Mutational Analysis of 48G7 Reveals that Somatic Hypermutation Affects Both Antibody Stability and Binding Affinity

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Supplementary Information

Materials and methods

Cloning of 48G7 mutants

The heavy and light chain variable regions of mature 48G7 and germline 48G7g were synthesized by GenScript USA, Inc. (Piscataway, NJ). These were amplified using oligomers purchased from Integrated DNA Technologies, Inc., (Coralville, IA) and purified with the Nucleospin® Extract II kit (Macherey-Nagel GmbH & Co. KG; Düren, Germany) (Table S3). These variable region cassettes were then ligated into pFUSE plasmids (InvivoGen; San Diego, CA; www.invivogen.com) with the IL-2 signal sequence. All mutations were generated through site-directed mutagenesis with Pfu UltraII HotStart DNA polymerase (Agilent Technologies; Santa Clara, CA). pFUSE vectors for each heavy and light chain mutant were transformed into TOP10 *E. coli* cells (Life Technologies Co.; Carlsbad, CA) and grown in 200 mL 2xYT medium overnight at 37 °C and 270 RPM. Plasmid was then purified using the Qiagen EndoFree Plasmid Maxi Kit (Venlo, Netherlands).

Fab expression and purification

Fab fragments were expressed using the 293 FreeStyle™ expression system following the commercial protocol (Life Technologies). pFUSE vectors containing the 48G7 heavy and light chain Fab fragments were co-transfected into FreeStyle™ 293-F cells using 293Fectin™. 293-F cells were cultured in FreeStyle™ 293 Expression Medium to a density of 10^6 cells/mL. 10 μg of heavy chain and 10 μg of light chain pFUSE vectors were added into 1 mL Opti-MEM® (Life Technologies). 40 μL 293Fectin was added to 1 mL Opti-MEM and incubated at room temperature for 5 minutes, followed by gentle mixing with the plasmid DNA diluent and incubation for an additional 30 minutes. The mixture was transferred into 20 mL of 293-F cell culture and incubated at 37 °C, 100 RPM, and 5% CO_2. The culture supernatant was harvested after 48 hours. Cells were resuspended in fresh media and cultured for another 48 hours for a second harvest. The supernatant was adjusted to pH 8.0 with a solution of 2 M Tris-HCl, pH 8.0, and then loaded onto a Ni-NTA column (Qiagen) and washed twice with 5 column volumes of wash buffer (50 mM Tris-HCl, 20 mM imidazole, 300 mM NaCl, pH 8.0). The pure Fab fragment was eluted by 5 column volumes of elution buffer (50 mM Tris-HCl, 400 mM imidazole, 300 mM NaCl, pH 8.0) and then buffer-exchanged to a low salt buffer (25 mM Tris-HCl, 50 mM NaCl, pH 8.0). Final yields for each mutant ranged from 10-50 μg from a 20 mL culture.

Extrinsic fluorescence assay to estimate stability (High-Throughput Thermal Scanning, HTTS)

SYPRO™ Orange Protein Gel Stain (Life Technologies Co., Carlsbad, CA) was supplied in DMSO at 5000× the working concentration for PAGE staining. Spectra were obtained on a Bio-Rad CFX96 thermal cycler Real-Time Detection System. Samples with a working volume of 20 μL per well were prepared by mixing 1 μL of 200× SYPRO™ Orange (final

concentration 10×) with 19 µL of protein (0.1 mg/mL or 2.1 µM) in low-salt buffer (25 mM Tris-HCl, 50 mM NaCl, pH 8.0), loaded into Bio-Rad 96-well 0.2 thin-wall PCR plates, and sealed with optical-quality sealing tape (Bio-Rad Laboratories, Inc.; Hercules, CA). In assays testing thermal stability in the presence of hapten, JWJ1 was added at a final concentration of 1 mM, which should saturate the binding sites for all of the mutants since their measured K_D values are well below millimolar range. Thermal denaturations (ramp rate of 1 °C/min at 0.2 °C intervals with an equilibration of 5 seconds at each temperature prior to measurement) were acquired by measuring fluorescence intensities using the FRET channel with excitation from 450-490 nm and detection from 560-580 nm (also referred to as differential scanning fluorimetry, DSF). All data were exported and plotted in Microsoft Excel 2010.

Binding affinity measurements

K_D values were measured with bio-layer interferometry technology on the Octet RED96 system (ForteBio, Inc.; Menlo Park, CA). Biotinylated JWJ1 hapten was dissolved at a concentration of 10 µg/mL in 1x kinetics buffer (1xPBS pH 7.4, 0.01% BSA, 0.002% Tween20) for immobilization on Dip and Read™ Streptavidin biosensors (ForteBio). All binding data were collected at 30 °C. Each 48G7 mutant Fab was first analyzed at 100 nM in a single-pass analysis. Then, 4-5 concentrations of each mutant were used in triplicate, with the highest concentration adjusted to the measured K_D in the single-pass analysis and then serially diluted down 2-fold for the lower concentrations. Baseline, wash, and dissociation steps were performed in 1x kinetics buffer alone. The K_D values reported in this study were calculated from the ratio of k_{off} to k_{on}.

