Engineered charge redistribution of Gp2 proteins through guided diversity for improved PET imaging of epidermal growth factor receptor

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Library	Pre-sorting Population	Post-sorting Population	T (°C)	Target (nM)	Parental Clone T_m (°C)	Parental Clone K_D (nM)
EGFR	Naïve	#1	60	20	71 ± 2	18 ± 8
	#1	#2a	60	2		
	#1	#2b	80	20		
	#2(a+b)	#3	80	2		
	#3	Final	4	deplete non-specific		
InsR	Naïve	#1	50	20	78	2.4 ± 0.4
	#1	#2a	50	2		
	#1	#2b	60	20		
	#2(a+b)	Final	4	deplete non-specific		
rIgG	Naïve	#1	50	2	80 ± 2	2.3 ± 1.4
	#1	#2	60	2		
	#2	Final	4	deplete non-specific		

Table S1. Library sorting conditions for charge mutated Gp2 libraries with parental clone *in vitro* characteristics.

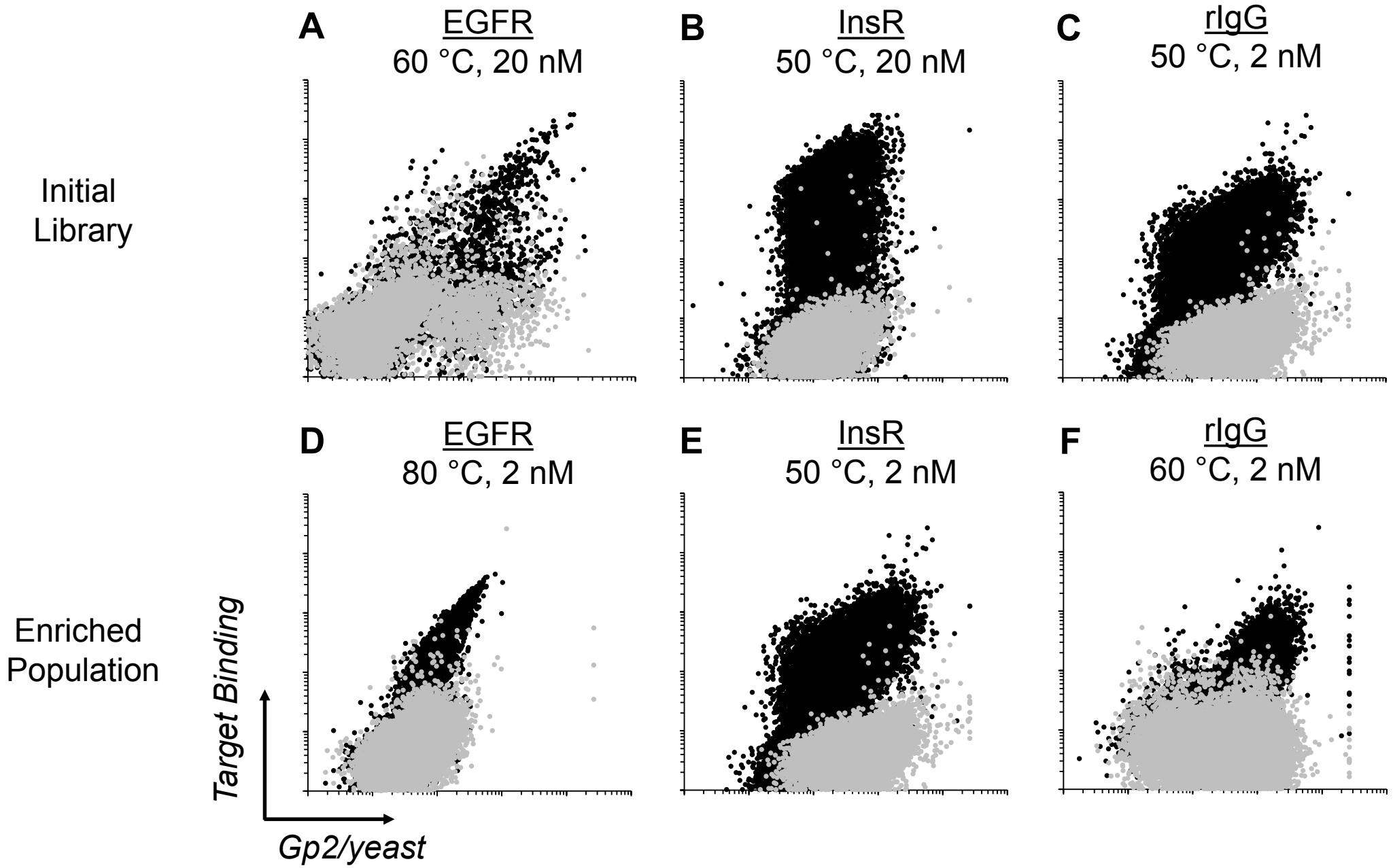


Figure S1. First Generation Library Selections. Naïve **(A, B, C)** and terminal **(D, E, F)** GαE, GαI, and GαR populations sorted for stability and binding with (black) and without (gray) their respective recombinant target.

