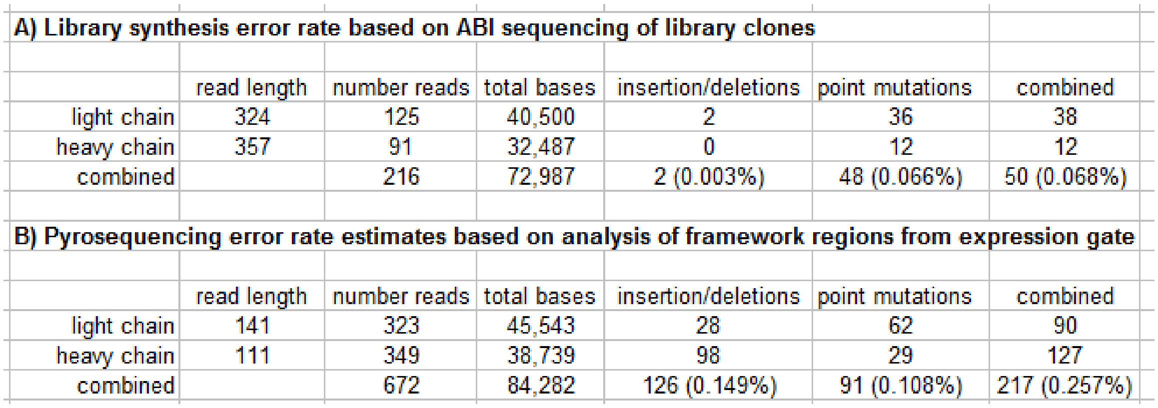


Supplemental Figure S1. Plasmid segregation over time to ensure every cell expressed a single clonal variant. Plasmids displaying two different antibodies are mixed equally and transformed into 293c18. Cells are removed from culture at various time points post-transfection and stained with a mix of the two antigens directly labeled with different fluorescent tags. Panel A shows cells expressing only one plasmid and incubated with 5nM AF647-antigen 1 and 5nM phycoerythrin (PE)-labeled antigen 2 on day 9. The percent double positive in the gate is show below the staining day. Antibody 1 has a lower surface expression level as compared to antibody 2, resulting in a reduced binding to antigen 1 and a lower fluorescence intensity. Panel B shows the mix of two antibodies segregating over time when incubated with both antigens on day 9, 16, 23, and 42.



Supplemental Figure S2. FACS titration of displayed hu225 wild type. Cells expressing hu225 wild type were incubated with AF647-EGFR-Cλ and phycoerythrin (PE)-labeled anti-human kappa to normalize for IgG expression, then analyzed by FACS. K_D=1.9 nM (95% confidence interval of 1.3nM - 2.8nM).



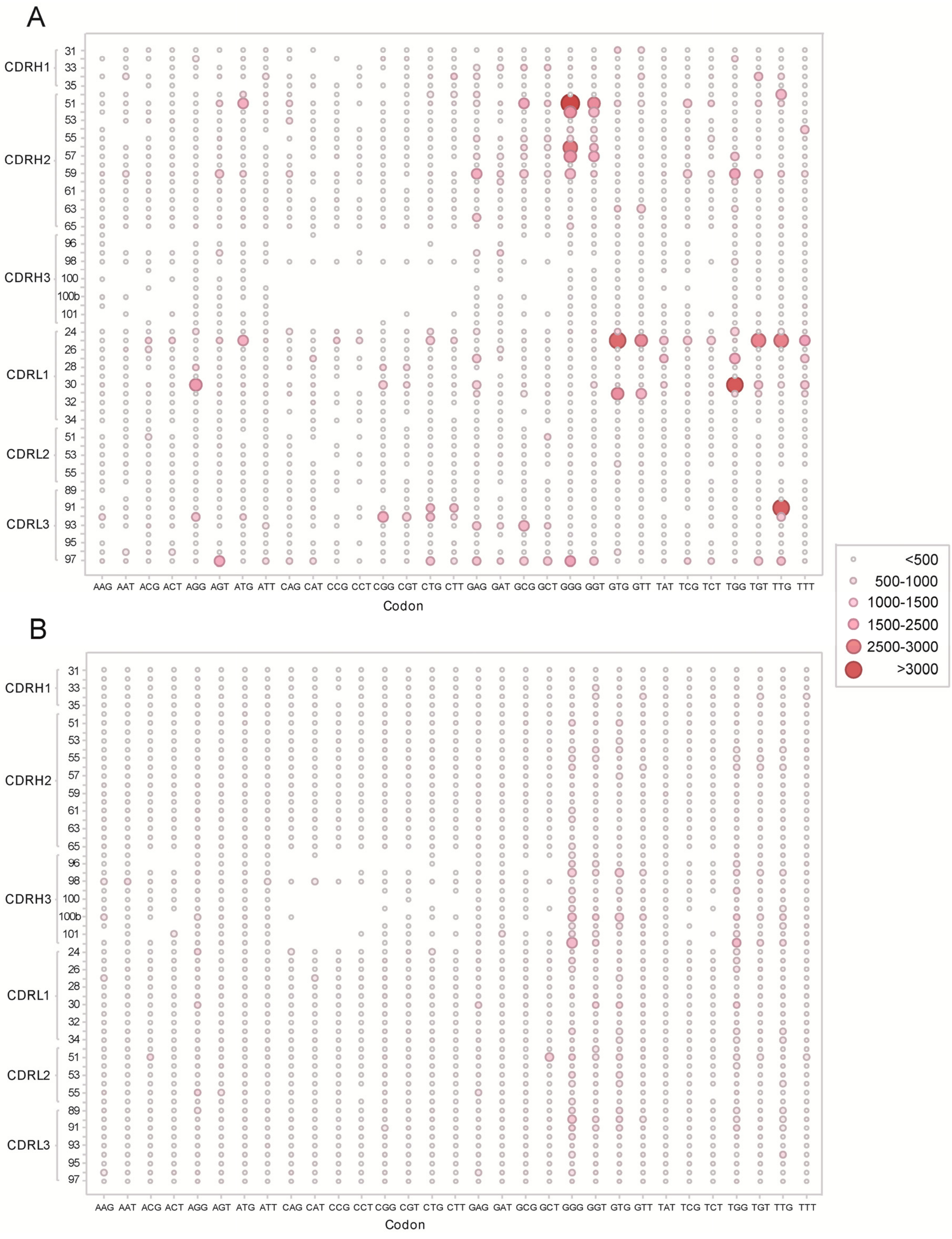
A) Library synthesis error rate based on ABI sequencing of library clones

	read length	number reads	total bases	insertion/deletions	point mutations	combined
light chain	324	125	40,500	2	36	38
heavy chain	357	91	32,487	0	12	12
combined		216	72,987	2 (0.003%)	48 (0.066%)	50 (0.068%)

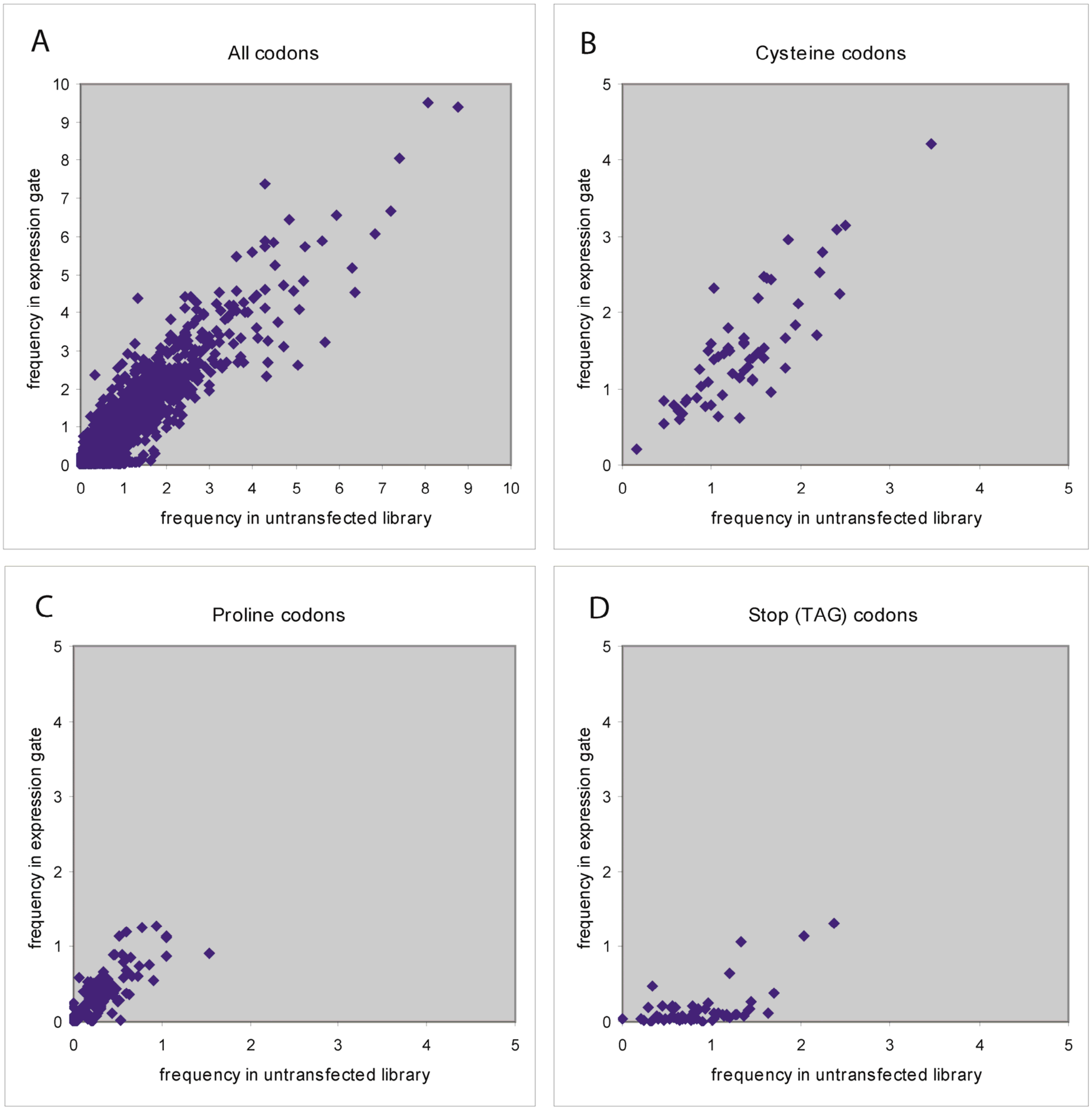
B) Pyrosequencing error rate estimates based on analysis of framework regions from expression gate

	read length	number reads	total bases	insertion/deletions	point mutations	combined
light chain	141	323	45,543	28	62	90
heavy chain	111	349	38,739	98	29	127
combined		672	84,282	126 (0.149%)	91 (0.108%)	217 (0.257%)

