Supporting Information

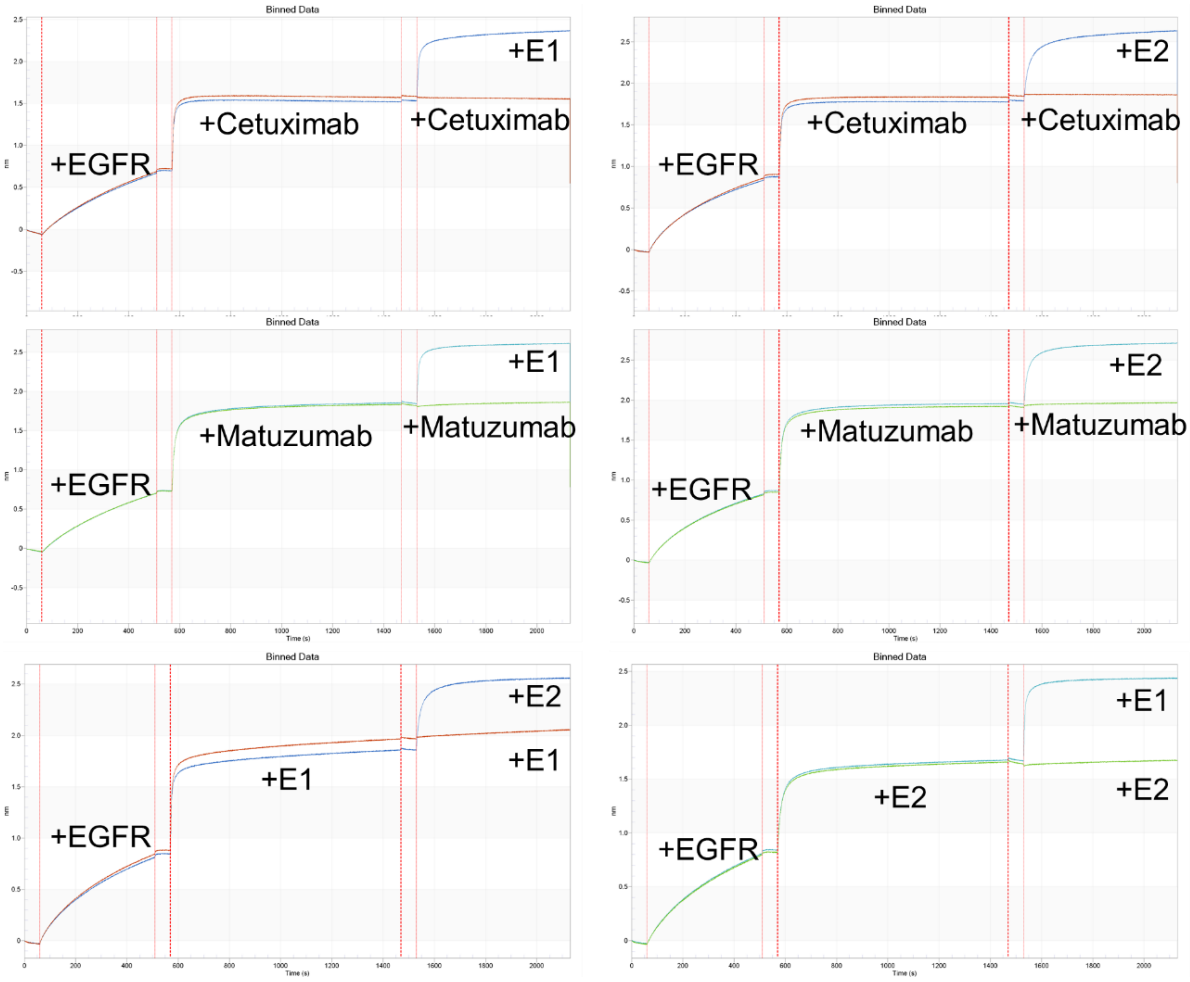


Figure S1: Epitope binning of chicken-derived E1 and E2 against Cetuximab, Matuzumab and each other in the Tandem Setup. Ni-NTA tips were loaded with 10 µg/mL His-Tagged EGFR (produced in house). Subsequently, 400 nM of the first antibody was applied for 900 seconds followed by applying either the same antibody sample again (control for saturation) or the second antibody of interest. An increment in layer thickness indicate orthogonal epitopes. E1 and E2 scFv-Fc fusions overlap neither with Cetuximab nor Matuzumab nor with each other.