Differential scanning calorimetry

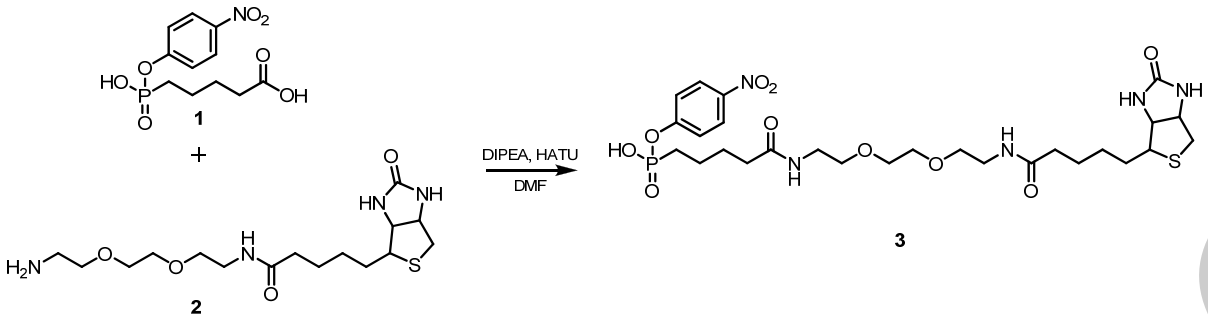
Differential scanning calorimetry (DSC) measurements were performed using a VP-Capillary DSC system (MicroCal, Inc.; Piscataway, NJ). The antibody concentrations were adjusted to 0.3 mg/mL prior to the measurement. The corresponding buffer (25 mM Tris-HCl, 50 mM NaCl, pH 8.0) was used as a reference. The samples were heated from 14 °C to 95 °C at a rate of 1 °C/min after initial 8 minutes of equilibration at 14 °C. A filtering period of 10 seconds was used and data were analyzed using Origin 7.0 software (OriginLab Corporation; Northampton, MA). Thermograms were corrected by subtraction of buffer-only scans and then normalized to the molar concentration of the protein. The final excess heat-capacity thermogram was obtained by interpolating a cubic baseline in the transition region. The midpoint of a thermal transition temperature (T_m) was obtained by analyzing the data using Origin 7.0 software provided with the instrument and using a two state non-ΔC_p fit.

Conjugation of JWJ1 hapten to biotinylated linker

To a solution of JWJ1 phosphonate **1**[1] (17 mg, 0.057 mmol) in DMF (1 mL) was added diisopropylethylamine (13 µL, 0.074 mmol) and HATU (26 mg, 0.068 mmol) at 0 °C. The reaction mixture was stirred for 20 min, followed by addition of commercially available EZ-Link Amine-PEG_2-Biotin (Thermo Fisher Scientific, Inc.; Rockford, IL) at 0 °C. The reaction mixture was stirred for an additional 2 hours and then quenched with saturated aqueous NH_4Cl and concentrated *in vacuo.* The residue was purified by HPLC on an Agilent Prep C-18 reverse phase column, 10 mm X 250mm (CH_3CN with 0.035% TFA: H_2O with 0.05% TFA = 98:2 to 2:98, Running time: 30min, 30 mL/min, 254 nm, retention time 25.7 min). CH_3CN, TFA and H_2O were removed *in vacuo* to give compound **3**. (12 mg, 0.018 mmol, 32% from **1**). ^{1}H-NMR (CD_3OD, 400 MHz) δ 8.28 (d, 2H, J = 9.1 Hz), 7.42 (d, 2H, J = 8.7 Hz), 4.50 (dd, 1H, J = 7.5, 4.8 Hz), 4.31 (dd, 1H, J = 7.8, 4.5 Hz), 3.61 (s, 4H), 3.54 (m, 4H), 3.36 (m, 4H), 3.21 (m, 1H), 2.93 (ddd, 1H, J = 12.8, 5.0, 2.2 Hz), 2.71 (d, 1H, J = 12.7 Hz), 2.30 - 2.17 (m, 4H), 2.07 – 1.90 (m, 2H), 1.75 – 1.42 (m, 10H).

LR-MS (FAB) m/z ($M+H^+$) 660.