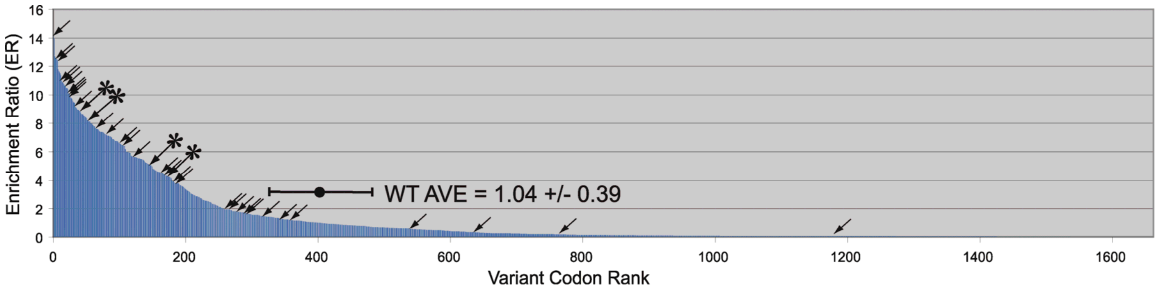
Supplemental Figure S3. Error rate analysis for library synthesis and pyrosequencing. A) Library error rate was determined from ABI sequences of 216 complete variable regions, where any differences outside of a single variant CDR codon in each variable region were scored as synthesis errors. Of 216 library clones, 7 (3.2%) had more than one mutant codon within a CDR. B) Pyrosequencing apparent errors (which are the sum of sequencing errors plus library synthesis errors) were determined by analysis of framework regions from 672 sequence reads from the expression gate.



Supplemental Figure S4. Frequencies of codon variants within the binding (A) and expression (B) gates from hu225 library FACS sorting as determined by pyrosequencing. X axis show 31 possible NNK codons (excluding TAG nonsense codon); Y axis shows identity of 59 CDR positions in the library. Number of sequence reads for each variant is shown as a bubble plot where size and color of bubble corresponds to number of reads.



Supplemental Figure S5. Comparison of codon frequencies in the untransfected library vs. the expression gate. All values are in frequency per 1000. An aliquot of the untransfected library was pyrosequenced similarly to plasmids recovered from the FACS gates. Comparisons are shown for (A) all 1865 NNK codons (B) all cysteine codons (C) all proline codons and (D) nonsense (TAG) codons. In general, codons are found in the expression gate at similar frequencies to their appearance in the untransfected library, indicating no expression bias against particular substitution types, even unpaired cysteine residues or prolines which might be suspected to disrupt folding or structure. A clear bias is seen against nonsense codons in the expression gate, as expected.

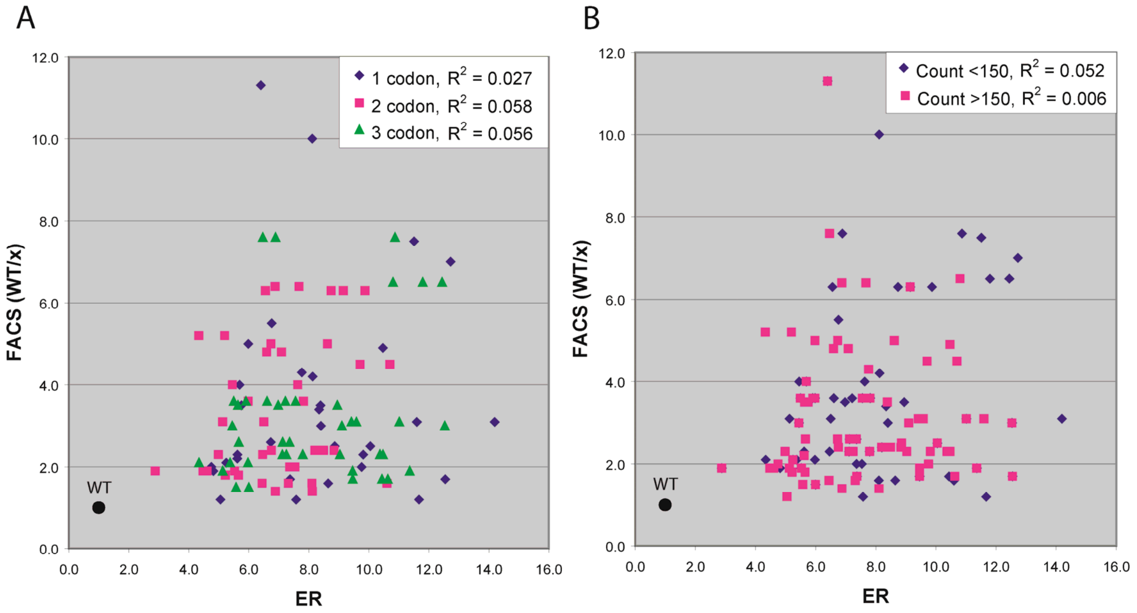


Supplemental Figure S6. 1662 hu225 codon variants ranked by enrichment ratio (ER). Average ER for the set of 71 synonymous wild type codons (1.04) is indicated. 37 codons for amino acid mutations previously found in higher affinity variants of Mab 225 CDRs are indicated. Arrows indicate variants from phage display affinity maturation[10]; arrows plus stars indicate variants identified from in silico design.[9]

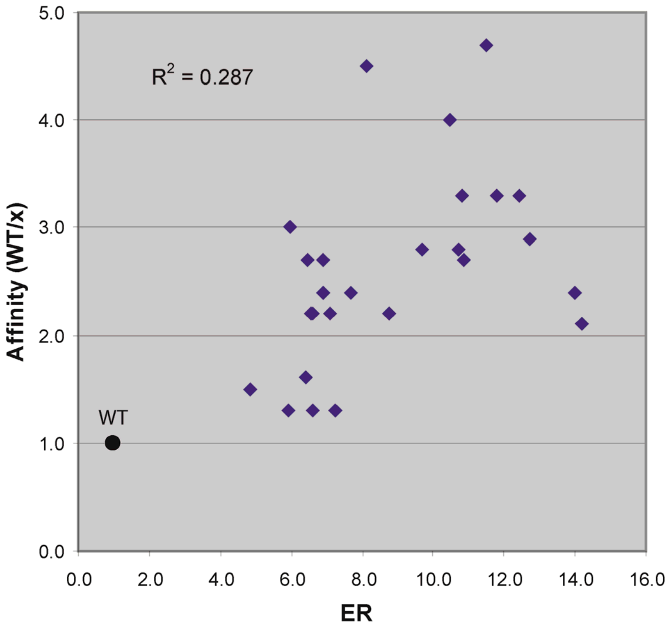
Supplemental Figure S7

position	mutant	ER(s)	total reads		position	mutant	ER(s)	total reads
VH:N31	V	7.81, 5.98	1663		VL:R24	P	7.54, 7.37	732
	I	5.53	265			I	4.68	214
	T	5.29	85			D	4.44	219
	D	4.59	162			F	4.33	334
VH:Y32	R	7.21, 6.61, 5.90	1278		VL:A25	C	11.60	2993
	W	4.48	884			V	10.70, 9.71	6364
						L	10.63, 10.44, 9.46	5032
VH:G33	A	9.87, 9.15	1897			F	9.81	2230
	D	8.11	614			M	9.76	1847
	E	5.63, 6.66	1102			T	8.93, 8.55	1994
						I	8.52	402
VH:V34	N	8.13	516			Y	7.44	1450
	L	8.94, 6.98, 5.64	2276			S	7.80, 7.25, 7.12	3398
	E	6.66	593			P	7.38, 5.51	1767
	Q	4.12	116					
					VL:S26	D	4.23	553
VH:V50	Q	12.73	205			T	5.08	629
	I	10.09	370					
	L	10.87, 6.89, 6.46	2703		VL:Q27	F	10.87	1277
	E	7.11	674			Y	10.47	1562
						W	8.86	1978
VH:I51	Q	8.34	798			E	7.20	1215
	C	7.96	846			D	4.22	484
	G	7.67, 6.88	7378					
	A	7.97, 5.69	3236		VL:S28	R	5.99, 5.57, 5.57	2986
	S	7.36, 7.13, 5.66	3368					
	M	5.75	2023		VL:G30	F	12.55	1340
						Y	11.51	903
VH:W52	G	8.62, 6.74	4460			T	10.61, 8.10	506
	T	7.63, 5.45	320			K	8.63	365
	I	4.70	249			H	7.87	375
						R	7.96, 7.34	2007
VH:S53	N	7.40	148			M	7.57	450
	T	6.50, 5.13	601			C	6.75	1407
	Q	5.69	748			Q	4.76	217
						W	4.75	4454
VH:G55	D	6.76	343					
	H	6.16	159		VL:T31	V	8.11, 6.88	4629
	E	5.43	730			E	5.05	776
	A	5.48, 4.60	1373			S	4.51, 4.26	714
						Q	4.19	473
VH:N56	G	5.19, 4.33	5329					
	A	4.30	520		VL:N32	H	12.63	434
VH:T57	P	8.74, 6.56	447		VL:N91	L	10.47, 10.37, 9.03	6885
	E	7.02	645					
	G	7.09, 6.60	4721		VL:N92	R	11.01, 9.58, 9.42	4550
	D	5.62	558			L	12.53, 9.10, 5.45	3665
	A	5.52	712			K	7.05	719
	S	5.97, 5.37, 4.34	906			M	8.92	889
	W	4.57	1464					
					VL:N93	E	10.05	1266
VH:Y59	H	8.65	127			A	8.84, 8.20	2660
	E	7.76	1971			D	7.58	801
	W	6.73	2276			T	4.66, 4.49	709
	S	7.56, 5.91, 5.50	3125					
	P	7.07, 5.08	327		VL:T96	L	6.20, 5.17, 4.27	511
	Q	5.95	776					
	A	6.46, 4.98	2008		VL:T97	C	14.00	1173
	C	5.62	1420			S	12.44, 11.80, 10.80	3689
	F	5.24	765			H	11.67	718
	M	4.60	1002			L	11.36, 9.46, 5.13	3645
	G	4.62, 4.48	3010			E	8.39	1441
	N	4.55	672			A	8.49, 6.76	2627
	D	4.17	946			I	7.37	817
						N	7.20	393
VH:N60	D	8.40	638			G	7.32, 6.45	3812
						Q	6.79	762
VH:F63	V	5.65, 5.22	2254			K	6.41	419
						D	5.98	1116
VH:T64	E	5.53	1356			Y	4.42	384
VH:T97	D	6.40	981					
	S	5.04	608					
VH:Y98	W	4.82	506					
VH:F100c	Y	14.19	166					