Name	1	14	20	23	24	27	30	31	42	44	Loop 1	Loop 2	Charge (+/-)	Net Charge	Total Charge	T_m (°C)	K_D (nM)	Yield (mg/L)
GαE22	L	E	T	Y	A	L	H	S	R	P	SRGDSYW	PMYWHIYY	2/2	0	4	60	6.3 ± 1.4	1.8 ± 0.4
GαE24	L	E	Q	D	T	V	E	W	R	Q	SRGDSYW	PMYWHIYY	2/4	-2	6	ND	ND	< 50
GαE25A	L	E	P	D	A	E	E	T	R	P	SRGDSYW	PMYWHIYY	2/5	-3	7	64	4.9 ± 0.7	1.8 ± 1.2
GαE25B	I	E	E	D	P	Q	E	R	Q	P	SRGDSYW	PMYWHIYY	2/5	-3	7	ND	ND	< 50
GαE35	L	E	Q	D	E	K	E	T	R	P	SRGDSYW	PMYWHIYY	3/5	-2	8	87 ± 1	8.8 ± 0.6	2.1 ± 0.5
GαE36	K	E	E	D	E	Q	E	W	R	Q	SRGDSYW	PMYWHIYY	3/6	-3	9	59	2.3 ± 1.1	5.0 ± 0.3
GαE57	K	E	E	D	E	E	E	R	R	R	SRGDSYW	PMYWHIYY	5/7	-2	12	79	9.4 ± 4.1	2.7 ± 0.1

Table S2. Mutated positions, paratope (loop) sequences, and *in vitro* characteristics of Gp2 clones recovered in flow cytometry sorts against EGFR derived from A431 cell lysate. K_D values represent equilibrium dissociation constants from titrations of mutants against A431 cells using flow cytometry (n = 3). Midpoint denaturation temperatures (T_m) were determined with circular dichroism spectroscopy of purified proteins (n = 1 - 3). Yield is reported as recovered mass of purified protein per L of culture (n = 3).