HR-MS (ESI-TOF) calculated for ($M+H^+$) 660.2463; found 660.2465



Scheme S1. Conjugation of JWJ1 phosphonate hapten to Amine-PEG2-Biotin

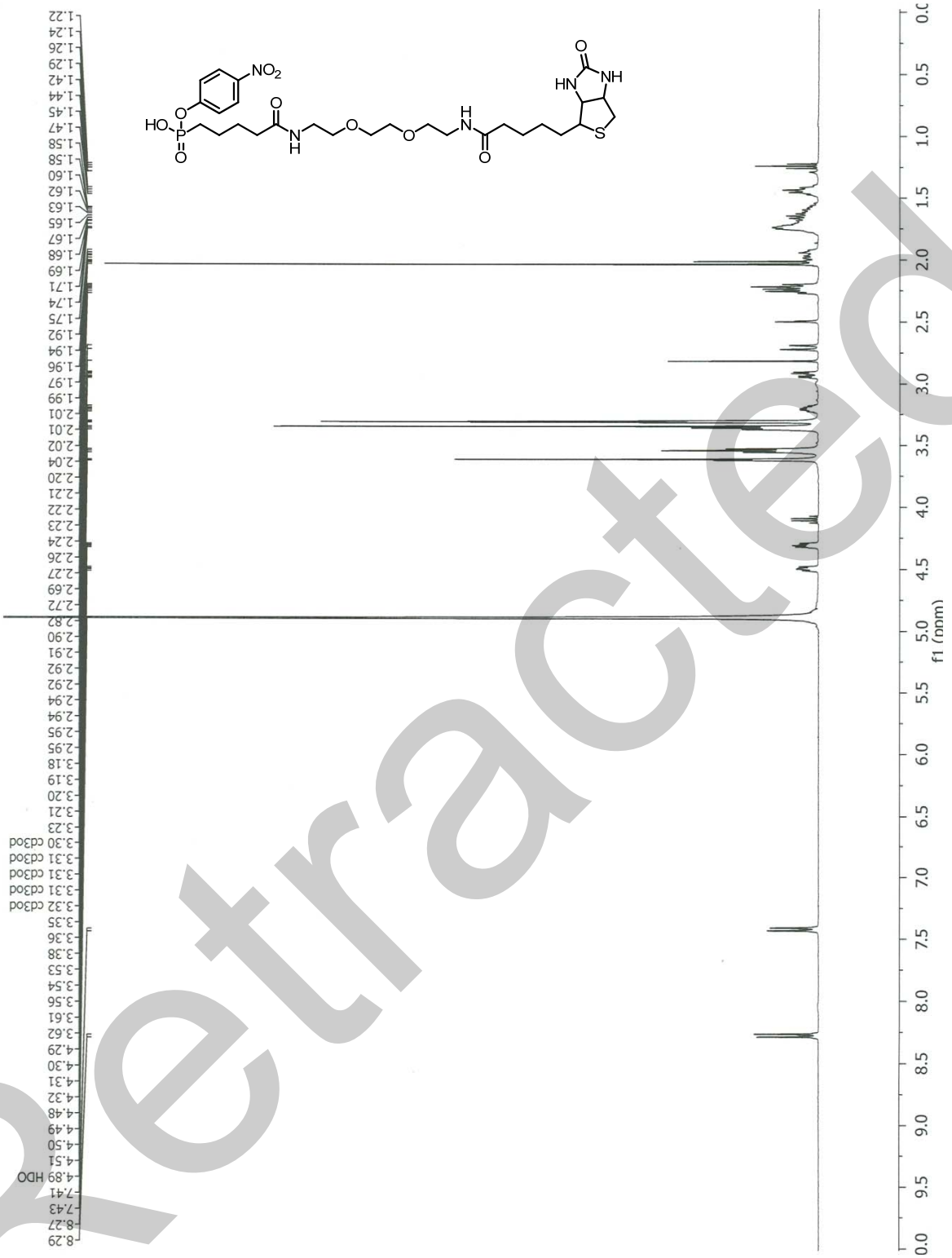


Figure S1. ^{1}H-NMR (Methanol-d_4, 400 MHz) of JWJ1-biotin conjugation

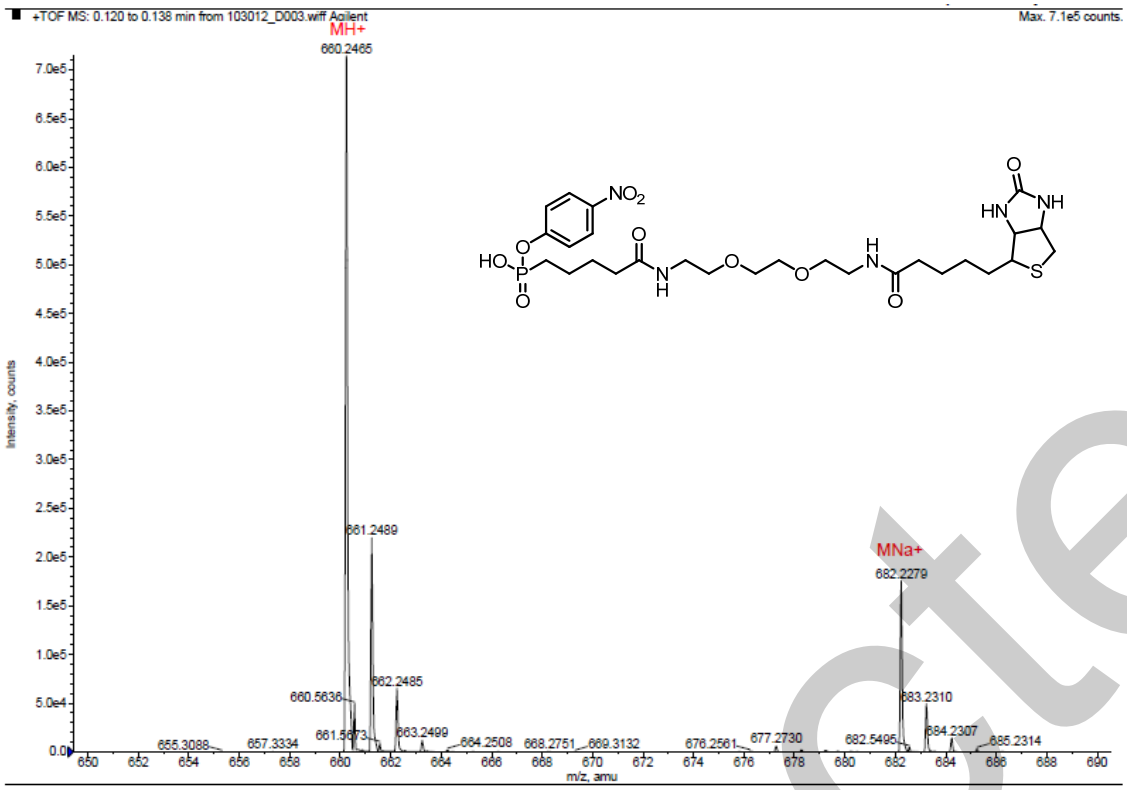


Figure S2. ESI-TOF MS of JWJ1-biotin conjugate

(A) Heavy chain

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          1         11        21        31        41        51
48G7g     EVQLQQSGAELVKPGASVKLSCTAS GFNIKDTYMH WVKQRPEQGLEWIG RIDPANGNTKY
48G7      Q*********************** ********** ******K******* ******VD***
                                    CDR1                     CDR2

          61        71        81        91       101       111
48G7g     DPKFQG KATITADTSSNTAYLQLSSLTSEDTAVYYCAS YYGIY WGQGTTLTVSS
48G7      *****D **********K*T******************** ***** ***********
                                                   CDR3