Supplemental Figure S7. Selection criteria for 67 higher affinity candidates for verification. The set of 179 codon variants with ER greater than 4 were used to identify 31 positions containing one or more candidate mutations. In order to look at as wide a range of positions as possible, within each position the top 1 to 4 candidates with highest ERs were selected for confirmation (rather than select candidates strictly by ER within the overall set). As a first strategy, these 49 variants (shown in blue) were pursued by gene synthesis of the individual variants. As a second parallel strategy (to reduce gene synthesis costs) at 6 of the ‘hotspot’ positions with multiple potential candidates (VH:I51, VH:T57, VH:Y59, VL:A25, VL:G30, and VL:T97) 96 individual clones from the corresponding positional sub library were sequenced to identify as many clones of interest as possible. The 18 variants thus identified (shown in grey) were also selected for verification, although they were not always the highest remaining ER candidates within their respective position. A few exceptions to the highest ER criteria for the first strategy were made as follows. At positions VH:I51, VL:A25, and VL:T97, Cys substitutions were skipped over in favor of other high ER variants due to a concern about potential problems from unpaired cysteines in the CDRs (although VL:A25C was nonetheless identified from the second strategy). At position VH:V50, Ile was passed over in favor of the conservative variant Leu, based on multiple codons and higher overall number of reads for Leu. At position VH:S53, Asn was passed over in favor of the conservative variant Gln for verification, due to the relatively low number of reads for Asn (148). At position VH:T57, identification of Asp from strategy 2 lead to dropping the conservative substitution Glu from the strategy 1 pool. At position VL:Q27, Tyr was selected for verification instead of the conservative substitution Phe, as they had nearly identical ERs and tyrosine residues are more frequently found in antibody CDRs. The His substitution at VL:N32 was not pursued as preliminary experiments showed no benefit over wild type, and closer inspection of the 454 sequencing data showed that the enriched His codon was found in only one of direction sequencing reads, suggesting some sort of sequencing artifact. No variants were selected for confirmation at positions VL:S26 and VL:T96 due to limitations in the number of variants that could be pursued, and because the best variants at these positions barely passed the 5.0 ER level.



Supplemental Figure S8. FACS based affinity and enrichment ratio relationship. (A) FACS binding of the variant over wild type verses ER where either one, two, or three codons encode for the same variant. (B) FACS verses ER where expression counts for a given codon have more or less than 150 sequences. Location of the average ER for 71 synonymous wild type codons is indicated.



Supplemental Figure S9. BIAcore based affinity and enrichment ratio relationship. BIAcore binding affinity over wild type verses ER. Location of the average ER for 71 synonymous wild type codons is indicated.

TABLE S1

Number of occurrences of 1806 possible NNK variant codons in the expression gate. Grey shade indicates codons with 10 or fewer occurrences; black indicates wild type codon.

position	WT codon	AAG	AAT	ACG	ACT	AGG	AGT	ATG	ATT	CAG	CAT	CCG	CCT	CGG	CGT	CTG	CTT	GAG	GAT	GCG	GCT	GGG	GGT	GTG	GTT	TAT	TCG	TCT	TGG	TGT	TTG	TTT
VH:N31	AAC	10	61	15	18	49	26	38	45	1	13	14	24	56	49	55	54	84	32	65	55	176	125	121	112	32	81	41	123	135	182	119
VH:Y32	TAC	46	33	45	30	77	22	56	25	46	4	8	19	59	53	63	55	66	35	70	86	203	87	140	79	244	61	31	178	122	162	132
VH:G33	GGC	46	37	42	12	65	61	77	109	65	53	29	22	96	102	125	94	85	75	108	94	156	504	284	231	71	173	146	251	132	300	254
VH:V34	GTC	23	63	19	28	84	165	66	141	25	75		83	24	33	60	186	86	139	59	74	173	480	198	531	244	32	303	248	418	82	415
VH:H35	CAC	32	54	40	21	101	33	74	117	79	128	36	20	150	104	100	76	51	93	131	95	321	215	341	232	96	146	96	210	253	222	270
VH:V50	GTG	14	22	33	24	85	62	279	38	17	15	29	31	60	67	68	44	94	50	210	83	283	274	■	285	88	63	74	224	193	268	141
VH:I51	ATA	95	44	108	63	151	131	338	125	97	89	96	18	287	190	188	228	210	110	376	127	652	371	643	327	61	229	146	400	107	367	112
VH:W52	TGG	42	21	33	12	88	80	94	49	60	18	72	5	228	83	43	37	145	32	189	76	398	205	296	153	61	191	44	■	228	371	163
VH:S53	TCA	18	20	58	43	136	72	127	105	126	60	206	29	252	103	185	124	96	77	205	73	210	276	405	234	101	178	112	334	242	364	219
VH:G54	GGC	14	21	35	64	86	107	109	58	82	32	61	32	195	122	230	115	89	82	275	201	468	579	535	332	58	228	150	426	361	409	374
VH:G55	GGA	56	25	32	39	118	133	85	106	95	25	52	71	161	146	142	131	128	50	114	144	544	480	376	247	110	99	130	458	407	332	239
VH:N56	AAC	30	180	101	49	147	163	98	108	92	68	145	59	242	283	220	177	210	186	255	110	647	372	385	438	115	166	109	538	497	463	226
VH:T57	ACA	29	48	83	99	118	76	134	96	96	19	42	13	101	85	147	88	91	95	123	115	372	307	456	281	60	71	23	295	155	237	142
VH:D58	GAC	214	122	128	127	280	195	167	66	141	76	56	42	119	71	110	37	273	252	172	93	332	286	272	130	114	92	101	292	201	219	140
VH:Y59	TAC	164	136	89	111	222	220	201	169	126	15	33	17	140	104	125	70	255	205	257	97	385	226	279	275	363	147	116	333	242	290	138
VH:N60	AAC	160	148	64	76	151	105	85	81	85	58	85	24	72	66	52	71	124	77	130	74	219	173	105	99	79	75	61	229	121	119	76
VH:T61	ACT	149	133	188	■	354	202	147	143	130	63	20	21	229	82	124	21	193	104	171	130	477	199	258	105	122	83	48	301	133	147	71
VH:P62	CCT	199	165	115	183	281	205	191	162	168	231	203	■	197	188	107	194	220	130	115	118	513	274	97	178	121	163	127	303	176	256	102
VH:F63	TTT	159	56	94	56	209	105	172	129	68	45	68	37	143	98	77	88	176	105	174	89	253	238	175	221	152	103	123	209	273	213	■
VH:T64	ACC	124	114	159	184	253	153	184	87	98	58	59	86	168	82	86	50	234	101	204	64	307	161	256	17	97	123	34	232	86	99	39
VH:S65	TCC	223	120	110	40	283	147	174	127	167	90	99	12	191	79	121	48	214	117	188	84	489	212	249	137	86	110	105	242	135	197	92
VH:A95	GCA	5	11			37	7	7	2		11					11		49	34	24	1	415	212	131	101	35			376	219	217	71
VH:L96	TTG	12	10			164	56	64	22							13		32	61			714	542	323	185	51	96	2	543	258	■	317
VH:T97	ACC	42	33	46	85	346	109	256	127				13	3	6		1	214	144	7		1017	629	1001	491	94	35	2	664	458	505	196
VH:Y98	TAT	437	610	246	268	259	241	203	401	133	430	134	173	86	120	130	118	134	279	124	167	89	72	147	118	■	137	208	94	380	150	135
VH:Y99	TAC	15	32	33	2	179	90	50	58					6	2		2	63	14	5		542	251	407	296	175	19	3	658	247	342	104
VH:D100	GAC	3	17	3	33	162	59	54	9						1			37	96	7		631	271	378	143	34	2		336	174	206	41
VH:Y100a	TAC	67	4	33	1	157	95	82	98					1		1		158	64	4	3	640	400	397	303	149	5	10	384	161	416	183
VH:E100b	GAG	527	18	1		452	304	305	157	8					4	2	3	■	83	3		1246	911	939	652	53			888	488	709	168
VH:F100c	TTC	55	14			211	99	137	115					3	1			106	44			469	352	503	304	12	51		327	31	416	348
VH:A101	GCT		9		415	161	64	59	22				1	1	7	1	33	66	491	18	■	583	452	292	353	39		79	504	171	295	133
VH:Y102	TAC	55	41	35	4	224	229	119	51					2	3			184	44	5	6	1472	815	209	268	130	29		1456	652	677	314
VL:R24	CGG	48	22	76	5	726	20	120	44	410	46	64	38	■	300	409	60	235	47	120	36	398	214	385	55	31	82	46	456	98	138	73
VL:A25	GCC	87	50	130	113	247	146	204	50	157	40	142	137	353	199	132	85	313	138	281	327	704	375	361	312	203	184	149	559	283	312	245
VL:S26	AGT	85	256	121	17	331	■	156	110	91	48	102	37	152	64	172	76	182	123	224	73	517	295	391	247	64	232	194	574	381	246	145
VL:Q27	CAG	481	91	132	100	222	196	96	125	■	480	86	176	343	203	119	168	175	108	247	204	125	113	403	328	162	141	123	238	191	237	128
VL:S28	AGC	46	92	104	57	191	249	126	107	119	118	123	113	201	133	110	183	200	63	288	156	363	323	264	311	106	93	137	55	215	257	206
VL:I29	ATT	51	140	56	74	293	194	121	■	72	46	78	28	287	120	102	59	76	45	139	96	360	361	244	199	79	73	37	357	216	270	168
VL:G30	GGG	45	33	38	22	681	107	62	73	44	50	50	37	155	118	84	87	690	170	248	119	■	900	860	391	86	50	43	905	214	226	118
VL:T31	ACT	127	32	59	■	130	87	97	192	106	65	46	45	207	95	85	54	150	146	185	155	225	218	357	280	100	164	68	293	248	287	218
VL:N32	AAT	129	■	60	50	159	235	151	89	29	38	90	62	166	163	74	135	105	116	22	51	300	298	273	285	113	106	81	358	237	300	254
VL:I33	ATC	116	180	87	51	178	185	170	160	130	66	76	56	293	308	176	105	213	137	139	32	615	295	429	173	124	238	109	503	359	493	291
VL:H34	CAC	139	126	81	59	203	273	219	123	105	194	89	40	208	188	279	151	287	147	232	73	353	325	416	324	79	165	162	407	258	482	282
VL:Y50	TAC	78	111	42	54	138	198	143	154	8	19	18	31	78	90	35	38	142	105	66	134	394	505	343	337	199	98	92	361	174	256	262
VL:A51	GCG	87	96	670	90	271	220	100	211	167	98	67	78	383	224	192	96	336	166	■	1020	837	523	629	400	221	185	131	493	428	366	425
VL:S52	AGC	63	48	40	19	227	369	138	100	18	32	23	5	134	68	120	19	77	80	118	96	355	272	304	150	82	63	47	409	196	316	159
VL:E53	GAA	109	48	87	19	172	143	200	79	119	23	93	82	275	128	153	191	251	124	81	103	738	336	605	239	174	140	72	355	136	336	115
VL:S54	TCA	82	63	73	38	171	110	172	170	72	93	42	7	195	117	98	49	151	134	241	99	480	336	527	286	121	131	126	286	197	448	183
VL:I55	ATT	350	85	137	88	792	494	318	■	172	48	136		325	160	95	17	525	135	213	36	249	301	308	169	47	66		385	92	65	10
VL:S56	TCA	104	40	79	63	234	199	104	65	18	5	66	63	202	112	148	68	169	131	173	125	510	281	311	222	58	202	99	296	141	189	113
VL:Q89	CAG	292	102	243	31	483	155	164	214	■	137	15	2	319	117	320	164	166	223	302	97	615	338	582	307	133	131	126	483	222	476	221
VL:Q90	CAA	112	85	95	38	240	203	257	167	195	99	73	74	175	125	280	112	225	209	271	329	1114	666	684	454	110	131	79	478	320	403	253
VL:N91	AAC	137	119	161	119	290	75	205	152	126	111	71	181	428	287	162	179	213	220	340	282	864	578	578	373	72	186	68	641	330	403	43
VL:N92	AAC	106	116	108	25	157	136	107	69	82	28	51	86	221	113	137	148	133	142	166	165	610	263	353	183	88	107	77	372	272	155	164
VL:N93	AAT	105	■	101	49	139	125	116	341	49	62	56	47	124	72	122	84	137	111	211	119	369	212	208	133	89	66	62	184	131	78	83
VL:W94	TGG	125	86	147	32	209	131	188	53	203	67	114	59	293	109	168	28	244	150	255	105	512	230	318	284	120	129	112	■	232	680	108
VL:P95	CCT	73	50	109	109	91	129	117	97	63	191	92	■	136	129	95	166	131	119	170	211	313	347	328	224	144	206	230	218	240	201	156
VL:T96	ACA	407	138	235	354	391	212	131	15	372	134	171	104	312	192	36	30	463	294	313	112	576	367	254	118	124	62	21	136	119	30	28
VL:T97	ACT	67	57	140	■	268	203	148	116	116	68	133	70	261	210	132	143	183	189	202	164	366	216	326	210	83	91	64	325	94	176	78