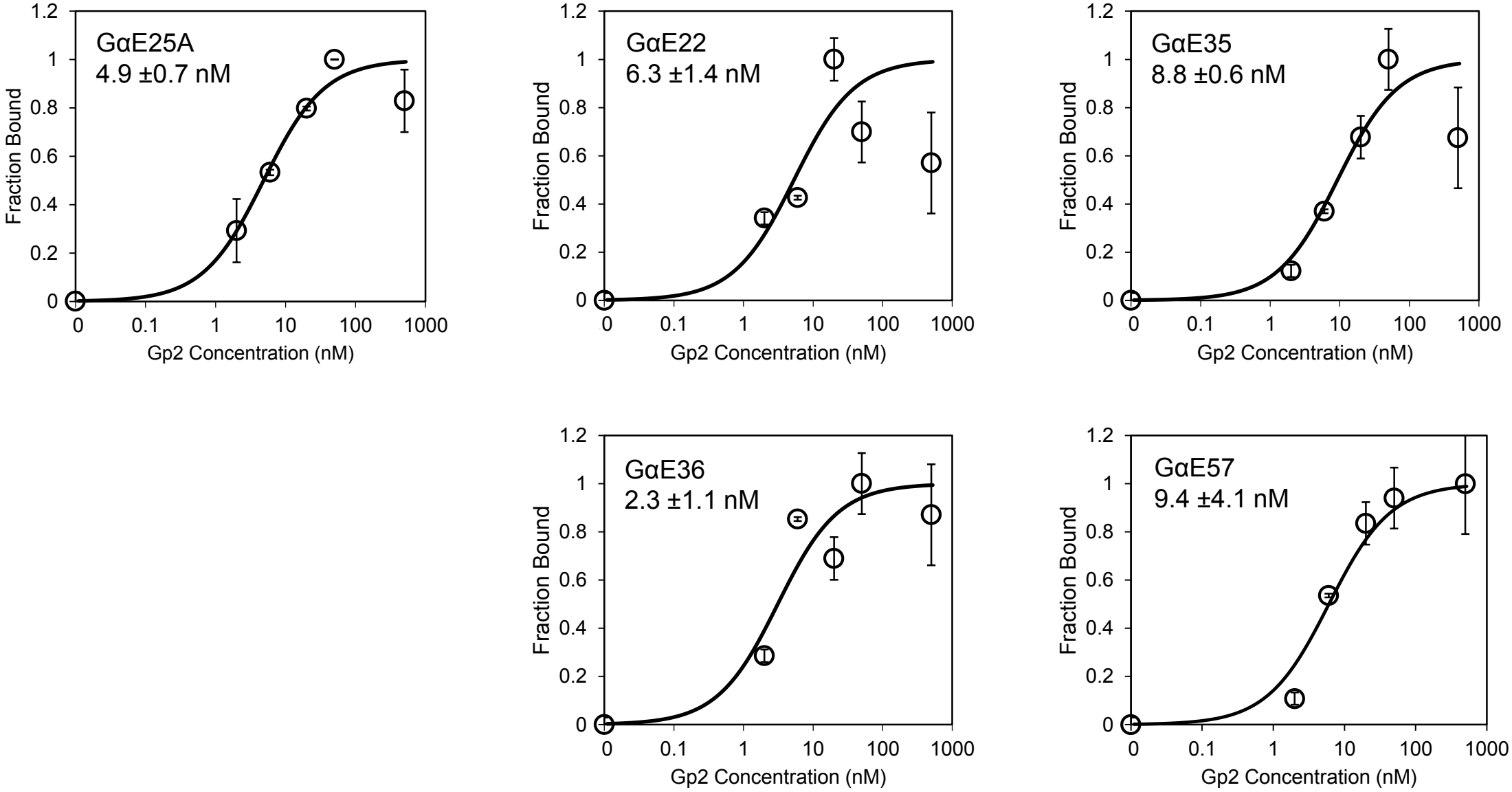


Figure S2. Anti-EGFR Gp2 Affinity titrations. A431 cells were incubated on ice with 2, 6, 20, 50, or 500 nM Gp2 ligand followed by labeling with fluorescein-conjugated anti-His_6 antibody and median fluorescence analysis via flow cytometry. Equilibrium dissociation constants were calculated using a 1:1 binding model (n = 3).