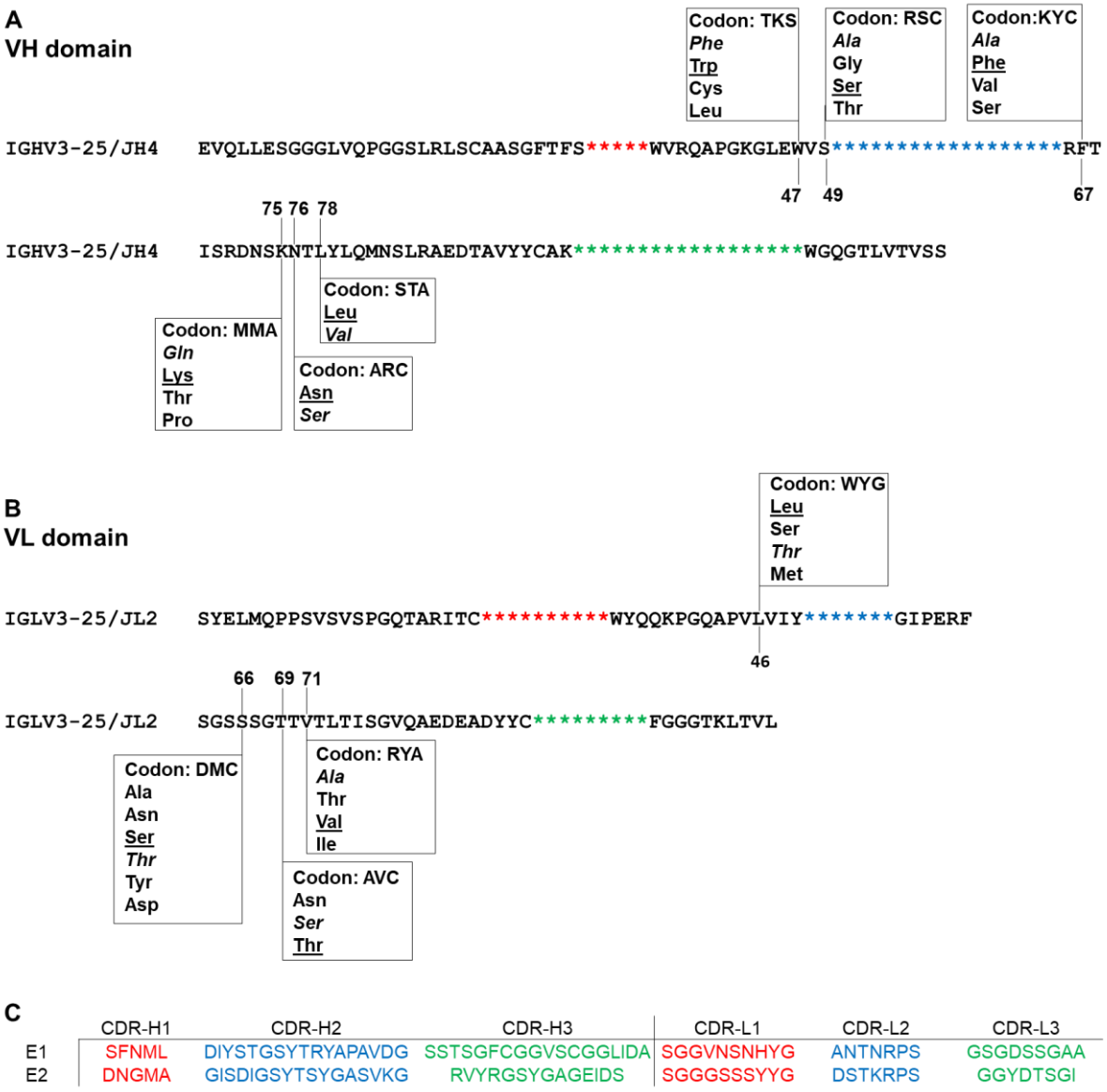


Figure S2: Library design. The human acceptor germline sequences for VH **(A)** and VL **(B)** are shown. CDRs a highlighted in red (CDR1), blue (CDR2) and green (CDR3). Vernier residues are marked in respect to the Kabat numbering scheme. Codons for partial randomization of Vernier residues are specified. Possible encoded residues are shown in italic (chicken germline), underlined (human germline) or plain (non-chicken/non-human residue). **(C)** CDR sequences of E1 and E2.

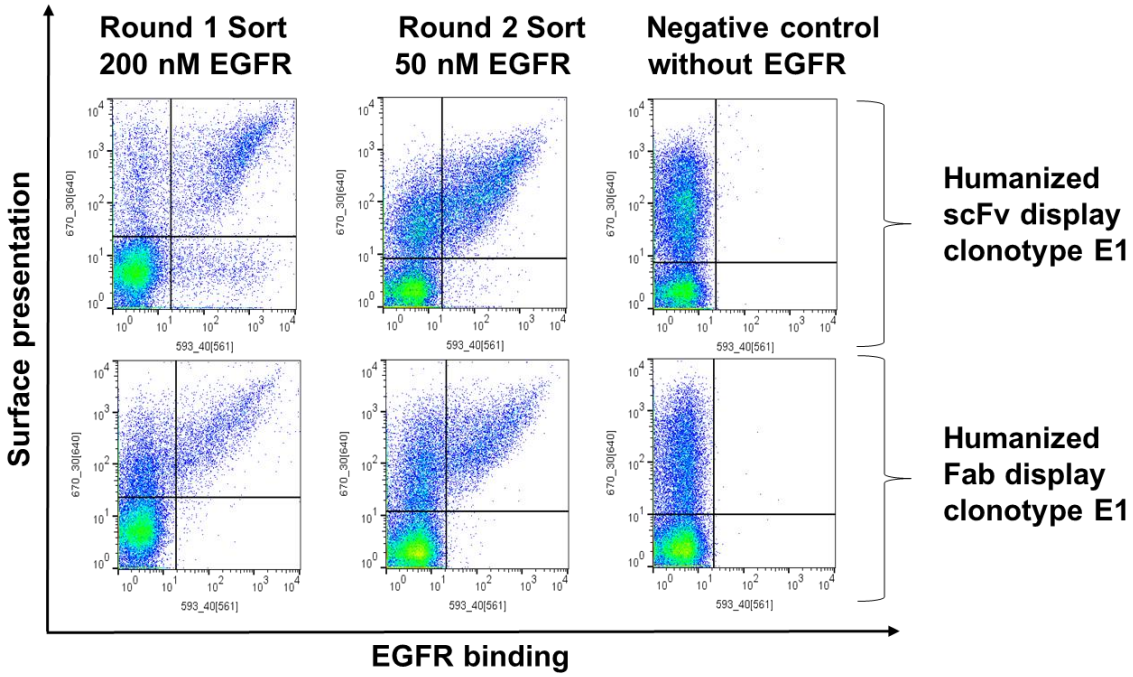


Figure S3: YSD Library screening of humanized chicken antibodies of the E1 clonotype. Cells showing both surface presentation (c-myc tag detection for scFvs or anti-λ staining for Fabs) and EGFR-Fc binding signal (Fc domain detection) were sorted according to the depicted gating strategy.