TABLE S2

Number of occurrences of 1806 possible NNK variant codons in the binding gate. Grey shade indicates codons with 10 or fewer occurrences; black indicates wild type codon.

position	WT codon	AAG	AAT	ACG	ACT	AGG	AGT	ATG	ATT	CAG	CAT	CCG	CCT	CGG	CGT	CTG	CTT	GAG	GAT	GCG	GCT	GGG	GGT	GTG	GTT	TAT	TCG	TCT	TGG	TGT	TTG	TTT
VH:N31	AAC		56	70	56	7	13	20	220	1	10			12	2	7	23	10	130	33	26	28	9	837	593	10	19	26	81	13	14	49
VH:Y32	TAC	1	21	6	3	402		151	3	2		2		377	310	4	4		3	5	2	9	1	11	3	207	11	2	706	5	14	9
VH:G33	GGC		2		1	5	5	2	4				1	1	1	8	2	424	539	874	821	271	393	6	4	2	3	8	10	1	13	7
VH:V34	GTC	51	453	1	17	2	284	107	498	91	1		1		7	371	928	507	11	33	32	13	40	268	651	3	30	313	7	1272	649	21
VH:H35	CAC			7	1				4	16	155	2	5	7	2	3	1	5	6	6	3	17	12	10	8	7	12	6	12	6	17	7
VH:V50	GTG	10	72		1	7	2	678	332	188		1			1	406	415	580		44	8	12	13	-10	230	2		2	12	64	1502	5
VH:I51	ATA	3	129	14	12	23	836	1685	181	701	90	1	2	62	43	361	326	448	349	1855	878	3887	2468	709	409	45	1124	902	66	739	640	15
VH:W52	TGG	1	48	218	57	12	9	19	200	11	1	1		49	4	12	6	5	1	1	3	2325	1532	140	112	8	7		-10	17	103	55
VH:S53	TCA		128	258	242	4	44	4	1	622	9	10		22	2	7	1	9	58	7	5	10	17	86	63	9	251	109	25	5	18	17
VH:G54	GGC	2			2	21	1	5	4		2	17	1	19	5	10	9	6		87	39	590	598	26	63	9	4	9	178	16	20	1078
VH:G55	GGA	7		15	26	6	400	79		59	134	2	4	30	24	53	19	602	293	541	574	668	672	10	17	133	287	422	78	87	31	217
VH:N56	AAC	3	117	112	2	74	237	10	4		11	6	1	74	20	6	8	6	5	838	410	2913	1397	11	16	4	212	112	51	30	12	13
VH:T57	ACA	5	59	113	77	8	286	75	19	94	45	318	74	10	12	9	9	554	463	589	287	2286	1756	177	125	60	331	119	1169	32	41	223
VH:D58	GAC	7	4	3	103	8	1	14	2	47			1	9	1	5		14	206	10	5	48	16	7	4	1	5		8		3	1
VH:Y59	TAC	362	536	275	197	155	1126	801	20	650	112	202	75	89	81	273	173	1716	741	1111	543	1494	905	178	173	398	963	553	1943	1178	811	627
VH:N60	AAC	7	137	82	52	10	90	10	81	13	1	3	5	7		13	6	2	561	106	55	26	43	46	37	24	56	17	764	10	8	39
VH:T61	ACT	37	51	151	-10	81	319	98	52	118		1		44	26	72	15	321	202	184	220	82	27	66	19	8	127	96	26	15	150	52
VH:P62	CCT	10	8	3	18	10	5	14	17	17	6	251	-10	11	8	19	118	12	14	7	10	67	16	16	3	7	6	45	15	8	15	21
VH:F63	TTT	27	2	63	40	46	41	235	194	18	12	11	4	22	20	133	154	27	14	161	164	90	83	858	1000	52	19	67	557	20	334	-10
VH:T64	ACC	17	192	215	200	44	250	337	104	58	15	12	52	16	11	77	62	1122	332	298	99	328	144	211	32	195	119	46	185	3	91	23
VH:S65	TCC	241	164	114	100	331	172	240	107	192	82	47	26	287	95	182	57	320	185	223	97	643	273	202	168	197	150	136	352	31	303	261
VH:A95	GCA	3									23									18	2	7	4	11	4	1			8	20	8	1
VH:L96	TTG	1				5	4	14	2							14			218			13	17	14	5	6	25		18	18	-10	12
VH:T97	ACC	7	2	9	78	10	499	10	6				23					466	837			47	23	47	21	15	2		33	31	32	7
VH:Y98	TAT	11	15	11	13	16	8	10	40	7	43	7	4	6	8	7	12	1	15	2	14			6	3	-10	1	8	412	39	14	15
VH:Y99	TAC			24		1		1										1	5			15	14	14	15	166	1		26	15	12	2
VH:D100	GAC	7			24	7	2	2											99			24	6	3	9				6	6	10	7
VH:Y100a	TAC			25		5	3	3	2									4				17	20	9	5	137	4		170	4	60	8
VH:E100b	GAG	84	5			14	38	17	2									-10	41	4		75	45	22	17				15	13	33	1
VH:F100c	TTC	1				2	3		1									1	2			12	18	18	11	154	25		203		18	345
VH:A101	GCT		20		122	26	24	12	1						6		18	10	20	3	-10	36	21	28	93	24	1	33	25	106	9	16
VH:Y102	TAC			18		7	14	8	5					1				3	1			41	31	48	21	113	1		53	29	18	267
VL:R24	CGG	42	18	66	9	606	24	167	170	449	4	399	231	-10	168	613	96	476	172	155	76	386	336	876	145	95	57	9	973	178	412	261
VL:A25	GCC	1	11	918	833	16	858	1643	352	3	52	865	623	11	21	1031	733	24	2	203	159	49	64	3190	2501	1247	1102	959	400	2710	2739	1985
VL:S26	AGT	17	366	508	37	39	-10	4	2	18	1	16		26	3	4	4	57	430	21	2	56	170	12	20	5	155	80	27	34	26	6
VL:Q27	CAG	17	26	66	73	6	39	21	9	-10	763	12	11	26	10	60	9	1040	376	74	41	11	3	208	325	1400	55	17	1740	10	6	1149
VL:S28	AGC	90	27	57	21	878	151	150	102	208	118	45	23	925	658	131	65	72		166	57	52	33	107	163	106	117	78	44	96	164	152
VL:I29	ATT	1	1		12	15	13	9	-10		41	2		22	10	7	11		3	54	3	17	3	7	26	12	9	9	17	16	91	203
VL:G30	GGG	320	49	254	192	2134	265	388	177	173	325			1019	715	143	214	935	85	362	108	-10	585	324	260	817	125	135	3549	1193	526	1222
VL:T31	ACT	144	8	112	-10	92	306	228	166	367	210	9	9	90	142	26	47	626	99	420	287	101	59	2391	1591	267	343	253	711	643	85	653
VL:N32	AAT	4	-10	1	7	11	73	4	10		396	3	2	3	5	8	1	3	17		8	6	16	10	29	2	4	1	21	42	10	3
VL:I33	ATC	4	6	2	4	2	5	34	113	3	7		2	14	17	15		5	2	7	1	17	16	68	17	8	9	7	15	6	68	4
VL:H34	CAC	1	1	6	1	3	8	7	2		104	9	3	11		12	4	5	7		1	9	7	6	5	3	11	4	10	14	14	16
VL:Y50	TAC		7	2		11	11	2	3		5			4	5	2	1	7	4	1		9	15	10	9	158	15	1	16		1	2
VL:A51	GCG	2	8	463	3	4	16		6	3	5		2	13	10	9	1	29	4	-10	777	101	22	234	15	1	231	25	29	14	2	9
VL:S52	AGC	1	8	1	1	51	248	55	17	17		2		2	4	68	10	82	34	122	37	121	71	55	35	17	35	40	26	106	64	25
VL:E53	GAA	5	1	6	1	1	6	7	7	3		3	3	9	3	11	7	140	203	1	12	21	32	25	8	6	6	3	32	16	55	5
VL:S54	TCA	62	138	32	12	28	76	166	15	38	42		1	19	2	10	11	198	55	64	61	195	128	456	278	30	74	61	166	60	34	90
VL:I55	ATT	5	4	3	69	24	28	25	-10	19	3	15		19		3	10	22	5	18		7	9	193	90	6	1		7	9		
VL:S56	TCA	44	26	36	25	93	105	56	42	11	13	69	36	38	33	150	22	84	45	70	64	153	80	203	86	32	163	73	77	26	96	44
VL:Q89	CAG	6		10	1	6	4	11	4	-10	43	2		31	4	9	7	14		17	4	41	12	8	12	5	11	4	17	4	12	8
VL:Q90	CAA		1	1	1	5	3	7	8	89	6				3	9	2	1	3	17	8	56	20	15	33	5	4	3	23	8	18	2
VL:N91	AAC	2	113	9	121	4	3	15	6	4	268		6	8	5	1390	1325	14	8	7	10	20	27	19	16		12	1	8	2	3426	4
VL:N92	AAC	613	111	43	5	1233	10	782	4	35	5	10	10	1706	1020	1408	661	9	7	56	24	15	18	15	10	1		2	10	22	1156	8
VL:N93	AAT	14	-10	372	187	8	96	8	486	21	5	3		5	6	6	3	1129	690	1530	800	13	12	136	109	6	85	93	14	7	6	2
VL:W94	TGG			2		10		7	3	3				35	2	5		1	1	3	4	71	8	12	4	9	4		-10	10	245	5
VL:P95	CCT	6		1	16	5	5	5	6	1	4	51	-10	6	25	6	129	5		13	4	13	19	8	8	1	7	88	6	10	2	8
VL:T96	ACA	13	429	178	466	19	13	1	24	51	9	4		10	6	183	127	24	12	10	2	12	25	480	243	8	5	4	1	3	105	1
VL:T97	ACT	352	336	116	-10	188	1798	75	701	646	650	3	15	173	221	1229	601	1258	927	1119	1142	1934	1296	46	22	301	880	653	398	1079	1364	228