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(B) Light chain

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          1         11        21        31        41        51
48G7g     DIQMTQSPSSLSASLGERVSLTC RASQEISGYLS WLQQKPDGTIKRLIY AASTLDS GVPK
48G7      ********************** ******N***G *************** *****H* ****
                                   CDR1                     CDR2

          61        71        81        91       101
48G7g     RFSGSRSGSDYSLTISSLESEDFADYYC LQYASYPRT FGGGTKVEIK
48G7      *************************** ********* **********
                                      CDR3
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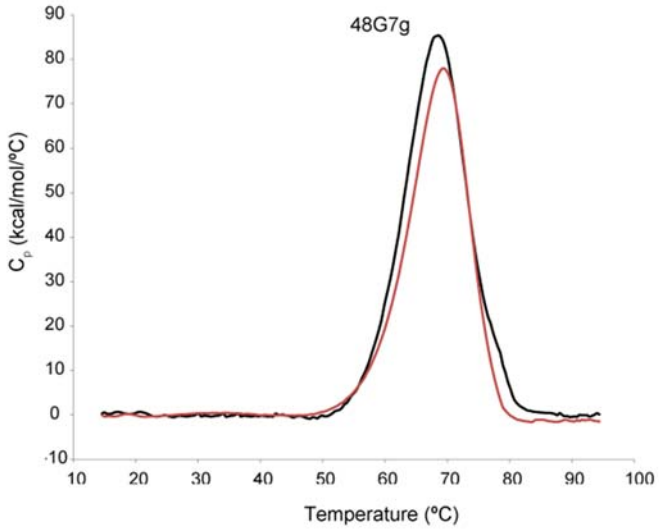
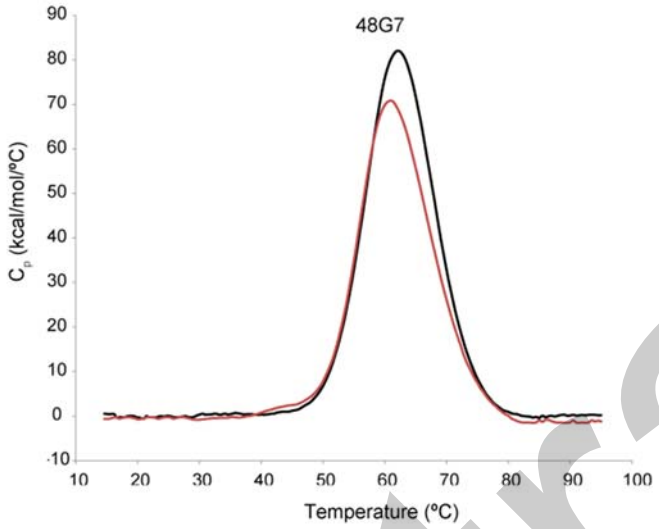
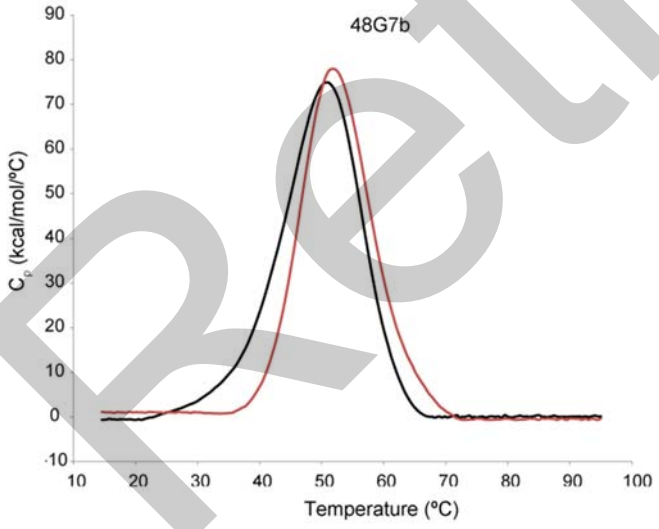
Figure S3. Sequence alignment of germline 48G7g with mature 48G7 (A) heavy chain and (B) light chain variable regions. Complementarity determining regions (CDRs) are underlined in color.