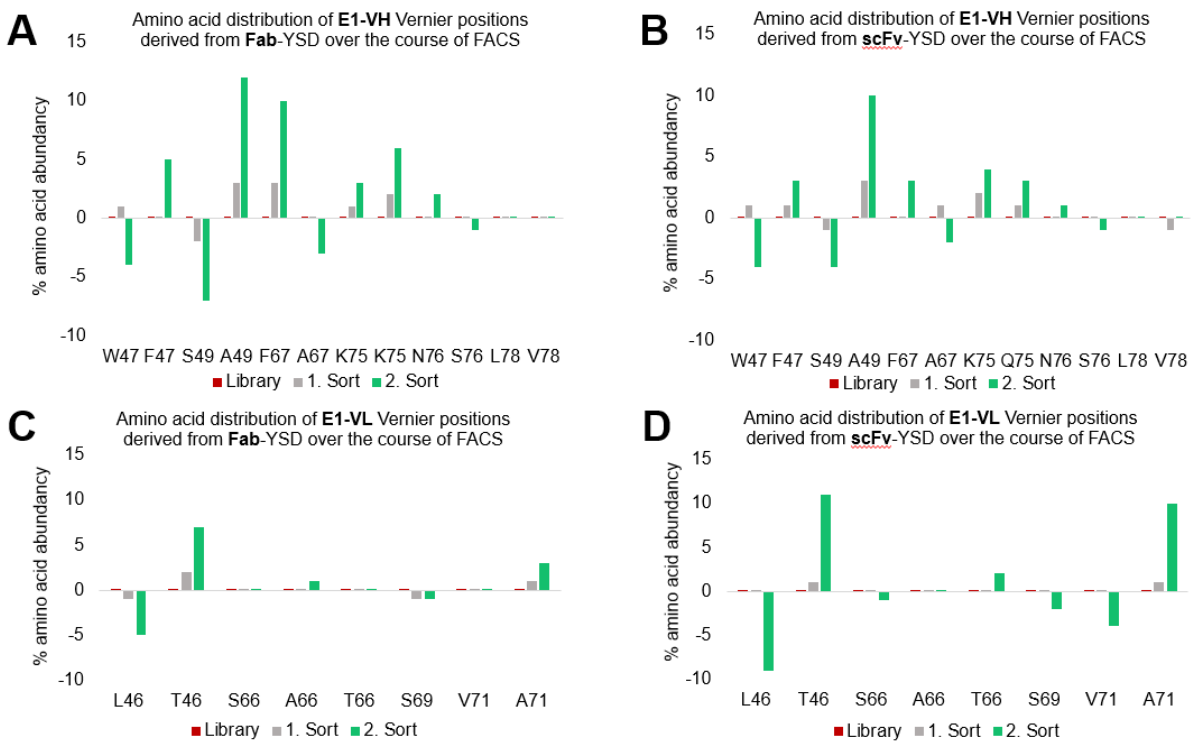


Figure S4: Next generation sequencing analysis of the yeast surface display library, 1st and 2nd sort outcome populations corresponding to clonotype E1. Amino acid abundancy, after one and two FACS rounds of E1-derived humanized variants corresponding to antibody formats Fab **(A, C)** and scFv **(B, D)**, relative to the respective amino acid abundancy of the initial library. **(A)** VH-E1 amino acid abundancies derived from Fab-sequencing and **(B)** VH-E1 amino acid abundancies corresponding to scFv-sequencing. **(C)** VL-E1 amino acid abundancies derived from Fab-sequencing and **(D)** VH-E1 amino acid abundancies corresponding to scFv-sequencing.

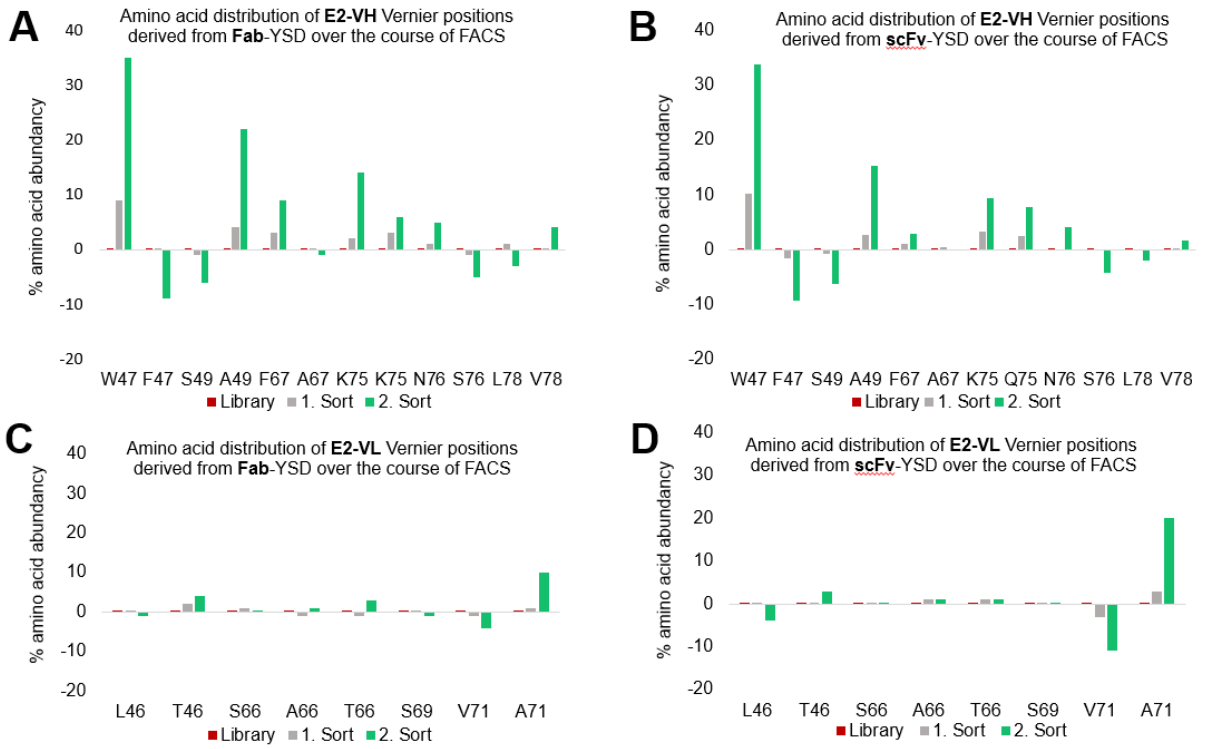


Figure S5: Next generation sequencing analysis of the yeast surface display library, 1st and 2nd sort outcome populations corresponding to clonotype E2. Amino acid abundancy, after one and two FACS rounds of E2-derived humanized variants corresponding to antibody formats Fab **(A, C)** and scFv **(B, D)**, relative to the respective amino acid abundancy of the initial library. **(A)** VH-E2 amino acid abundancies derived from Fab-sequencing and **(B)** VH-E2 amino acid abundancies corresponding to scFv-sequencing. **(C)** VL-E2 amino acid abundancies derived from Fab-sequencing and **(D)** VH-E2 amino acid abundancies corresponding to scFv-sequencing.