TABLE S3

Hu225 enrichment ratios. Grey shade indicates codons with 10 or fewer occurrences; black indicates wild type codon. Distance to EGRF is shown in angstroms; contact residues (<5 angstroms) are shown in bold.

position	WT codon	distance	AAG	AAT	ACG	ACT	AGG	AGT	ATG	ATT	CAG	CAT	CCG	CCT	CGG	CGT	CTG	CTT	GAG	GAT	GCG	GCT	GGG	GGT	GTG	GTT	TAT	TCG	TCT	TGG	TGT	TTG	TTT
VH:N31	AAC	**4.6**	0.00	1.04	5.29	3.50	0.16	0.56	0.59	5.53	▨	0.87	0.00	0.00	0.24	0.05	0.14	0.48	0.14	4.59	0.57	0.53	0.18	0.08	7.82	5.98	0.35	0.27	0.72	0.74	0.11	0.09	0.47
VH:Y32	TAC	6.8	0.03	0.72	0.15	0.11	5.90	0.00	3.05	0.14	0.05	▨	0.28	0.00	7.22	6.61	0.07	0.08	0.00	0.10	0.08	0.03	0.05	0.01	0.09	0.04	0.96	0.20	0.07	4.48	0.05	0.10	0.08
VH:G33	GGC	8.8	0.00	0.06	0.00	0.10	0.09	0.09	0.03	0.04	0.00	0.00	0.00	0.05	0.01	0.01	0.07	0.02	5.63	8.11	9.15	9.87	1.96	0.88	0.02	0.02	0.03	0.02	0.06	0.05	0.01	0.05	0.03
VH:V34	GTC	13.5	2.50	8.13	0.06	0.69	0.03	1.94	1.83	3.99	4.12	0.02	▨	0.01	0.00	0.24	6.98	5.64	6.66	0.09	0.63	0.49	0.08	0.09	1.53	1.39	0.01	1.06	1.17	0.03	3.44	8.95	0.06
VH:H35	CAC	7.4	0.00	0.00	0.20	0.06	0.00	0.00	0.00	0.04	0.23	1.37	0.06	0.28	0.05	0.02	0.03	0.02	0.11	0.07	0.05	0.04	0.06	0.06	0.03	0.04	0.08	0.09	0.07	0.06	0.03	0.09	0.03
VH:V50	GTG	6.0	0.83	3.77	0.00	0.05	0.09	0.04	2.80	10.09	12.73	0.00	0.04	0.00	0.00	0.02	6.89	10.87	7.11	0.00	0.24	0.11	0.05	0.05	■	0.93	0.03	0.00	0.03	0.06	0.38	6.46	0.04
VH:I51	ATA	7.4	0.04	3.38	0.15	0.22	0.18	7.36	5.75	1.67	8.34	1.17	0.01	0.13	0.25	0.26	2.22	1.65	2.46	3.66	5.69	7.97	6.88	7.67	1.27	1.44	0.85	5.66	7.13	0.19	7.96	2.01	0.15
VH:W52	TGG	**3.1**	0.03	2.63	7.63	5.45	0.16	0.13	0.23	4.70	0.21	0.06	0.02	▨	0.25	0.06	0.32	0.19	0.04	0.04	0.01	0.05	6.74	8.62	0.55	0.84	0.15	0.04	0.00	■	0.09	0.32	0.39
VH:S53	TCA	**4.0**	0.00	7.40	5.13	6.50	0.03	0.71	0.04	0.01	5.69	0.17	0.06	0.00	0.10	0.02	0.04	0.01	0.11	0.87	0.04	0.08	0.06	0.07	0.24	0.31	0.10	1.63	1.12	0.09	0.02	0.06	0.09
VH:G54	GGC	**4.0**	0.16	0.00	0.00	0.04	0.28	0.01	0.05	0.08	0.00	0.07	0.32	0.04	0.11	0.05	0.05	0.09	0.08	0.00	0.36	0.22	1.45	1.19	0.06	0.22	0.18	0.02	0.07	0.48	0.05	0.06	3.33
VH:G55	GGA	5.1	0.14	0.00	0.54	0.77	0.06	3.47	1.07	0.00	0.72	6.16	0.04	0.07	0.21	0.19	0.43	0.17	5.43	6.76	5.48	4.60	1.42	1.61	0.03	0.08	1.39	3.34	3.74	0.20	0.25	0.11	1.05
VH:N56	AAC	**3.8**	0.12	0.75	1.28	0.05	0.58	1.68	0.12	0.04	0.00	0.19	0.05	0.02	0.35	0.08	0.03	0.05	0.03	0.03	3.79	4.30	5.19	4.33	0.03	0.04	0.04	1.47	1.19	0.11	0.07	0.03	0.07
VH:T57	ACA	8.1	0.20	1.41	1.57	0.90	0.08	4.34	0.65	0.23	1.13	2.74	8.74	6.56	0.11	0.16	0.07	0.12	7.02	5.62	5.52	2.88	7.09	6.60	0.45	0.51	1.15	5.37	5.97	4.57	0.24	0.20	1.81
VH:D58	GAC	**3.0**	0.04	0.04	0.03	0.94	0.03	0.01	0.10	0.03	0.38	0.00	0.00	0.03	0.09	0.02	0.05	0.00	0.06	0.94	0.07	0.06	0.17	0.06	0.03	0.04	0.01	0.06	0.00	0.03	0.00	0.02	0.01
VH:Y59	TAC	11.0	2.55	4.55	3.56	2.05	0.81	5.91	4.60	0.14	5.95	8.65	7.07	5.08	0.73	0.90	2.52	2.85	7.76	4.17	4.98	6.46	4.48	4.62	0.74	0.73	1.26	7.56	5.50	6.73	5.62	3.23	5.24
VH:N60	AAC	7.8	0.05	1.07	1.48	0.79	0.08	0.99	0.14	1.15	0.18	0.02	0.04	0.24	0.11	0.00	0.29	0.10	0.02	8.40	0.94	0.86	0.14	0.29	0.51	0.43	0.35	0.86	0.32	3.85	0.10	0.08	0.59
VH:T61	ACT	6.6	0.29	0.44	0.93	■	0.26	1.82	0.77	0.42	1.05	0.00	0.06	0.00	0.22	0.37	0.67	0.82	1.92	2.24	1.24	1.95	0.20	0.16	0.30	0.21	0.08	1.77	2.30	0.10	0.13	1.18	0.84
VH:P62	CCT	9.6	0.06	0.06	0.03	0.11	0.04	0.03	0.08	0.12	0.12	0.03	1.43	■	0.06	0.05	0.20	0.70	0.06	0.12	0.07	0.10	0.15	0.07	0.19	0.02	0.07	0.04	0.41	0.06	0.05	0.07	0.24
VH:F63	TTT	10.9	0.20	0.04	0.77	0.82	0.25	0.45	1.58	1.74	0.30	0.31	0.19	0.13	0.18	0.23	1.99	2.02	0.18	0.15	1.07	2.12	0.41	0.40	5.65	5.22	0.39	0.21	0.63	3.07	0.08	1.81	■
VH:T64	ACC	6.3	0.16	1.94	1.56	1.25	0.20	1.88	2.11	1.38	0.68	0.30	0.23	0.70	0.11	0.15	1.03	1.43	5.53	3.79	1.68	1.78	1.23	1.03	0.95	2.17	2.32	1.12	1.56	0.92	0.04	1.06	0.68
VH:S65	TCC	12.6	1.25	1.58	1.20	2.88	1.35	1.35	1.59	0.97	1.33	1.05	0.55	2.49	1.73	1.39	1.73	1.37	1.73	1.82	1.37	1.33	1.52	1.49	0.94	1.41	2.64	1.57	1.49	1.68	0.26	1.77	3.27
VH:A95	GCA	8.1	▨	0.00	▨	▨	0.00	▨	▨	▨	▨	2.30	▨	▨	▨	▨	0.00	▨	0.00	0.00	0.83	▨	0.02	0.02	0.09	0.04	0.03	▨	▨	0.02	0.10	0.04	0.02
VH:L96	TTG	8.1	0.09	0.00	▨	▨	0.03	0.08	0.24	0.10	▨	▨	▨	▨	▨	▨	1.18	▨	0.00	3.93	▨	▨	0.02	0.03	0.05	0.03	0.13	0.29	▨	0.04	0.08	■	0.04
VH:T97	ACC	**2.9**	0.18	0.07	0.22	1.01	0.03	5.04	0.04	0.05	▨	▨	▨	1.94	▨	▨	▨	▨	2.40	6.40	▨	▨	0.05	0.04	0.05	0.05	0.17	0.06	▨	0.05	0.07	0.07	0.04
VH:Y98	TAT	**3.9**	0.03	0.03	0.05	0.05	0.07	0.04	0.05	0.11	0.06	0.11	0.06	0.03	0.08	0.07	0.06	0.11	0.01	0.06	0.02	0.09	0.00	0.00	0.05	0.03	■	0.01	0.04	4.82	0.11	0.10	0.12
VH:Y99	TAC	**2.7**	0.00	0.00	0.80	▨	0.01	0.00	0.02	0.00	▨	▨	▨	▨	▨	▨	▨	▨	0.02	0.39	▨	▨	0.03	0.06	0.04	0.06	1.04	0.06	▨	0.04	0.07	0.04	0.02
VH:D100	GAC	**2.9**	2.63	0.00	0.00	0.80	0.05	0.04	0.04	▨	▨	▨	▨	▨	▨	▨	▨	▨	0.00	1.14	▨	▨	0.04	0.02	0.01	0.07	0.00	▨	▨	0.02	0.04	0.05	0.19
VH:Y100a	TAC	**3.0**	0.00	▨	0.83	▨	0.03	0.03	0.04	0.02	▨	▨	▨	▨	▨	▨	▨	▨	0.03	0.00	▨	▨	0.03	0.06	0.02	0.02	1.01	▨	0.00	0.49	0.03	0.16	0.05
VH:E100b	GAG	**4.2**	0.18	0.30	▨	▨	0.03	0.14	0.06	0.01	▨	▨	▨	▨	▨	▨	▨	▨	■	0.54	▨	▨	0.07	0.05	0.03	0.03	0.00	▨	▨	0.02	0.03	0.05	0.01