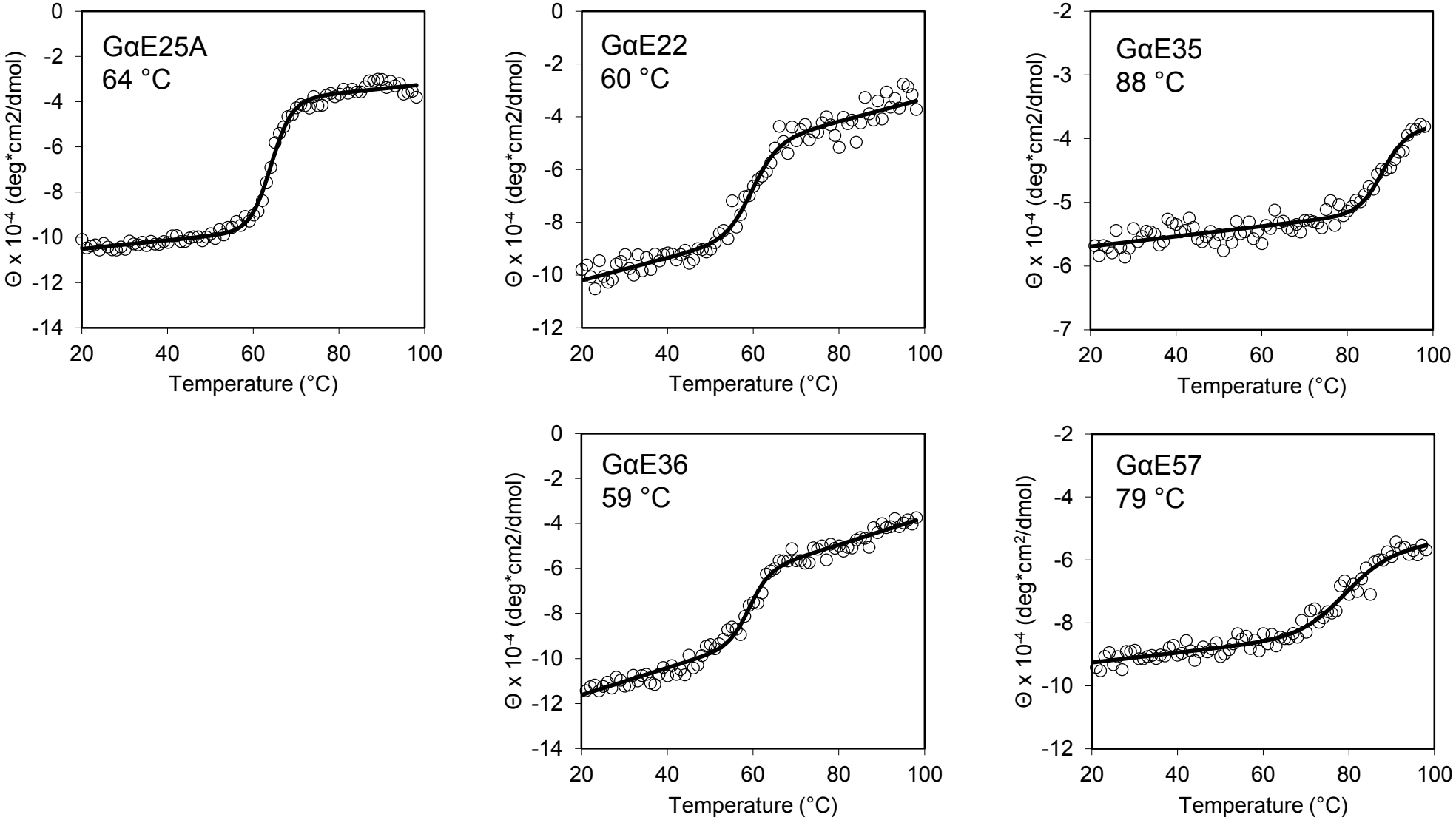


Figure S3. Anti-EGFR Gp2 Thermal denaturation curves. Purified ligands were scanned at a wavelength of 220 nm during heating from 20 to 98 °C (2 °C/min). The midpoint of denaturation (T_m) was determined using a two-state protein unfolding model.