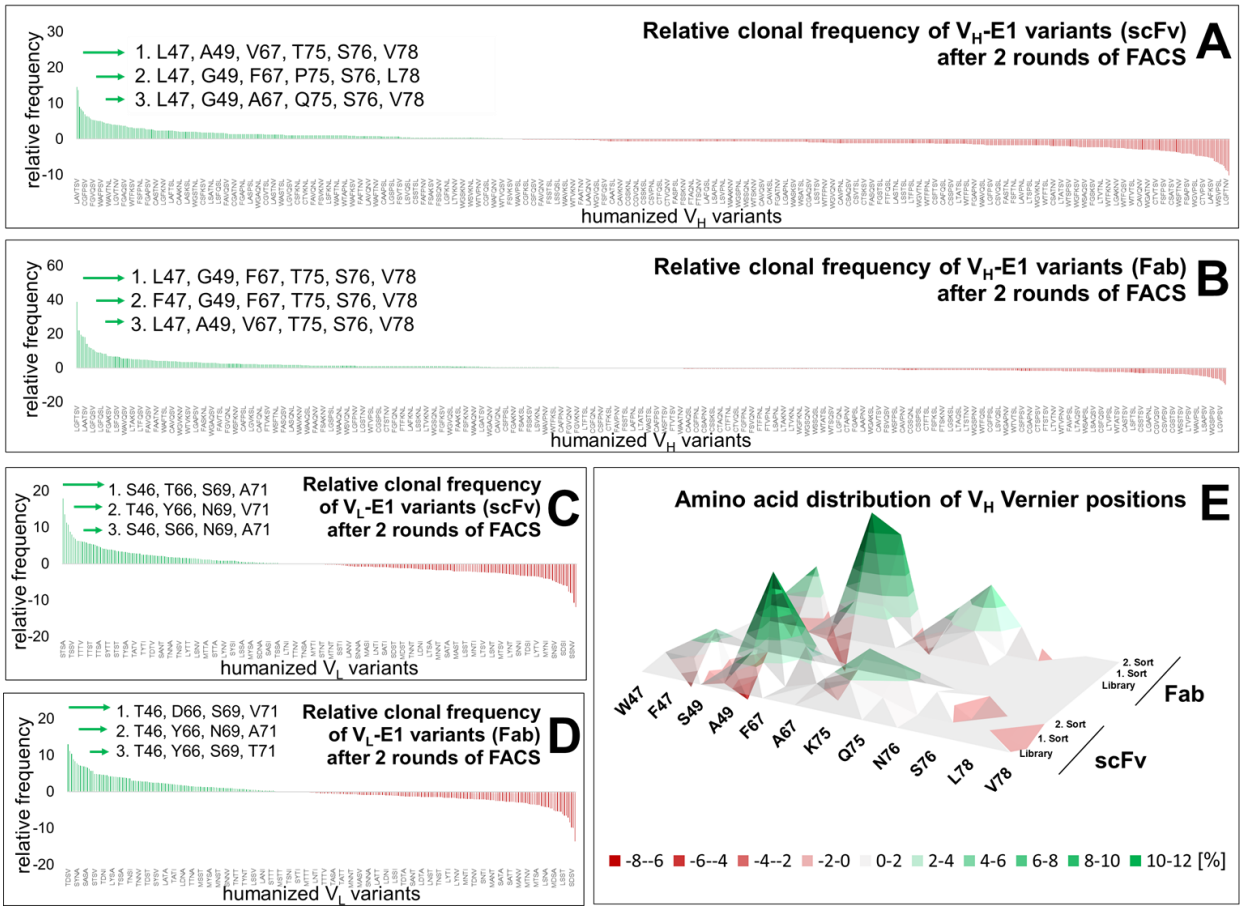


Figure S6: Next generation sequencing analysis of humanized clones corresponding to clonotype E1. Clonal frequencies, after two FACS rounds of VH-E1 variants corresponding to antibody formats scFv **(A)** and Fab **(B)**, relative to the respective clonal frequencies of the initial library sequencing. Clonal frequencies, after two FACS rounds of VL-E1 variants corresponding to **(C)** scFv and **(D)** Fabs, relative to the respective clonal frequencies of the initial library sequencing. Most frequent humanized VH and VL variants (represented by their different Vernier position amino acids) are highlighted by green arrows. **(E)** Analysis of amino acid distribution corresponding to VH Vernier positions. Sort-subordinated enriched (green) or decreased (red) proportions [%] of human- and chicken-germline amino acids on Vernier positions, normalized to their respective initial library abundancies.

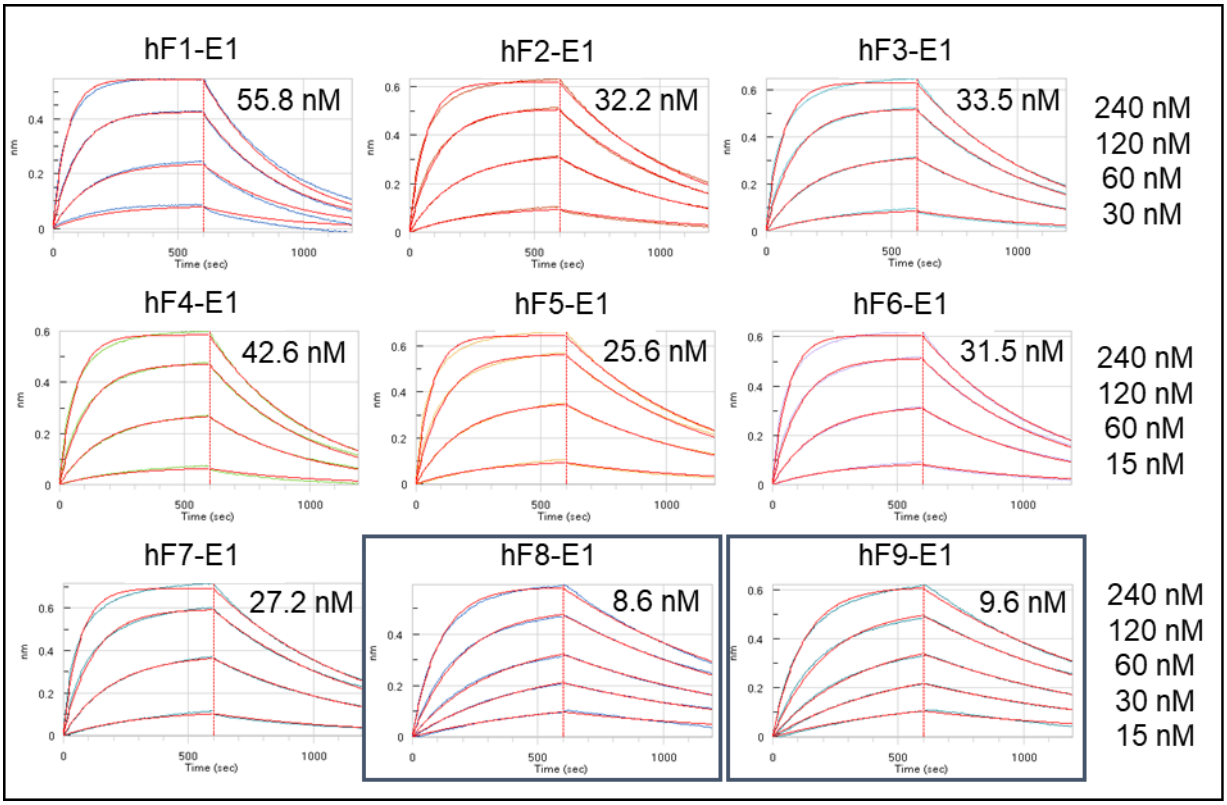


Figure S7: Determination of binding kinetics of humanized full-length antibodies corresponding to clonotype E1 (hF(1-9)-E1). Cell culture supernatant was loaded to Anti-human IgG Fc Capture (AHC) Biosensors. Binding kinetics of highlighted variants (hF8-E1, hF9-E1) were evaluated using purified protein samples. Quenching and dissociation steps were performed using Kinetics buffer. Binding intensities corresponding to EGFR-ECD concentrations ranging from 15 nM to 240 nM, depending on the affinity of the respective antibody variant was used for the determination of binding kinetics. Binding kinetics were determined based on Savitzky-Golay filtering and a 1:1 Langmuir binding model using FortéBio data analysis software 9.0.