VH:F100c	TTC	9.4	0.02	0.00	▨	▨	0.01	0.03	0.00	0.01	▨	▨	▨	▨	▨	▨	▨	▨	0.01	0.05	▨	▨	0.03	0.06	0.04	0.04	14.19	0.54	▨	0.68	0.00	0.05	1.09
VH:A101	GCT	11.4	▨	2.45	▨	0.32	0.18	0.41	0.22	0.05	▨	▨	▨	▨	▨	0.96	▨	0.60	0.17	0.04	0.18	■	0.07	0.05	0.11	0.29	0.67	▨	0.46	0.05	0.68	0.03	0.13
VH:Y102	TAC	12.5	0.00	0.00	0.57	▨	0.03	0.07	0.07	0.11	▨	▨	▨	▨	▨	▨	▨	▨	0.02	0.02	▨	▨	0.03	0.04	0.25	0.09	0.96	0.04	▨	0.04	0.05	0.03	0.94
VL:R24	CGG	13.5	1.06	0.99	1.05	2.22	1.01	1.45	1.69	4.68	1.33	0.10	7.54	7.37	■	0.68	1.82	1.94	2.45	4.44	1.56	2.56	1.17	1.90	2.76	3.20	3.71	0.84	0.24	2.58	2.20	3.62	4.33
VL:A25	GCC	10.4	0.01	0.27	8.55	8.93	0.08	7.12	9.76	8.52	0.02	1.58	7.38	5.51	0.04	0.13	9.46	10.44	0.09	0.02	0.88	0.59	0.08	0.21	10.70	9.71	7.44	7.25	7.80	0.87	11.60	10.63	9.81
VL:S26	AGT	7.1	0.24	1.73	5.08	2.64	0.14	■	0.03	0.02	0.24	0.03	0.19	0.00	0.21	0.06	0.03	0.06	0.38	4.23	0.11	0.03	0.13	0.70	0.04	0.10	0.09	0.81	0.50	0.06	0.11	0.13	0.05
VL:Q27	CAG	**3.7**	0.04	0.35	0.61	0.88	0.03	0.24	0.27	0.09	■	1.93	0.17	0.08	0.09	0.06	0.61	0.07	7.20	4.22	0.36	0.24	0.11	0.03	0.63	1.20	10.47	0.47	0.17	8.86	0.06	0.03	10.87
VL:S28	AGC	6.7	2.37	0.36	0.66	0.45	5.57	0.73	1.44	1.15	2.12	1.21	0.44	0.25	5.57	6.00	1.44	0.43	0.44	0.00	0.70	0.44	0.17	0.12	0.49	0.64	1.21	1.52	0.69	0.97	0.54	0.77	0.89
VL:I29	ATT	7.3	0.02	0.01	0.00	0.20	0.06	0.08	0.09	■	0.00	1.08	0.03	0.00	0.09	0.10	0.08	0.23	0.00	0.08	0.47	0.04	0.06	0.01	0.03	0.16	0.18	0.15	0.30	0.06	0.09	0.41	1.46
VL:G30	GGG	9.0	8.63	1.79	8.10	10.61	3.80	3.00	7.57	2.94	4.76	7.87	0.00	0.00	7.96	7.34	2.06	2.98	1.64	0.61	1.77	1.10	■	0.79	0.46	0.81	11.51	3.03	3.80	4.75	6.75	2.82	12.55
VL:T31	ACT	8.0	1.37	0.30	2.30	■	0.86	4.26	2.85	1.05	4.19	3.91	0.24	0.24	0.53	1.81	0.37	1.05	5.05	0.82	2.75	2.24	0.54	0.33	8.11	6.88	3.23	2.53	4.51	2.94	3.14	0.36	3.63
VL:N32	AAT	8.2	0.04	■	0.02	0.17	0.08	0.38	0.03	0.14	0.00	12.63	0.04	0.04	0.02	0.04	0.13	0.01	0.04	0.18	0.00	0.19	0.02	0.07	0.04	0.12	0.02	0.05	0.02	0.07	0.21	0.04	0.01
VL:I33	ATC	9.7	0.04	0.04	0.03	0.09	0.01	0.03	0.24	0.86	0.03	0.13	0.00	0.04	0.06	0.07	0.10	0.00	0.03	0.02	0.06	0.04	0.03	0.07	0.19	0.12	0.08	0.05	0.08	0.04	0.02	0.17	0.02
VL:H34	CAC	6.8	0.01	0.01	0.09	0.02	0.02	0.04	0.04	0.02	0.00	0.65	0.12	0.09	0.06	0.00	0.05	0.03	0.02	0.06	0.00	0.02	0.03	0.03	0.02	0.02	0.05	0.08	0.03	0.03	0.07	0.04	0.07
VL:Y50	TAC	**3.8**	0.00	0.07	0.05	0.00	0.09	0.07	0.02	0.02	▨	0.31	0.00	0.00	0.06	0.07	0.07	0.03	0.06	0.05	0.02	0.00	0.03	0.04	0.03	0.03	0.94	0.18	0.01	0.05	0.00	0.00	0.01
VL:A51	GCG	11.8	0.03	0.10	0.82	0.04	0.02	0.09	0.00	0.03	0.02	0.06	0.00	0.03	0.04	0.05	0.06	0.01	0.10	0.03	■	0.90	0.14	0.05	0.44	0.04	0.01	1.48	0.23	0.07	0.04	0.01	0.03
VL:S52	AGC	11.2	0.02	0.20	0.03	0.06	0.27	0.80	0.47	0.20	1.11	0.00	0.10	▨	0.02	0.07	0.67	0.62	1.26	0.50	1.22	0.46	0.40	0.31	0.21	0.28	0.24	0.66	1.01	0.08	0.64	0.24	0.19
VL:E53	GAA	7.0	0.05	0.03	0.08	0.06	0.01	0.05	0.04	0.10	0.03	0.00	0.04	0.04	0.04	0.03	0.09	0.04	0.66	1.94	0.02	0.14	0.03	0.11	0.05	0.04	0.04	0.05	0.05	0.11	0.14	0.19	0.05
VL:S54	TCA	13.4	0.89	2.60	0.52	0.38	0.19	0.82	1.14	0.10	0.63	0.54	0.00	▨	0.12	0.02	0.12	0.27	1.55	0.49	0.31	0.73	0.48	0.45	1.02	1.15	0.29	0.67	0.57	0.69	0.36	0.09	0.58
VL:I55	ATT	11.0	0.02	0.06	0.03	0.93	0.04	0.07	0.09	■	0.13	0.07	0.13	▨	0.07	0.00	0.04	0.69	0.05	0.04	0.10	0.00	0.03	0.04	0.74	0.63	0.15	0.02	▨	0.02	0.12	0.00	0.00
VL:S56	TCA	14.8	0.50	0.77	0.54	0.47	0.47	0.62	0.64	0.77	0.72	3.03	1.24	0.68	0.22	0.35	1.20	0.38	0.59	0.41	0.48	0.61	0.36	0.34	0.77	0.46	0.65	0.96	0.87	0.31	0.22	0.60	0.46
VL:Q89	CAG	8.4	0.03	0.00	0.05	0.04	0.02	0.03	0.08	0.02	■	0.38	0.16	▨	0.12	0.04	0.03	0.05	0.10	0.00	0.07	0.05	0.08	0.04	0.02	0.05	0.05	0.10	0.04	0.04	0.02	0.03	0.04
VL:Q90	CAA	6.5	0.00	0.01	0.01	0.03	0.03	0.02	0.03	0.06	0.56	0.07	0.00	0.00	0.00	0.03	0.04	0.02	0.01	0.02	0.08	0.03	0.06	0.04	0.03	0.09	0.05	0.04	0.05	0.06	0.03	0.05	0.01
VL:N91	AAC	**4.5**	0.02	1.16	0.07	1.24	0.02	0.05	0.09	0.05	0.04	2.94	0.00	0.04	0.02	0.02	10.47	9.03	0.08	0.04	0.03	0.04	0.03	0.06	0.04	0.05	0.00	0.08	0.02	0.02	0.01	10.37	0.11
VL:N92	AAC	5.6	7.05	1.17	0.49	0.24	9.58	0.09	8.92	0.07	0.52	0.22	0.24	0.14	9.42	11.01	12.53	5.45	0.08	0.06	0.41	0.18	0.03	0.08	0.05	0.07	0.01	0.00	0.03	0.03	0.10	9.10	0.06
VL:N93	AAT	**3.4**	0.16	■	4.49	4.66	0.07	0.94	0.08	1.74	0.52	0.10	0.06	0.00	0.05	0.10	0.06	0.04	10.05	7.58	8.84	8.20	0.04	0.07	0.80	1.00	0.08	1.57	1.83	0.09	0.07	0.09	0.03
VL:W94	TGG	**4.1**	0.00	0.00	0.02	0.00	0.06	0.00	0.05	0.07	0.02	0.00	0.00	0.00	0.15	0.02	0.04	0.00	0.00	0.01	0.01	0.05	0.17	0.04	0.05	0.02	0.09	0.04	0.00	■	0.05	0.44	0.06
VL:P95	CCT	8.2	0.10	0.00	0.01	0.18	0.07	0.05	0.05	0.08	0.02	0.03	0.68	■	0.05	0.24	0.08	0.95	0.05	0.00	0.09	0.02	0.05	0.07	0.03	0.04	0.01	0.04	0.47	0.03	0.05	0.01	0.06
VL:T96	ACA	**4.6**	0.04	3.79	0.92	1.61	0.06	0.07	0.01	1.94	0.17	0.08	0.03	0.00	0.04	0.04	6.20	5.17	0.06	0.05	0.04	0.02	0.03	0.08	2.31	2.51	0.08	0.10	0.23	0.01	0.03	4.27	0.04
VL:T97	ACT	9.2	6.41	7.20	1.01	■	0.86	10.80	0.62	7.37	6.79	11.67	0.03	0.26	0.81	1.28	11.36	5.13	8.39	5.98	6.76	8.49	6.45	7.32	0.17	0.13	4.42	11.80	12.44	1.49	14.00	9.46	3.56
AVERAGE ER			0.72	1.16	1.29	1.34	0.59	1.24	1.11	1.18	1.49	1.55	0.79	0.76	0.79	0.83	1.69	1.49	1.64	1.63	1.42	1.50	0.87	0.90	0.90	0.89	1.31	1.26	1.40	1.02	1.03	1.42	1.21