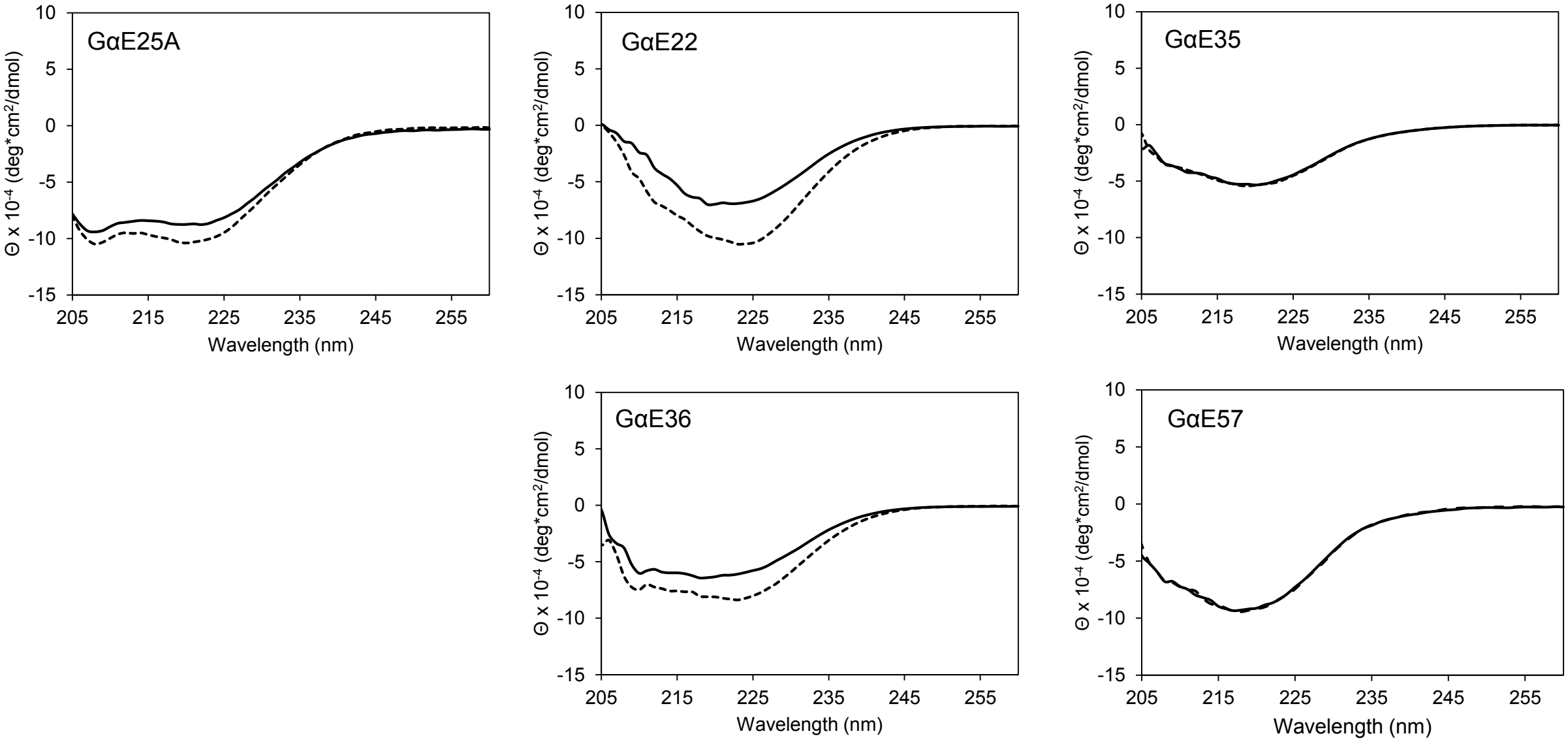
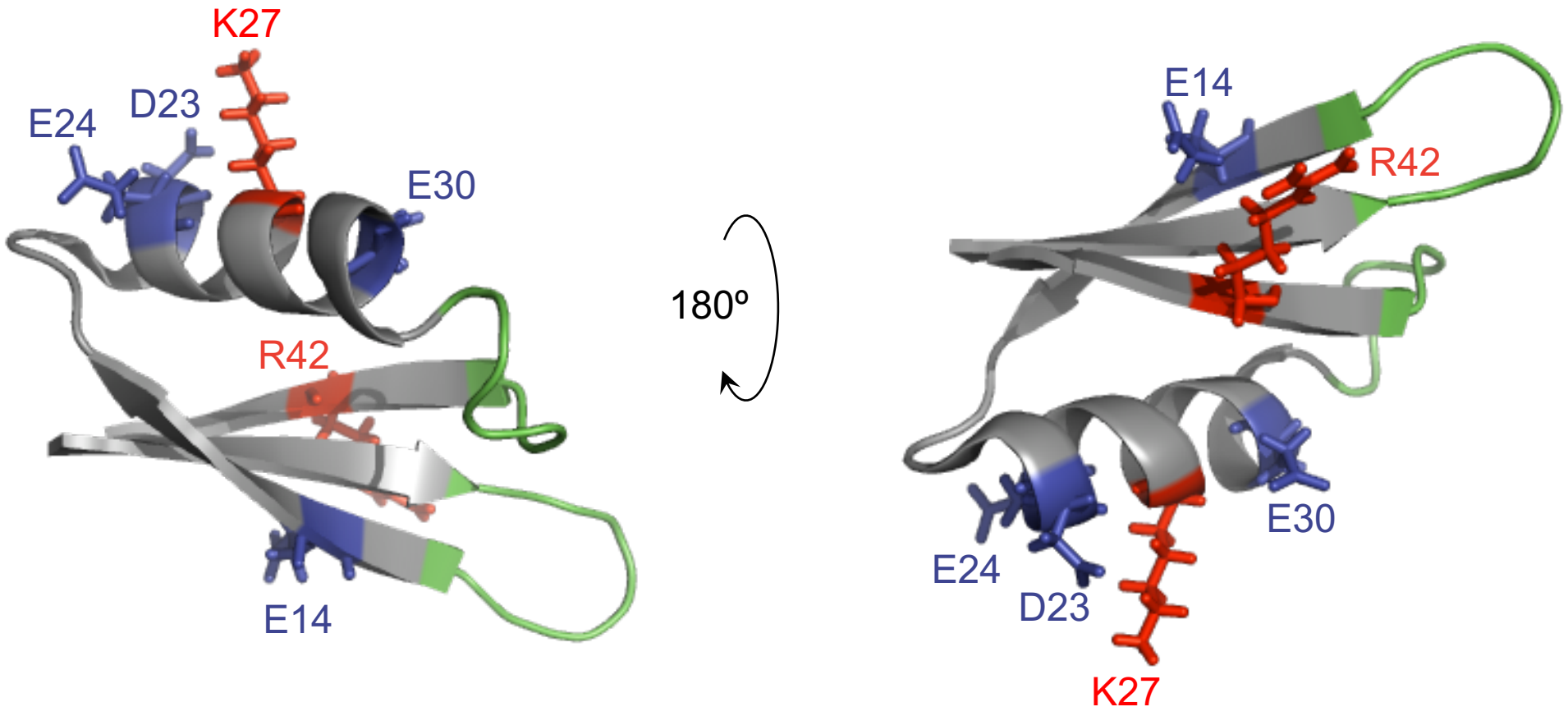