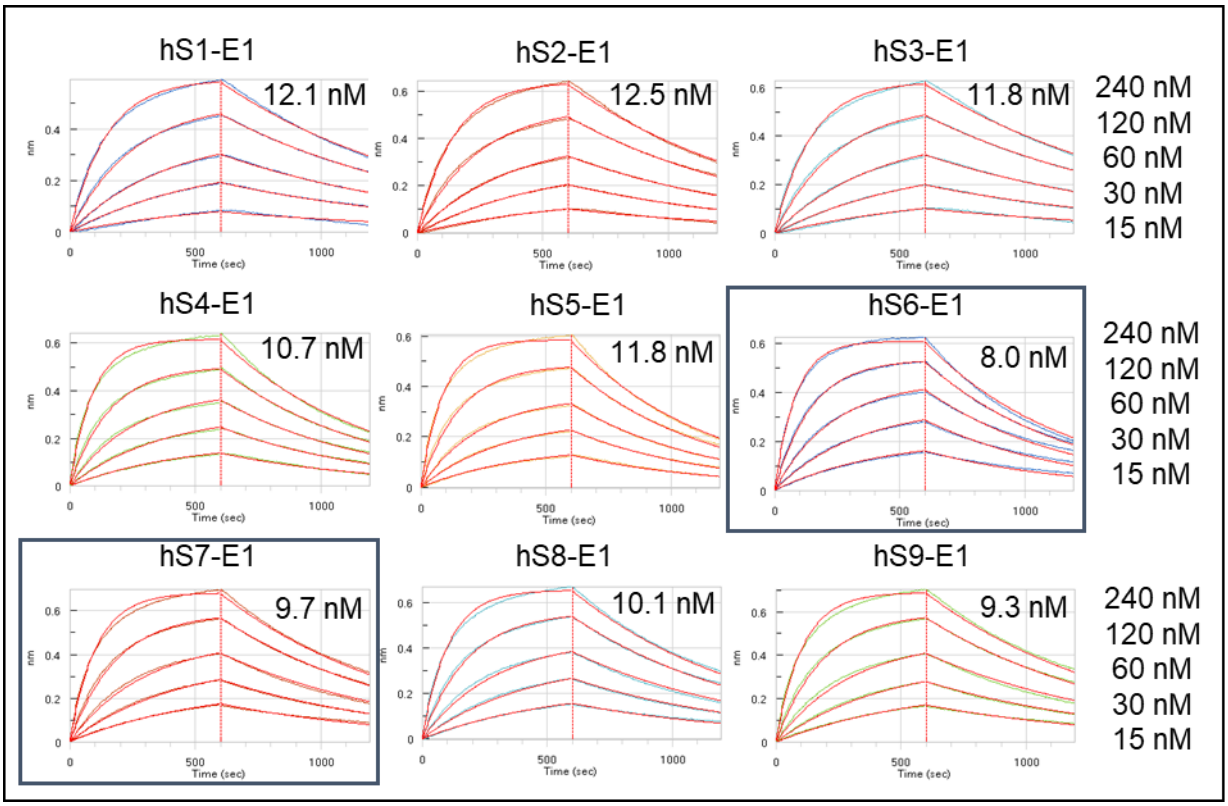


Figure S8: Determination of binding kinetics of humanized scFv-Fc variants corresponding to clonotype E1 (hS(1-9)-E1). Cell culture supernatant was loaded to Anti-human IgG Fc Capture (AHC) Biosensors. Binding kinetics of highlighted variants (hS6-E1, hS7-E1) were evaluated using purified protein samples. Quenching and dissociation steps were performed using Kinetics buffer. Binding intensities corresponding to EGFR-ECD concentrations ranging from 15 nM to 240 nM, depending on the affinity of the respective antibody variant was used for the determination of binding kinetics. Binding kinetics were determined based on Savitzky-Golay filtering and a 1:1 Langmuir binding model using FortéBio data analysis software 9.0.

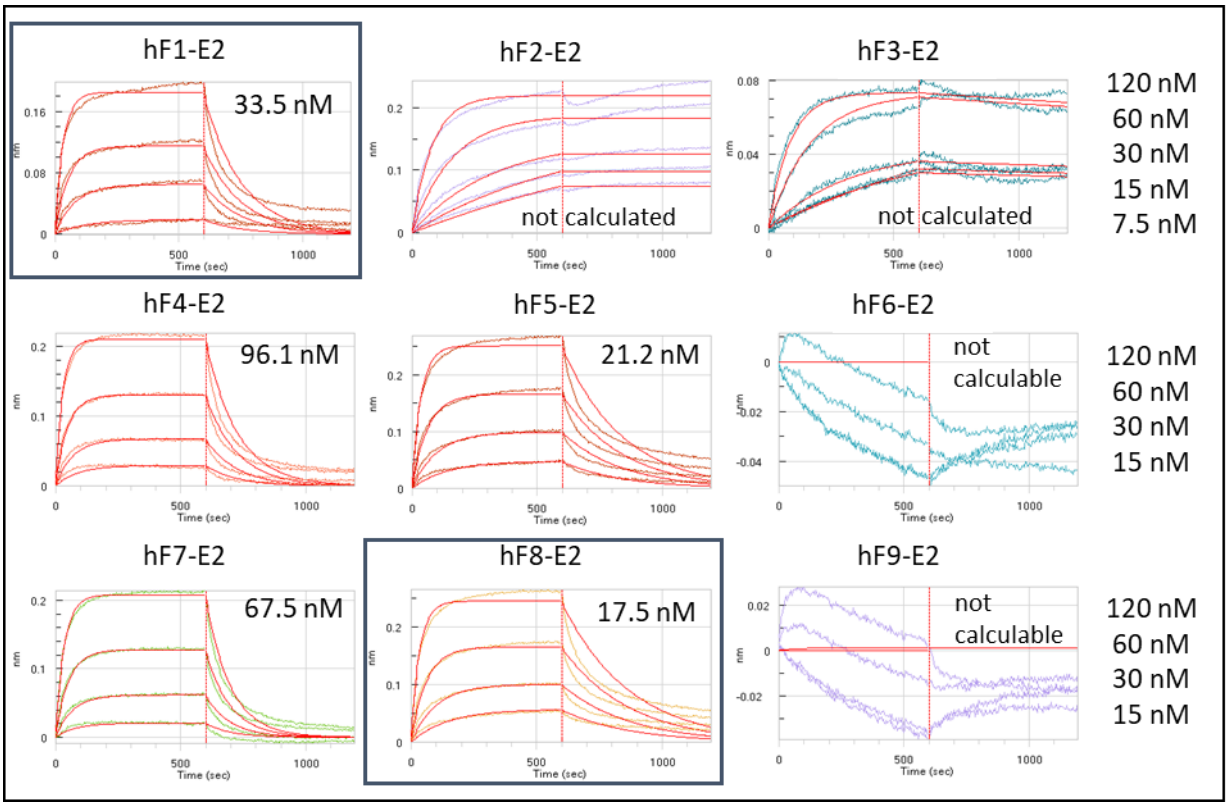


Figure S9: Determination of binding kinetics of humanized full-lenght antibodies corresponding to clonotype E2 (hF(1-9)-E2). Cell culture supernatant was loaded to Anti-human IgG Fc Capture (AHC) Biosensors. Binding kinetics of highlighted variants (hF1-E2, hF8-E2) were evaluated using purified protein samples. Quenching and dissociation steps were performed using Kinetics buffer. Binding intensities corresponding to EGFR-ECD concentrations ranging from 15 nM to 240 nM, depending on the affinity of the respective antibody variant was used for the determination of binding kinetics. Binding kinetics were determined based on Savitzky-Golay filtering and a 1:1 Langmuir binding model using FortéBio data analysis software 9.0.