	DSF T_m	Std error	DSC T_m	Std error
48G7g	70.5	0.1	69.4	0.4
48G7	63.5	0.1	62.2	0.6
48G7b	52	0.2	51.3	0.4

Figure S4. Differential scanning calorimetry (DSC) curves and comparison against differential scanning fluorimetry (DSF) values for 48G7g, 48G7, and 48G7b.

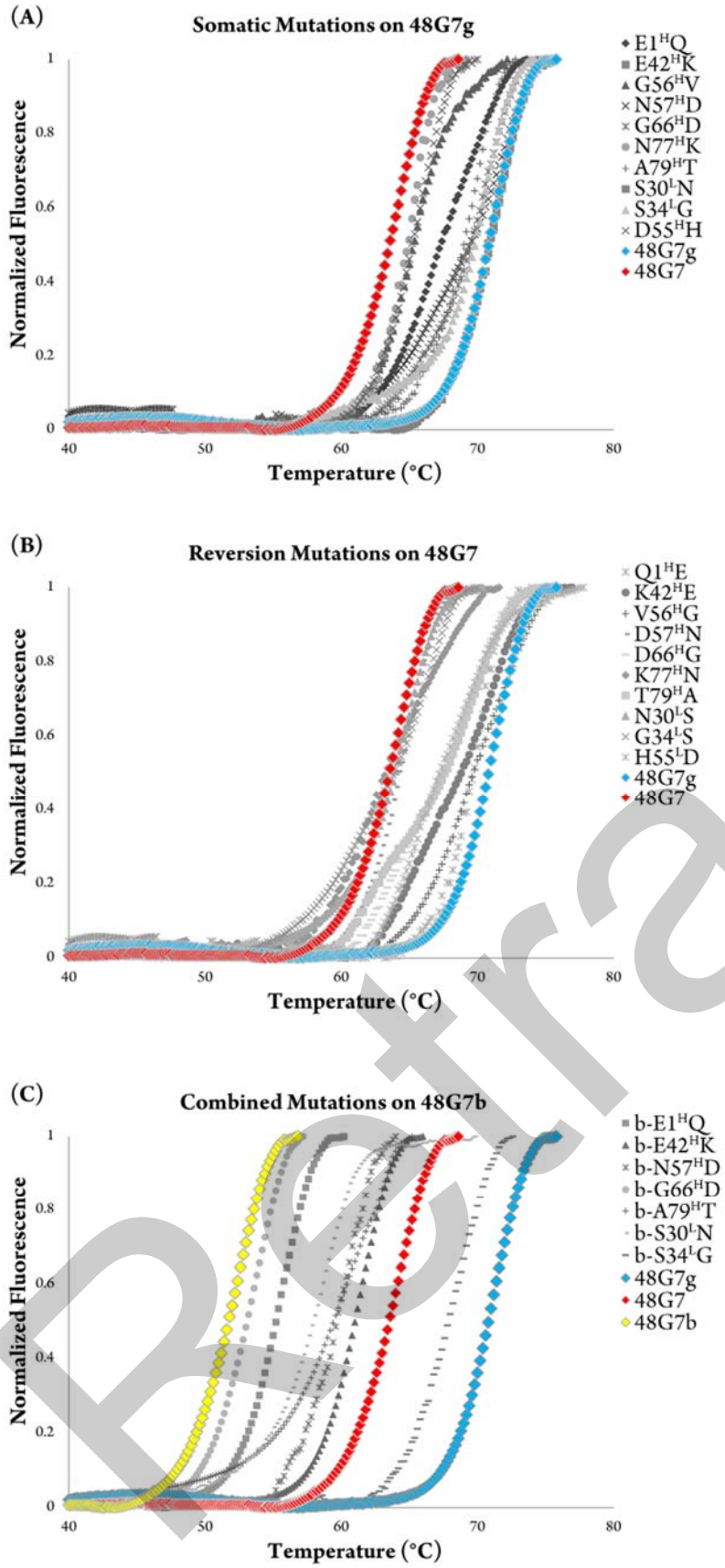


Figure S5. Sample thermal denaturation curves for mutants of 48G7. A lowercase 'b' indicates that mutations that follow have been added to the binding mutant 48G7b.