Figure S4. Circular dichroism spectroscopy of recovered mutants. Purified anti-EGFR Gp2 mutants were analyzed by circular dichroism spectroscopy in triplicate between 205 and 260 nm wavelengths before (- - -) and after (——) thermal denaturation and cooling.



1 10 20 30 40

GαE35: L F W A T V S R G D • S Y W F E V P V Y A Q T L D E A L K L A E T Q Y P M Y H • I Y Y V T R V P P

Figure S5. (A) Approximate structure, based on PDB: 2WNM, and sequence of the GαE35 ligand with acidic (blue), basic (red), and paratope (green) residues highlighted.

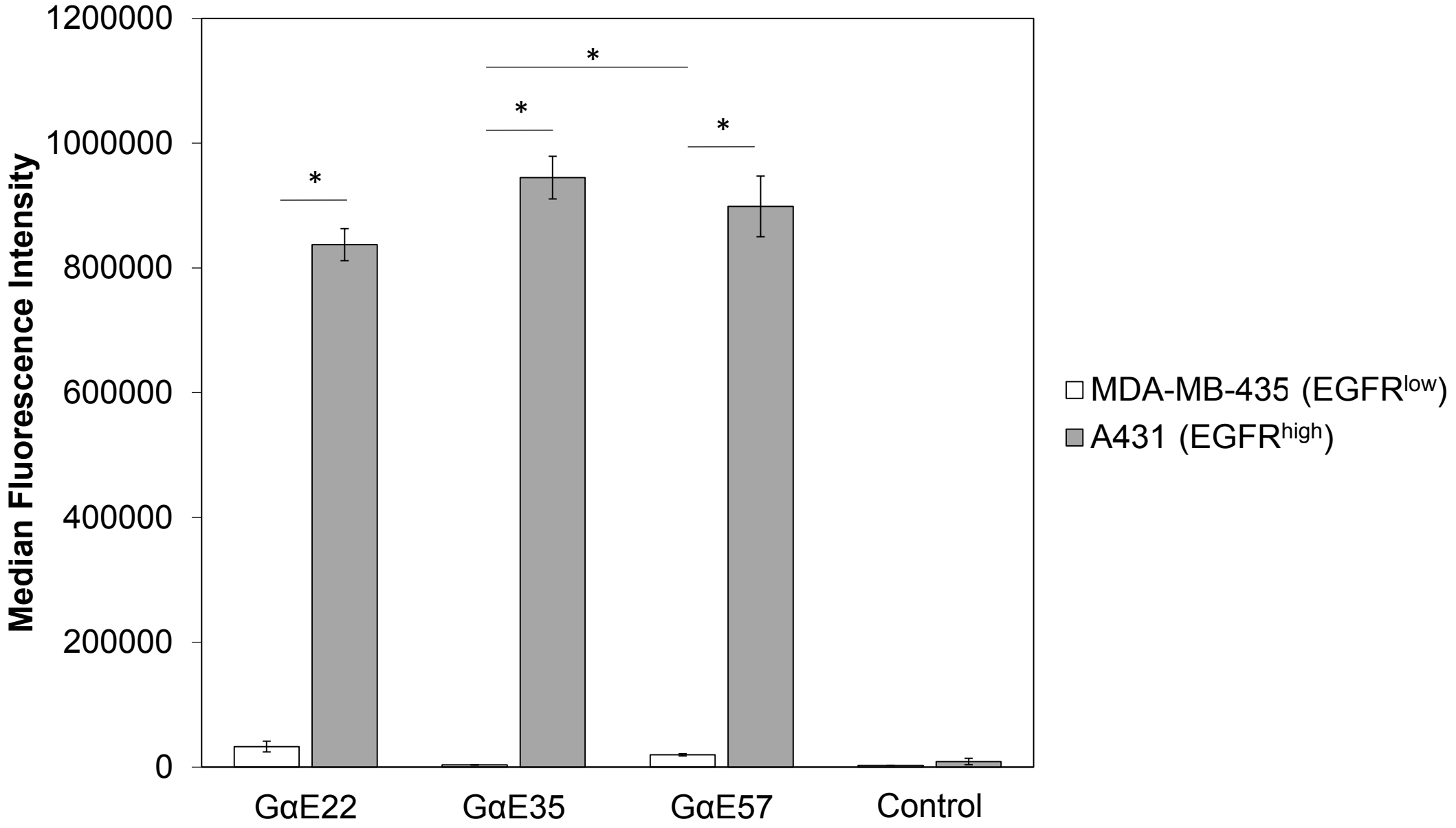


Figure S6. EGFR binding specificity. MDA-MB-435 ($EGFR^{low}$) and A431 ($EGFR^{high}$) cells were incubated on ice with 500 nM Gp2 ligand, labeled with fluorescein-conjugated anti-His_6 antibody, and median fluorescence signal determined with flow cytometry (n = 3) (* signifies $p \leq 0.001$).