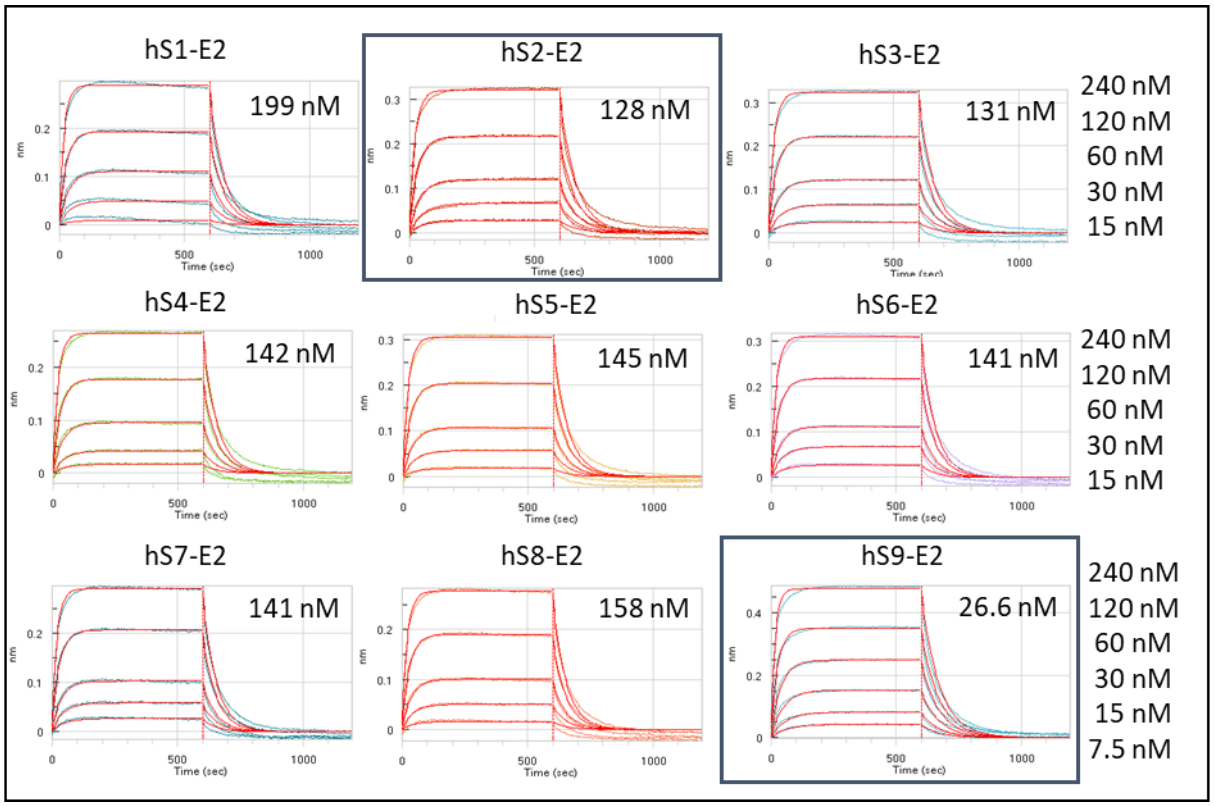


Figure S10: Determination of binding kinetics of humanized scFv-Fc variants corresponding to clonotype E2 (hS(1-9)-E2). Cell culture supernatant was loaded to Anti-human IgG Fc Capture (AHC) Biosensors. Binding kinetics of highlighted variants (hS2-E2, hS9-E2) were evaluated using purified protein samples. Quenching and dissociation steps were performed using Kinetics buffer. Binding intensities corresponding to EGFR-ECD concentrations ranging from 15 nM to 240 nM, depending on the affinity of the respective antibody variant was used for the determination of binding kinetics. Binding kinetics were determined based on Savitzky-Golay filtering and a 1:1 Langmuir binding model using FortéBio data analysis software 9.0.