TABLE S4
Sequencing data and enrichment ratios (ERs) for 71 synonymous wild type hu225 codons.

Position	WT AA	WT codon	synonymous codon	BIND count	BIND reads	BIND freq/1000	EXP count	EXP reads	EXP freq/1000	ER
VH:N31	N	AAC	AAT	56	152,333	0.368	61	172,135	0.354	1.04
VH:Y32	Y	TAC	TAT	207	152,333	1.359	244	172,135	1.417	0.96
VH:G33	G	GGC	GGG	271	152,333	1.779	156	172,135	0.906	1.96
			GGT	393	152,333	2.58	504	172,135	2.928	0.88
VH:V34	V	GTC	GTG	268	152,333	1.759	198	172,135	1.150	1.53
			GTT	651	152,333	4.274	531	172,135	3.085	1.39
VH:H35	H	CAC	CAT	155	152,333	1.018	128	172,135	0.744	1.37
VH:V50	V	GTG	GTT	230	139,375	1.65	285	160,766	1.773	0.93
VH:I51	I	ATA	ATT	181	139,375	1.299	125	160,766	0.778	1.67
VH:S53	S	TCA	AGT	44	139,375	0.316	72	160,766	0.448	0.71
			TCG	251	139,375	1.801	178	160,766	1.107	1.63
			TCT	109	139,375	0.782	112	160,766	0.697	1.12
VH:G54	G	GGC	GGG	590	139,375	4.233	468	160,766	2.911	1.45
			GGT	598	139,375	4.291	579	160,766	3.602	1.19
VH:G55	G	GGA	GGG	668	139,375	4.793	544	160,766	3.384	1.42
			GGT	672	139,375	4.822	480	160,766	2.986	1.61
VH:N56	N	AAC	AAT	117	139,375	0.839	180	160,766	1.120	0.75
H:T57	T	ACA	ACG	113	139,375	0.811	83	160,766	0.516	1.57
			ACT	77	139,375	0.552	99	160,766	0.616	0.90
VH:D58	D	GAC	GAT	206	139,375	1.478	252	160,766	1.567	0.94
VH:Y59	Y	TAC	TAT	398	139,375	2.856	363	160,766	2.258	1.26
VH:N60	N	AAC	AAT	137	139,375	0.983	148	160,766	0.921	1.07
VH:T61	T	ACT	ACG	151	139,375	1.083	188	160,766	1.169	0.93
VH:P62	P	CCT	CCG	251	139,375	1.801	203	160,766	1.263	1.43
VH:T64	T	ACC	ACG	215	139,375	1.543	159	160,766	0.989	1.56
			ACT	200	139,375	1.435	184	160,766	1.145	1.25
VH:S65	S	TCC	AGT	172	139,375	1.234	147	160,766	0.914	1.35
			TCG	150	139,375	1.076	110	160,766	0.684	1.57
			TCT	136	139,375	0.976	105	160,766	0.653	1.49
VH:A95	A	GCA	GCG	18	140,847	0.128	24	154,976	0.155	0.83
VH:L96	L	TTG	CTG	14	140,847	0.099	13	154,976	0.084	1.18
VH:T97	T	ACC	ACG	9	140,847	0.064	46	154,976	0.297	0.22
			ACT	78	140,847	0.554	85	154,976	0.548	1.01
VH:Y99	Y	TAC	TAT	166	140,847	1.179	175	154,976	1.129	1.04
VH:D100	D	GAC	GAT	99	140,847	0.703	96	154,976	0.619	1.14
VH:Y100a	Y	TAC	TAT	137	140,847	0.973	149	154,976	0.961	1.01
VH:F100c	F	TTC	TTT	345	140,847	2.449	348	154,976	2.246	1.09
VH:A101	A	GCT	GCG	3	140,847	0.021	18	154,976	0.116	0.18
VH:Y102	Y	TAC	TAT	113	140,847	0.802	130	154,976	0.839	0.96
VL:R24	R	CGG	AGG	606	127,465	4.754	726	154,397	4.702	1.01
			CGT	168	127,465	1.318	300	154,397	1.943	0.68
VL:A25	A	GCC	GCG	203	127,465	1.593	281	154,397	1.820	0.88
			GCT	159	127,465	1.247	327	154,397	2.118	0.59
VL:S26	S	AGT	TCG	155	127,465	1.216	232	154,397	1.503	0.81
			TCT	80	127,465	0.628	194	154,397	1.257	0.50
VL:S28	S	AGC	AGT	151	127,465	1.185	249	154,397	1.613	0.73
			TCG	117	127,465	0.918	93	154,397	0.602	1.52
			TCT	78	127,465	0.612	137	154,397	0.887	0.69
VL:G30	G	GGG	GGT	585	127,465	4.589	900	154,397	5.829	0.79
VL:T31	T	ACT	ACG	112	127,465	0.879	59	154,397	0.382	2.30
VL:I33	I	ATC	ATT	113	127,465	0.887	160	154,397	1.036	0.86
VL:H34	H	CAC	CAT	104	127,465	0.816	194	154,397	1.257	0.65
VL:Y50	Y	TAC	TAT	158	129,373	1.221	199	153,122	1.300	0.94
VL:A51	A	GCG	GCT	777	129,373	6.006	1020	153,122	6.661	0.90
VL:S52	S	AGC	AGT	248	129,373	1.917	369	153,122	2.410	0.80
			TCG	35	129,373	0.271	63	153,122	0.411	0.66
			TCT	40	129,373	0.309	47	153,122	0.307	1.01
VL:E53	E	GAA	GAG	140	129,373	1.082	251	153,122	1.639	0.66
VL:S54	S	TCA	AGT	76	129,373	0.587	110	153,122	0.718	0.82
			TCG	74	129,373	0.572	131	153,122	0.856	0.67
			TCT	61	129,373	0.472	126	153,122	0.823	0.57
VL:S56	S	TCA	AGT	105	129,373	0.812	199	153,122	1.300	0.62
			TCG	163	129,373	1.26	202	153,122	1.319	0.96
			TCT	73	129,373	0.564	99	153,122	0.647	0.87
VL:Q90	Q	CAA	CAG	89	123,483	0.721	195	150,630	1.295	0.56
VL:N91	N	AAC	AAT	113	123,483	0.915	119	150,630	0.790	1.16
VL:N92	N	AAC	AAT	111	123,483	0.899	116	150,630	0.770	1.17
VL:P95	P	CCT	CCG	51	123,483	0.413	92	150,630	0.611	0.68
VL:T96	T	ACA	ACG	178	123,483	1.441	235	150,630	1.560	0.92
			ACT	466	123,483	3.774	354	150,630	2.350	1.61
VL:T97	T	ACT	ACG	116	123,483	0.939	140	150,630	0.929	1.01

TABLE S5

Sequencing data, enrichment ratios (ERs) and rank out of 1662 total codon variants for amino acid mutations occurring in previously characterized higher affinity variants of the CDRs of cetuximab/Mab225. Beidler, amino acid variants from phage display affinity maturation; Lippow, variants from *in silico* prediction (see references in main text).