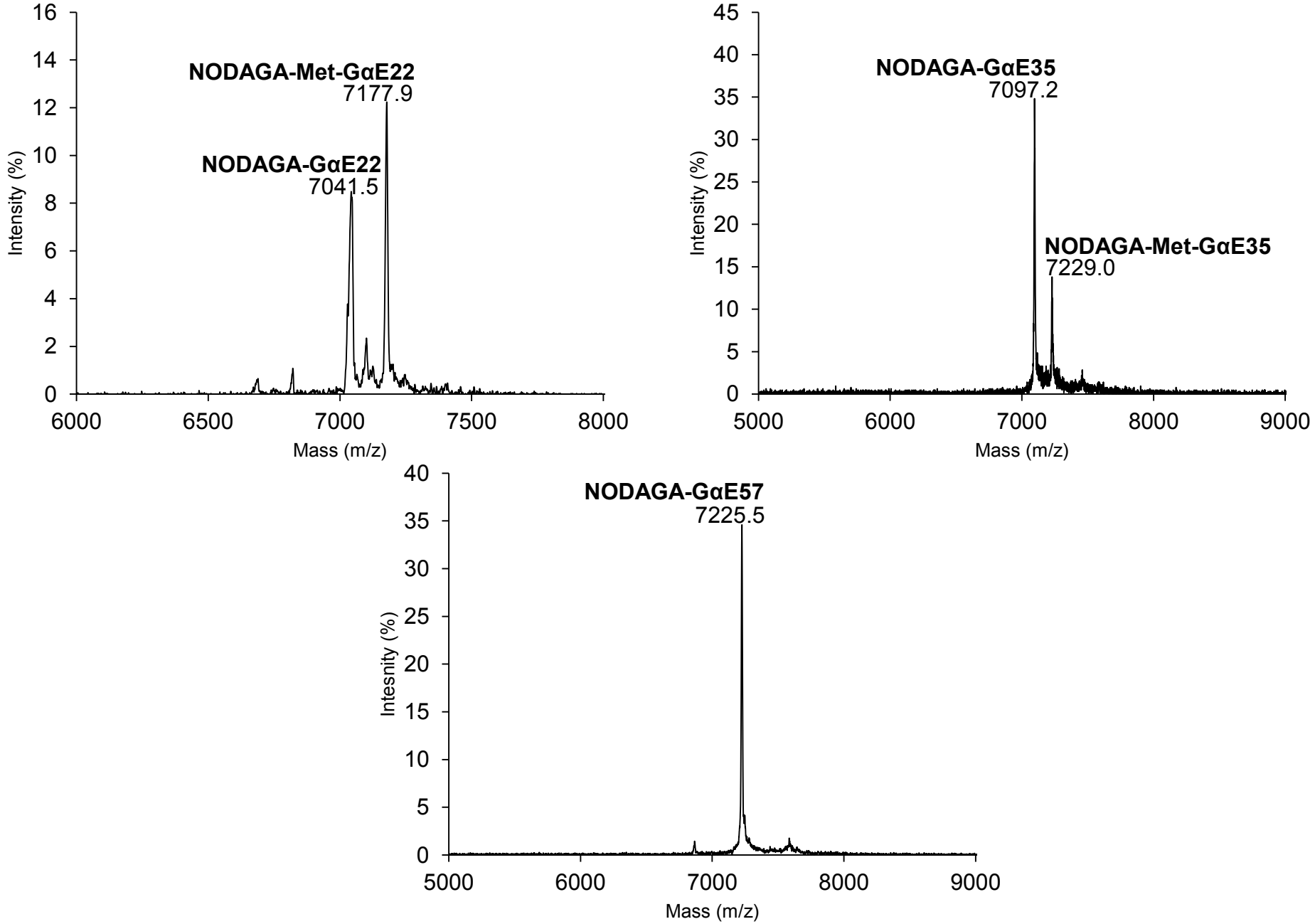


Figure S7. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) of NODAGA-conjugated GαE22, GαE23, and GαE57.