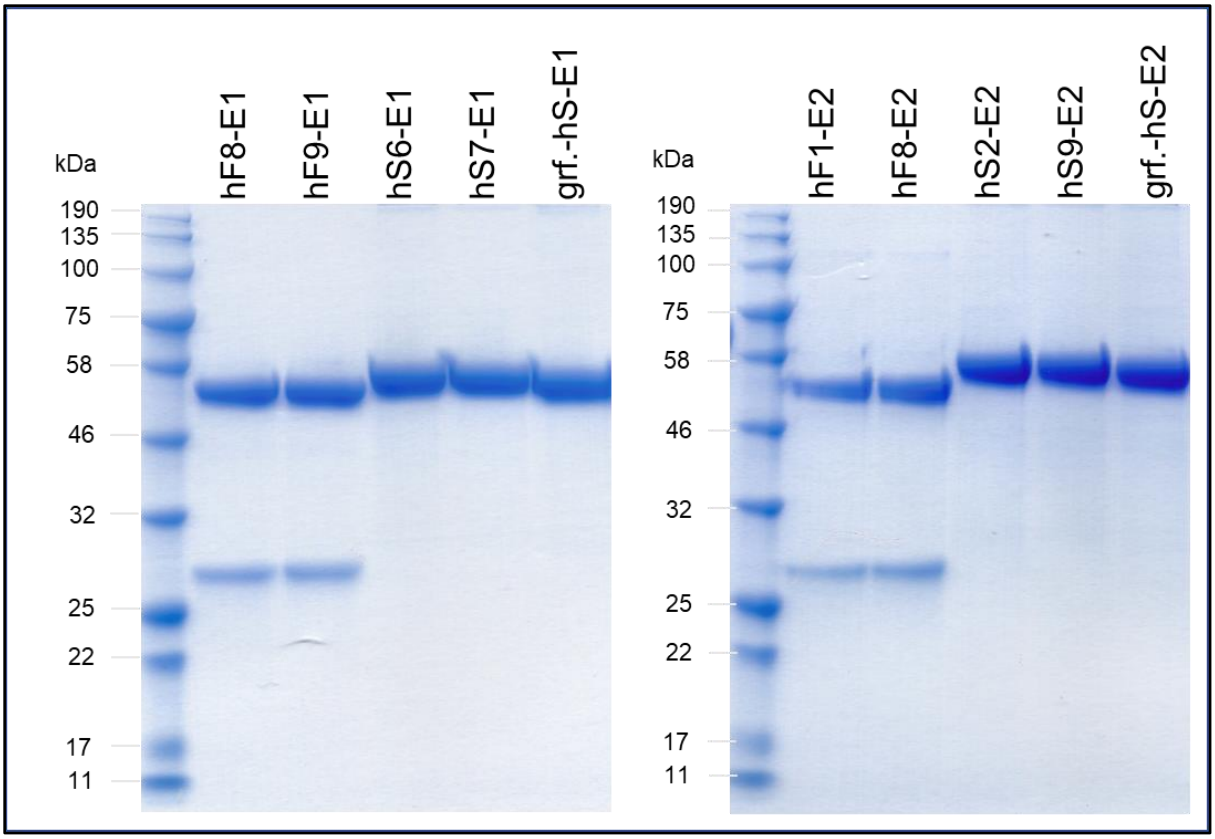


Figure S11: SDS PAGE analysis was performed using a 4–20% precast polyacrylamide gel (Biorad). 5 µg of protein sample was analyzed under reducing conditions. Blue Prestained Protein Standard Broad Range (11-190 kDa) (M) (Biorad) was used. Coomassie Brilliant Blue Staining was performed.

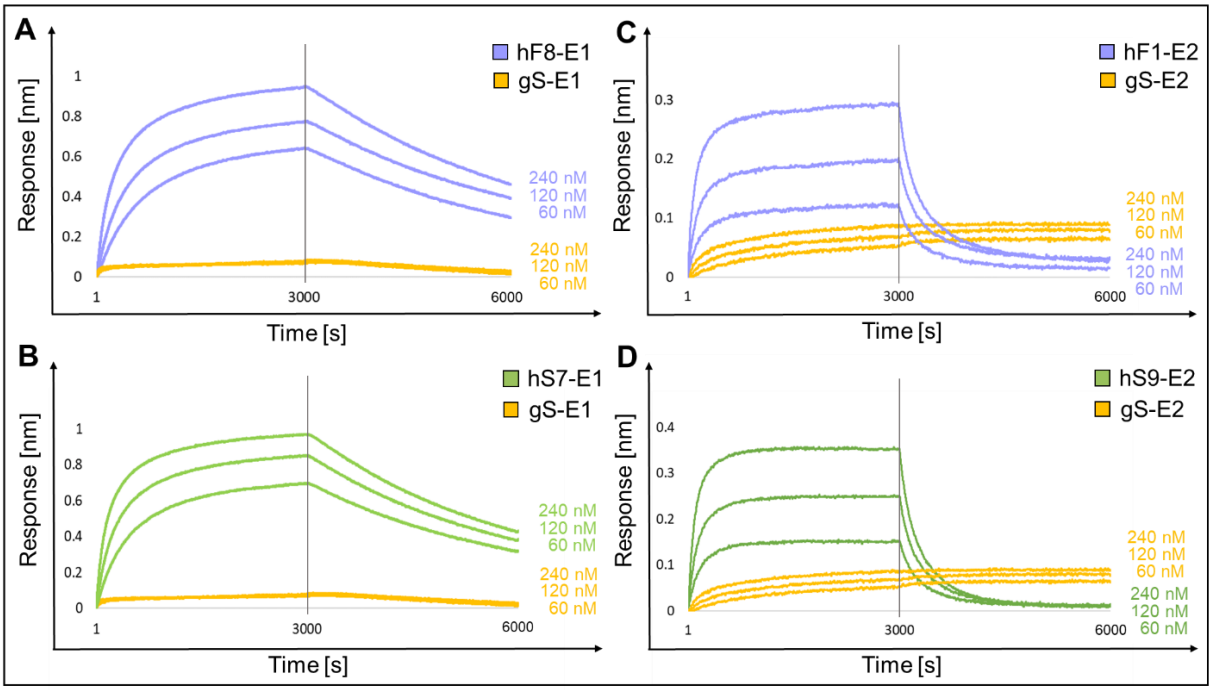


Figure S12. Biolayer interferometry analysis of the humanized full-length antibody hF8-E1 **(A)** and the humanized scFv-Fc fusion protein hS7-E1 **(B)** in comparison with the grafted scFv-E1 (gS-E1)**.** Biolayer interferometry analysis of the humanized full-length antibody hF1-E2 **(C)** and the humanized scFv-Fc fusion protein hS9-E2 **(B)** in comparison with the grafted scFv-E2 (gS-E2).

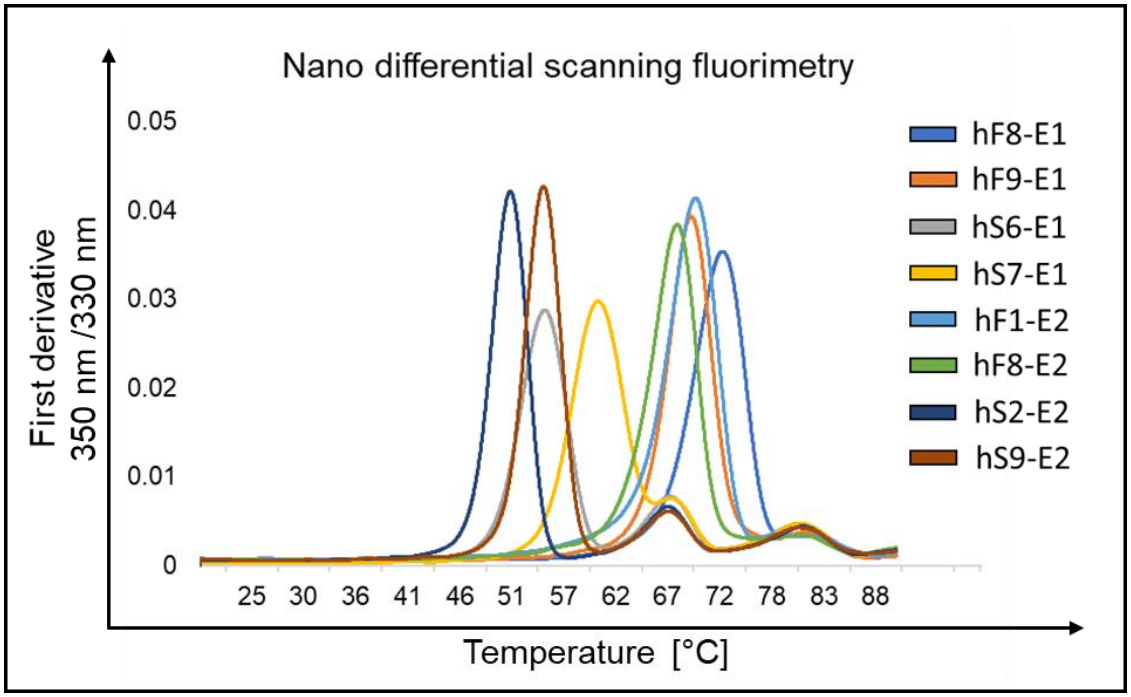
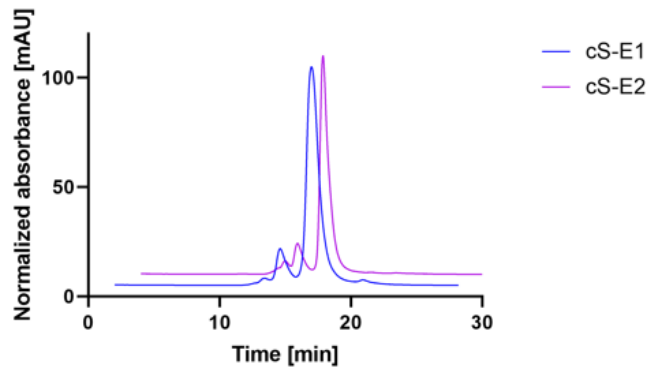