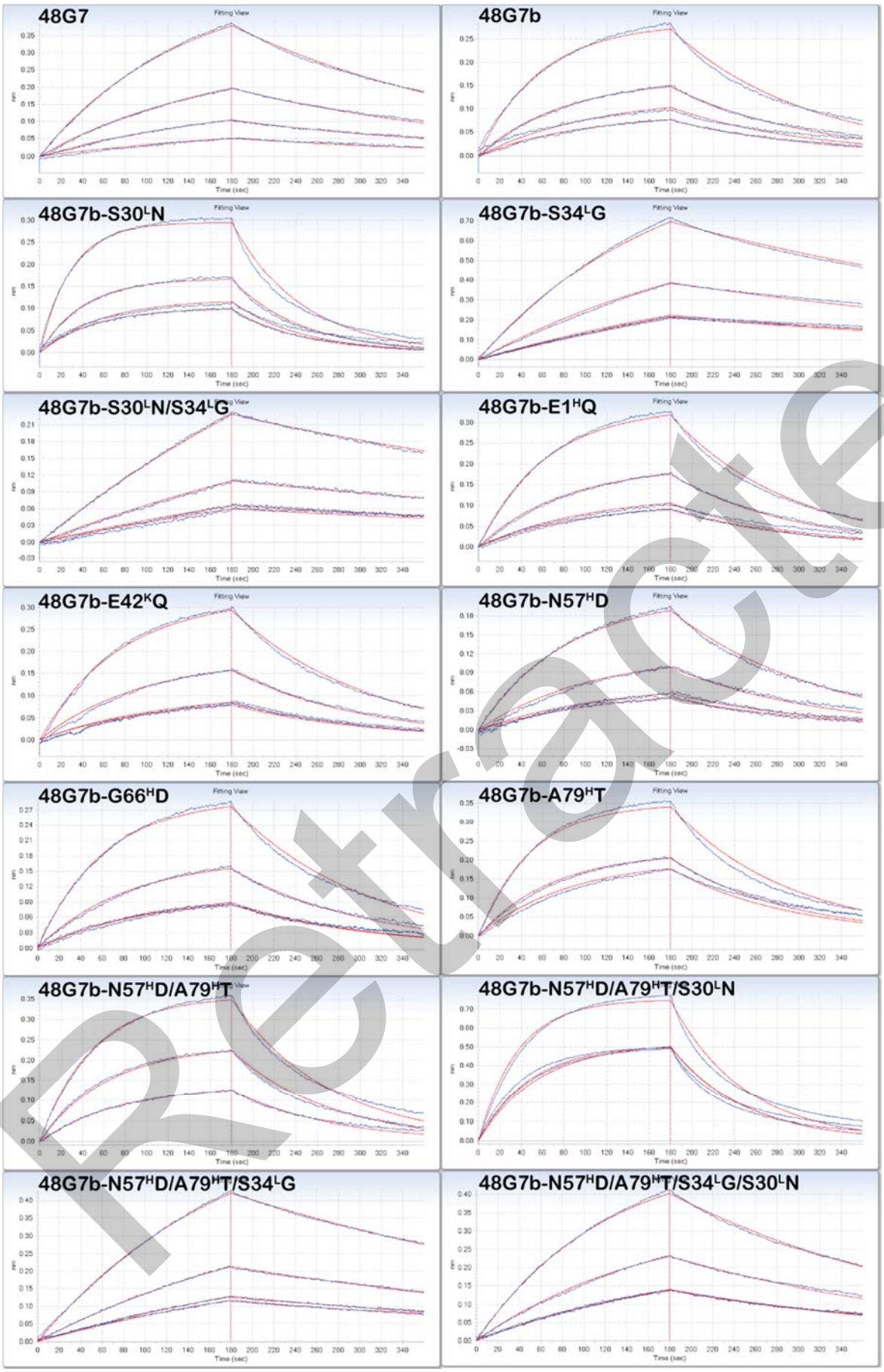


Figure S6. Sample binding curves used to determine K_D values

			Tm (°C)			
	Light chain	Heavy Chain	- hapten	Std error	+ hapten (1 mM)	Std error
	48G7g	**48G7g**	**70.5**	0.1	**71.2**	0.1
	48G7	**48G7**	**63.5**	0.1	**77.8**	0.0
Somatic mutations	48G7g	gE1Q	68.2	0.8	68.7	0.1
	48G7g	gE42K	71	0.1	73.1	0.2
	48G7g	**gG56V**	**65.2**	0.2	**72**	0.1
	48G7g	gN57D	69.9	0.3	71.5	0.0
	48G7g	gG66D	69.3	0.5	72.4	0.1
	48G7g	**gN77K**	**65.2**	0.1	**72.6**	0.3
	48G7g	gA79T	68.9	0.3	71.2	0.1
	gS30N	48G7g	71.1	0.0	73.4	0.1
	gS34G	48G7g	70	0.1	71.5	0.0
	gD55H	**48G7g**	**65.3**	0.2	**73.7**	0.1
Reversion mutations	48G7	Q1E	69.9	1.6		
	48G7	K42E	68.9	0.1		
	48G7	V56G	69.2	0.6		
	48G7	D57N	64.2	0.7		
	48G7	D66G	67.5	0.0	ND	
	48G7	K77N	64.9	0.0		
	48G7	T79A	67.1	0.5		
	N30S	48G7	64	0.1		
	G34S	48G7	64.4	0.3		
	H55D	48G7	67.7	0.5		
Combination mutations	**gD55H**	**gG56V,N77K**	**52**	0.2	**63.5**	0.1
	gD55H	gE1Q,G56V,N77K	55.4	0.1	72.7	0.1
	gD55H	gE42K,G56V,N77K	60.9	0.2	74.2	0.1
	gD55H	gG56V,N57D,N77K	59.9	0.2	75.6	0.1
	gD55H	gG56V,G66D,N77K	53.5	0.2	71	0.1
	gD55H	gG56V,N77K,A79T	59.2	0.1	73.9	0.1
	N30S	gG56V,N77K	68.1	0.1	73.2	0.1
	G34S	gG56V,N77K	57.7	0.1	72	0.0
	48G7	gG56V,N77K	71.9	0.1		
	gD55H	gG56V,N57D,N77K,A79T	62.5	0.3		
	N30S	gG56V,N57D,N77K,A79T	56.7	0.1		
	G34S	gG56V,N57D,N77K,A79T	48.7	0.2		
	48G7	gG56V,N57D,N77K,A79T	61.6	0.2		
	gD55H	gN77K	61.4	1.3	ND	
	gD55H	48G7	58.5	0.1		
	H55D	K77N	68.3	0.1		
	Germline	G56V,N57D	68.9	0.5		
	Germline	N77K,A79T	67.5	0.4		
	Mature	V56G, D57N	69.4	0.4		
	Mature	K77N, T79A	68.7	0.1		