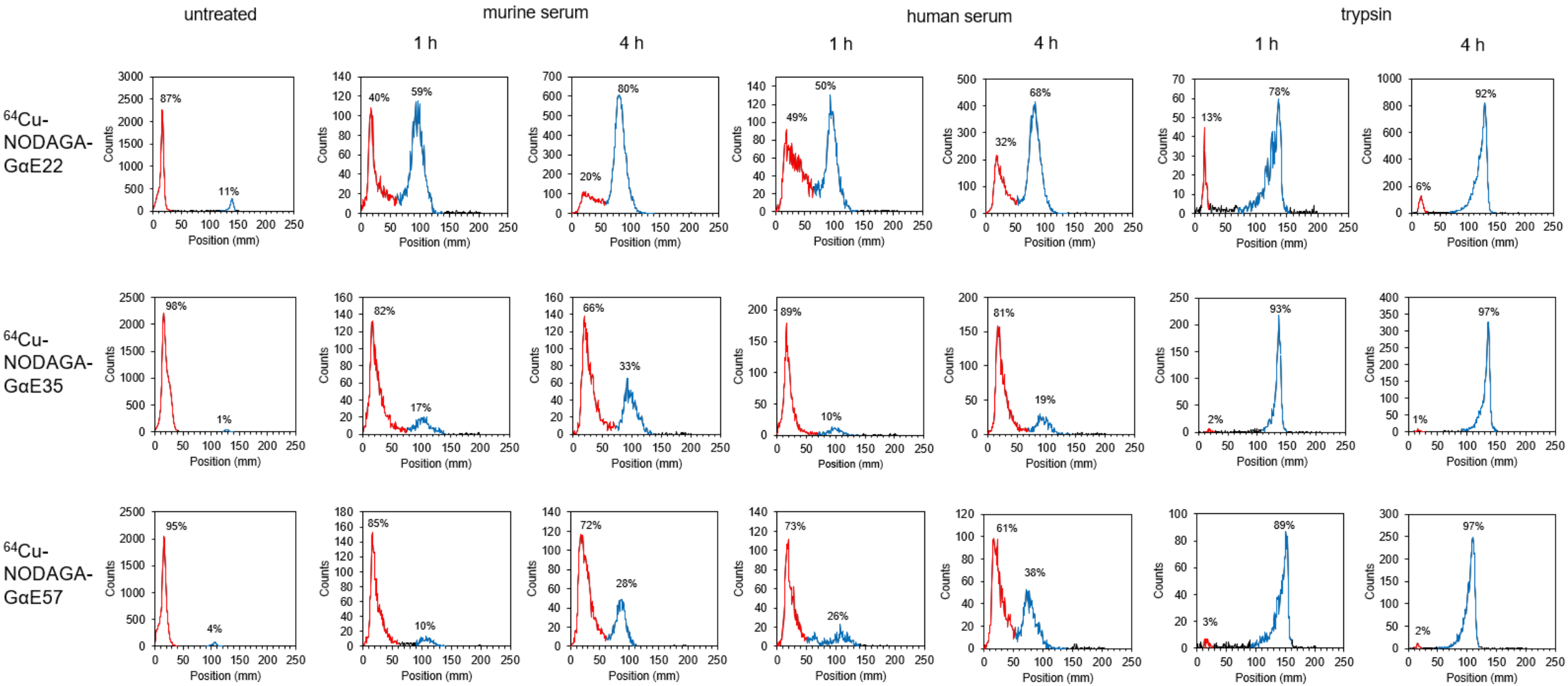


Figure S8. Radio-thin-layer chromatography of radiolabeled GαE clones left untreated, in serum, or trypsin.