MUTANT	WT codon	MUTANT codon	BIND count	BIND read	BIND freq./1000	EXP count	EXP read	EXP freq./1000	ER	rank/1662	REFERENCE
VH:Y32W	TAC	TGG	706	152,333	4.64	178	172,135	1.03	4.48	165	Beidler
VH:G33A	GGC	GCT	821	152,333	5.39	94	172,135	0.55	9.87	26	Beidler
		GCG	874	152,333	5.74	108	172,135	0.63	9.15	34	Beidler
VH:G33D	GGC	GAT	539	152,333	3.54	75	172,135	0.44	8.11	54	Beidler
VH:G33E	GGC	GAG	424	152,333	2.78	85	172,135	0.49	5.63	124	Beidler
VH:V50N	GTG	AAT	72	139,375	0.52	22	160,766	0.14	3.77	189	Beidler
VH:N56A	AAC	GCT	410	139,375	2.94	110	160,766	0.68	4.30	172	Beidler
		GCG	838	139,375	6.01	255	160,766	1.59	3.79	188	Beidler
VH:N56S	AAC	AGT	237	139,375	1.70	163	160,766	1.01	1.68	297	Beidler
		TCG	212	139,375	1.52	166	160,766	1.03	1.47	323	Beidler
		TCT	112	139,375	0.80	109	160,766	0.68	1.19	366	Beidler
VH:N56T	AAC	ACG	112	139,375	0.80	101	160,766	0.63	1.28	349	Beidler
		ACT	2	139,375	0.01	49	160,766	0.31	0.05	1179	Beidler
VH:T57P	ACA	CCG	318	139,375	2.28	42	160,766	0.26	8.74	42	Beidler
		CCT	74	139,375	0.53	13	160,766	0.08	6.56	102	Beidler
VH:T64A	ACC	GCT	99	139,375	0.71	64	160,766	0.40	1.78	284	Beidler
		GCG	298	139,375	2.14	204	160,766	1.27	1.68	295	Beidler
VH:T64D	ACC	GAT	332	139,375	2.38	101	160,766	0.63	3.79	186	Beidler
VH:T64N	ACC	AAT	192	139,375	1.38	114	160,766	0.71	1.94	266	Beidler
VH:T97D	ACC	GAT	837	140,847	5.94	144	154,976	0.93	6.40	108	Beidler
VH:E100bD	GAG	GAT	41	140,847	0.29	83	154,976	0.54	0.54	547	Beidler
VH:E100bN	GAG	AAT	5	140,847	0.04	18	154,976	0.12	0.30	644	Beidler
VH:F100cY	TTC	TAT	154	140,847	1.09	12	154,976	0.08	14.19	1	Beidler
VL:S26D	AGT	GAT	430	127,465	3.37	123	154,397	0.80	4.23	175	Lippow
VL:Q27E	CAG	GAG	1,040	127,465	8.16	175	154,397	1.13	7.20	80	Beidler
VL:Q27F	CAG	TTT	1,149	127,465	9.01	128	154,397	0.83	10.87	14	Beidler
VL:Q27H	CAG	CAT	763	127,465	5.99	480	154,397	3.11	1.93	271	Beidler
VL:Q27Y	CAG	TAT	1,400	127,465	10.98	162	154,397	1.05	10.47	21	Beidler
VL:T31E	ACT	GAG	626	127,465	4.91	150	154,397	0.97	5.05	148	Lippow
VL:N93A	AAT	GCG	1,530	123,483	12.39	211	150,630	1.40	8.84	41	Lippow
		GCT	800	123,483	6.48	119	150,630	0.79	8.20	52	Lippow
VL:N93D	AAT	GAT	690	123,483	5.59	111	150,630	0.74	7.58	66	Beidler
VL:N93E	AAT	GAG	1,129	123,483	9.14	137	150,630	0.91	10.05	25	Beidler
VL:N93K	AAT	AAG	14	123,483	0.11	105	150,630	0.70	0.16	773	Beidler
VL:T97S	ACT	TCT	653	123,483	5.29	64	150,630	0.43	12.44	7	Beidler
		TCG	880	123,483	7.13	91	150,630	0.60	11.80	8	Beidler
		AGT	1,798	123,483	14.56	203	150,630	1.35	10.80	16	Beidler

TABLE S6

FACS based affinity analysis of higher affinity hu225 variants. FACS (xWT), ratio of MFI for 1.0 nM EGFR-Cλ-AF647 antigen to compared to wild type hu225. Beidler, amino acid variants from phage display affinity maturation; Lippow, variants from in silico prediction (see references in main text).

CDR	MUTANT	reference	ER for codon(s)	codon rank(s)/1662	FACS (xWT)
H1	VH:N31V		7.82, 5.98	61, 113	3.6
	VH:Y32R		7.22, 6.61, 5.90	79, 100, 117	3.6
	VH:G33A	Biedler	9.87, 9.15	26, 34	6.3
	VH:G33D	Biedler	8.11	54	10.0
	VH:V34L		8.95, 6.98, 5.64	37, 89, 123	3.5
	VH:V34N		8.13	53	4.2
H2	VH:V50L		10.87, 6.89. 6.46	15, 90, 104	7.6
	VH:V50Q		12.73	3	7.0
	VH:I51G		7.67, 6.88	64, 92	6.4
	VH:I51M		5.75	118	3.5
	VH:I51Q		8.34	51	3.4
	VH:I51S		7.36, 7.13, 5.66	75, 82, 121	2.6
	VH:W52G		8.62, 6.74	45, 97	5.0
	VH:W52T		7.63, 5.45	65, 135	4.0
	VH:S53Q		5.69	119	4.0
	VH:S53T		6.50, 5.13	103, 145	3.1
	VH:G55D		6.76	94	5.5
	VH:N56G		5.19, 4.33	142, 170	5.2
	VH:T57A		5.52	131	1.9
	VH:T57D	Biedler	5.62	125	2.3
	VH:T57G		7.09, 6.60	85, 101	4.8
	VH:T57P	Biedler	8.74, 6.56	42, 102	6.3
	VH:T57S		5.97, 5.37, 4.34	114, 138, 169	2.1
	VH:Y59A		6.46, 4.98	105, 150	2.3
	VH:Y59C		5.62	126	2.2
	VH:Y59E		7.76	63	4.3
	VH:Y59F		5.24	140	2.1
	VH:Y59G		4.62, 4.48	157, 163	1.9
	VH:Y59H		8.56	43	1.6
	VH:Y59S		7.56, 5.91, 5.50	68, 116, 133	3.6
	VH:Y59W		6.73	98	2.6
	VH:N60D		8.4	49	3.0
	VH:F63V		5.65, 5.22	122, 141	1.8
	VH:T64E		5.53	120	1.9
H3	VH:T97D	Biedler	6.4	108	11.3
	VH:Y98W		4.82	151	1.9
	VH:F100cY	Biedler	14.19	1	3.1
L1	VL:R24P		7.54, 7.37	69, 74	2.0
	VL:A25C		11.6	10	3.1
	VL:A25F		9.81	27	2.3
	VL:A25L		10.63, 10.44, 9.46	18, 22, 31	1.7
	VL:A25M		9.76	28	2.0
	VL:A25S		7.80, 7.25, 7.12	62, 78, 83	2.3
	VL:A25V		10.70, 9.71	17, 29	4.5
	VL:Q27W		8.86	40	2.5
	VL:Q27Y	Biedler	10.47	21	4.9
	VL:S28R		6.00, 5.57, 5.57	111, 127, 128	1.5
	VL:G30F		12.55	5	1.7
	VL:G30M		7.57	67	1.2
	VL:G30T		10.61, 8.10	19, 56	1.6
	VL:G30W		4.75	153	2.0
	VL:G30Y		11.51	11	7.5
	VL:T31E	Lippow	5.05	148	1.2
	VL:T31V		8.11, 6.88	55, 91	1.4
L3	VL:N91L		10.47, 10.37, 9.03	20, 23, 36	2.3
	VL:N92L		12.53, 9.10, 5.45	6, 35, 136	3.0
	VL:N92R		11.01, 9.58, 9.42	13, 30, 33	3.1
	VL:N93A	Lippow	8.84, 8.20	41, 52	2.4
	VL:N93E	Biedler	10.05	25	2.5
	VL:T97A		8.49, 6.76	48, 95	2.4
	VL:T97D		5.98	112	5.0
	VL:T97E		8.39	50	3.5
	VL:T97G		7.32, 6.45	77, 106	1.6
	VL:T97H		11.67	9	1.2
	VL:T97I		7.37	73	1.7
	VL:T97L		11.36, 9.46, 5.13	12, 32, 144	1.9
	VL:T97S	Biedler	12.44, 11.80, 10.80	7, 8, 16	6.5