Table S1. Melting temperatures for mutants obtained through DSF. Notable reference points are highlighted in bold. A lowercase 'g' indicates that the mutations that follow are somatic mutations added to 48G7g. "ND" – not determined

Light chain	Heavy chain	T_m (°C)	K_D JWJ1 (M)	Std error (M)	ΔG (kcal/mol)
48G7	48G7	63.5	1.10×10^{-8}	4×10^{-10}	11.0
48G7b	48G7b	52.0	2.9×10^{-8}	6×10^{-9}	10.4
48G7b	48G7b-E1Q	55.4	2.6×10^{-8}	3×10^{-9}	10.5
48G7b	48G7b-E42K	60.9	1.3×10^{-8}	1×10^{-9}	10.9
48G7b	48G7b-N57D	59.9	3.6×10^{-8}	6×10^{-9}	10.3
48G7b	48G7b-G66D	53.5	2.5×10^{-8}	1×10^{-9}	10.5
48G7b	48G7b-A79T	59.2	3.30×10^{-8}	7×10^{-10}	10.4
48G7b-S30N	48G7b	57.7	3.1×10^{-8}	4×10^{-9}	10.4
48G7b-S34G	48G7b	68.1	8.7×10^{-9}	2×10^{-10}	11.2
48G7	48G7b	71.9	3.7×10^{-8}	2×10^{-9}	10.3
48G7b	48G7b-N57D/A79T	62.5	2.6×10^{-8}	2×10^{-9}	10.5
48G7b-S30N	48G7b-N57D/A79T	48.7	1.6×10^{-7}	2×10^{-8}	9.4
48G7b-S34G	48G7b-N57D/A79T	56.7	1.8×10^{-8}	1×10^{-9}	10.7
48G7	48G7b-N57D/A79T	61.6	3.9×10^{-8}	2×10^{-9}	10.3

Table S2. Binding affinity measurements for mutants on 48G7b ($G56^{H}V$, $N77^{H}K$, and $D55^{L}H$ in the context of 48G7g). K_D measurements were obtained through bio-layer interferometry measurements on ForteBio Octet RED96 using three independent datasets.

Primers to clone 48G7 into pFUSE vectors for Fab expression				Notes
48G7	Heavy chain	Forward	acttgtcacgaattcgcaggtgcagctgcagcAGTCTGGGGCAGAGCTTGTG	
		Reverse	gtctggccagctagcacttattagtgatgatgatggtgatggctgccgtcACAAGATTTGGGCTCAACTTTCTTGTCC	Adds His6 tag
	Light chain	Forward	acttgtcacgaattcgGATATCCAGATGACCCAGTCTCCATCC	Germline generated from mature
		Reverse	gtctggccagctagcacttattaacactctcccctgttgaagctctttgtgacgggCGAGCTCAGGCCCTGATGG	
48G7g	Heavy chain	Forward	acttgtcacgaattcgGAGGTGCAGCTGCAGCAG	Reverse primer same as for mature
Primers for site-directed mutagenesis to generate 48G7 mutants				
48G7g	$E1^{H}Q$	Forward	CACTAAGTCTTGCACTTGTCACGAATTCGCAGGTGCAG	
		Reverse	GACTGCTGCAGCTGCACCTGCGAATTCG	
	$E42^{H}K$	Forward	CTGGGTGAAGCAGAGGCCTAAACAGGGC	
		Reverse	CAATCCACTCCAGGCCCTGTTTAGGCCTCT	
	$G56^{H}V$	Forward	GGATTGGAAGGATTGATCCTGCGAATGTTAATACTA	
		Reverse	CTTGCCCTGGAACTTCGGGTCATATTTAGTATTAACATTCGC	
	$N57^{H}D$	Forward	GATTGGAAGGATTGATCCTGCGAATGGTGATACTAAA	
		Reverse	CCTGGAACTTCGGGTCATATTTAGTATCACCATTC	
	$G66^{H}D$	Forward	ATACTAAATATGACCCGAAGTTCCAGGACAAGGCCA	
		Reverse	GAGGATGTATCTGCTGTTATAGTGGCCTTGTCCTGGAAC	
	$N77^{H}K$	Forward	GCCACTATAACAGCAGATACATCCTCCAAGACAGCCT	
		Reverse	CTGAGCTGCAGGTAGGCTGTCTTGGAGG	
	$A79^{H}T$	Forward	CCACTATAACAGCAGATACATCCTCCAACACAACCTACCT	
		Reverse	GCTGCTGAGCTGCAGGTAGGTTGTGTTG	
	$G56^{H}V$, $N57^{H}D$	Forward	GGATTGGAAGGATTGATCCTGCGAATGTTGATACTAAA	
		Reverse	CCTGGAACTTCGGGTCATATTTAGTATCAACATTCGCA	
	$N77^{H}K$, $A79^{H}T$	Forward	GCCACTATAACAGCAGATACATCCTCCAAGACAACCTACCTG	
		Reverse	GCTGCTGAGCTGCAGGTAGGTTGTCTTGGAGGA	
48G7	$Q1^{H}E$	Forward	CACTAAGTCTTGCACTTGTCACGAATTCGGAGGTGCAG	
		Reverse	GACTGCTGCAGCTGCACCTCCGAATTCG	
	$K42^{H}E$	Forward	CTGGGTGAAGCAGAGGCCTGAACAGGGC	
		Reverse	CAATCCACTCCAGGCCCTGTTCAGGCCTCT	
	$V56^{H}G$	Forward	GGATTGGAAGGATTGATCCTGCGAATGGTGATACTA	
		Reverse	CTTGTCCTGGAACTTCGGGTCATATTTAGTATCACCATTCGCA	
	$D57^{H}N$	Forward	GATTGGAAGGATTGATCCTGCGAATGTTAATACTAAA	
		Reverse	CCTTGTCCTGGAACTTCGGGTCATATTTAGTATTAACATTCG	
	$D66^{H}G$	Forward	ATACTAAATATGACCCGAAGTTCCAGGGCAAGGCCA	
		Reverse	GAGGATGTATCTGCTGTTATAGTGGCCTTGCCCTGGAAC	
	$K77^{H}N$	Forward	GCCACTATAACAGCAGATACATCCTCCAACACAACCTA	
		Reverse	GCTGAGCTGCAGGTAGGTTGTGTTGGAGGA	
	$T79^{H}A$	Forward	CACTATAACAGCAGATACATCCTCCAAGACAGCCTACCTG	
		Reverse	GCTGCTGAGCTGCAGGTAGGCTGTCTTGG	
	$V56^{H}G$, $D57^{H}N$	Forward	GGATTGGAAGGATTGATCCTGCGAATGGTAATACTAAA	
		Reverse	CCTTGTCCTGGAACTTCGGGTCATATTTAGTATTACCATTCGCA	
	$K77^{H}N$, $T79^{H}A$	Forward	GCCACTATAACAGCAGATACATCCTCCAACACAGCCTACCTG	
		Reverse	GCTGCTGAGCTGCAGGTAGGCTGTGTTGGAGGA	
	$N30^{L}S$	Forward	CACTTGTCGGGCAAGTCAGGAAATTAGTGGTTACTTAGGC	
		Reverse	CTGCTGAAGCCAGCCTAAGTAACCACTAATTTCCTGACT	
	$H55^{L}D$	Forward	CCTGATCTACGCCGCATCCACTTTAGATTCTGG	
		Reverse	CCACTGAACCTTTTTGGGACACCAGAATCTAAAGTG	
	$G34^{L}S$	Forward	CGGGCAAGTCAGGAAATTAATGGTTACTTAAGCTGGCT	
		Reverse	CATCTGGTTTCTGCTGAAGCCAGCTTAAGTAAC	

Table S3. Primers used to clone 48G7 into pFUSE vectors and to perform site-directed mutagenesis to produce 48G7 mutants

Light Chain	Heavy Chain	Expected Mass	ESI Result
Germline	Germline	47557	47558
Germline	E1Q	47540	47543
Germline	E42K	47556	47560
Germline	G56V	47599	47604
Germline	N57D	47558	47563
Germline	G66D	47615	47620
Germline	N77K	47571	47576
Germline	A79T	47587	*49900**
Germline	G56V,N57D	47600	47606
Germline	N77K,A79T	47601	47606
Mature	Mature	47703	47703
S30N	Germline	47584	47588
S34G	Germline	47527	47532
D55H	Germline	47579	47584
N30S	Mature	47676	47681
G34S	Mature	47733	47737
H55D	Mature	47681	47685
Mature	Q1E	47720	47723
Mature	K42E	47704	47706
Mature	V56G	47661	47663
Mature	D57N	47702	47704
Mature	D66G	47645	47646
Mature	K77N	47689	*49943*
Mature	T79A	47673	47675
Mature	V56G, D57N	47660	47662
Mature	K77N, T79A	47659	47663
N30S	gG56V,N77K	47605	47605
G34S	gG56V,N77K	47662	47662
Mature	gG56V,N77K	47632	47632
gD55H	gG56V,N77K	47635	47636
gD55H	gE1Q,G56V,N77K	47618	47619
gD55H	gE42K,G56V,N77K	47634	47636
gD55H	gG56V,N57D,N77K	47636	47638
gD55H	gG56V,G66D,N77K	47693	47695
gD55H	gG56V,N77K,A79T	47665	47667
gD55H	gG56V,N57D,N77K,A79T	47666	47668
N30S	gG56V,N57D,N77K,A79T	47636	47637
G34S	gG56V,N57D,N77K,A79T	47693	47694
Mature	gG56V,N57D,N77K,A79T	47663	47664
gD55H	gN77K	47593	47598
gD55H	Mature	47706	47706
H55D	K77N	47667	*49913**

Table S4. ESI-TOF MS data. The N-terminal glutamines cyclize, reducing the mass by 16 Da. *Again, the combination of both N77 and T79 introduces a glycosylation site, which results in a higher mass (NetNGlyc 1.0 Server, http://www.cb.dtu.dk/services/NetNGlyc).