Figure S13: Melting point determination was performed using nano differential fluorimetry measurement. 10 µl of a 0.4 mg/ml protein solution was applied to the capillaries. First derivative of the ratio 350 nm / 330 nm was calculated and set in relation to temperature gradient 1°C/min ranging from 20°C to 90°C. Data processing was performed using the Prometheus ThermControl v.2.1.2 software.

A



B

Clonotype E1	Aggregates [%]
hF8-E1	0.00
hF9-E1	2.98
hS6-E1	21.44
hS7-E1	25.53
cS-E1	9.5

C

Clonotype E2	Aggregates [%]
hF1-E2	25.23
hF8-E2	44.29
hS2-E2	6.85
hS9-E2	3.55
cS-E2	8.70

Figure S14: (A) Determination of protein aggregation behavior by size exclusion chromatography of chicken scFv-Fc protein E1 and E2 (cS-E1, cS-E2) and the proportion of aggregates (%) **(B, C)**. 10 µg (25 µl of 0.4 mg/ml protein solution) was injected on a TSKgel SuperSW3000 column (Tosoh) applying a flow rate of 0.35 ml/min. Peak integration was performed using Agilent OpenLab CDS software. Chromatography data are subject from Grzeschik and coworkers (Reference 29). Proportion of aggregates (%) of humanized variants **(B)** hF8-E1, hF9-E1, hS6-E1, hS7-E1 and **(C)** hF1-E2, hF8-E2, hS2-E2, hS9-E2 corresponding to the respective size exclusion chromatograms (Figure 3).

Table S1. Oligonucleotide sequences for library generation.

Clonotype	Library type	Resulting template	Primer Name	Sequence (5'-3')
E1/E2	Fab/scFv	VH	E1+2 VH Fr1 for	GAGGTGCAGCTGTTGGAGTC TGGGGGAGGCTTGGTACAGC CTGGGGGGTCCCTGAGACTC TCC
E1	Fab/scFv	VH	E1 VH Fr1 CDR1 FR2 for	GCCTGGGGGGTCCCTGAGA CTCTCCTGTGCAGCCTCTGG ATTCACCTTTAGCAGCTTCAA CATGCTCTGGGTCCGCC
E1	Fab/scFv	VH	E1 VH CDR1 Fr2 CDR2 for	CAACATGCTCTGGGTCCGCC AGGCTCCAGGGAAGGGGCT GGAGTKSGTTRSCGACATTTA CAGCACTGGTAGTTAC
E1	Fab/scFv	VH	E1 VH CDR2 for	CAGCACTGGTAGTTACACGA GATACGCGCCGGCGGTGGAT GGCC
E1	Fab/scFv	VH	E1 VH CDR2 Fr3 for	CGCGCCGGCGGTGGATGGC CGGKYCACCATCTCCAGAGA CAATTCCMMAARCACGSTAT ATCTGCAAATGAACAGCCTG AGAGCCG
E1	Fab/scFv	VH	E1 VH Fr3 CDR3 for	CTGCAAATGAACAGCCTGAG AGCCGAGGACACGGCCGTAT ATTACTGTGCGAAAAGTTCTA CTAGTGGTTTTTGTGGTGGT GTTAGTTG
E1	Fab/scFv	VH	E1 VH CDR3 Fr4 rev	TGAGGAGACGGTGACCAGG GTTCCTTGGCCCCATGCGTC GATGAGTCCGCCACAACTAA CACCACCACAAAAACCACTA GTAGAACT
E1/E2	Fab	VH	VH chicken hum for GGA	GCGCGCGCGGTCTCAAGGT GAGGTGCAGCTGTTGGAGTC TGGGGG
E1/E2	scFv	VH	VH Chicken hum for scFv	GGTGGTGGTGGTTCTGGTGG TGGTGGTTCTGCTAGCGAGG TGCAGCTGTTGGAGTCTGGG GG
E1/E2	scFv	VH	VH chicken hum rev scFv	TCCGCCCCCCGACCCGCCG CCGCCTGAGCCGCCTCCCCC TGAGGAGACGGTGACCAGG GTTCCTTG
E1/E2	Fab	VH	VH chicken hum rev GGA	GCGCGCTGGTCTCTTAGTAG AAGCTGAGGAGACGGTGACC AGGGTTCCTTG

E1/E2	Fab/scFv	VL	E1+2 VL Fr1 for	TCCTATGAGCTGATGCAGCC ACCCTCGGTGTCAGTGTCCC CAGGACAGACGGCCAGGATC ACCTGCTCC
E1	Fab/scFv	VL	E1 VL Fr1 CDR1 Fr2 for	GACGGCCAGGATCACCTGCT CCGGGGGTGTTAACAGCAAC CACTATGGCTGGTACCAGCA GAAGCCAGGCCAGGCCC
E1	Fab/scFv	VL	E1 VL Fr2 CDR2 Fr3 for	GCAGAAGCCAGGCCAGGCC CCTGTGWYGGTGATATATGC TAACACCAACAGGCCCTCGG GGATCCCTGAGCGATTCTC
E1	Fab/scFv	VL	E1+2 VL Fr3 for	GGGATCCCTGAGCGATTCTC TGGCTCCDMCTCAGGGAVCA CARYAACGTTGACCATCAGT GGAGTCCAGGCAGAAG
E1	Fab/scFv	VL	E1 VL Fr3 CDR3 for	CAGTGGAGTCCAGGCAGAAG ATGAGGCTGACTATTACTGTG GGAGTGGAGACAGCAGTGGT GCTGCATTCGGCGG
E1	Fab/scFv	VL	E1 VL CDR3 Fr4 rev	TAGGACGGTCAGCTTGGTCC CTCCGCCGAATGCAGCACCA CTGCTGTCTCCACTCCCAC
E1/E2	Fab	VL	VL Chicken hum for GGA	GCGCGCGGTCTCTAAGCGTT CCTATGAGCTGATGCAGCCA CCC
E1/E2	scFv	VL	VL Chicken hum for scFv	GGCGGCTCAGGCGGCGGCG GGTCGGGGGGCGGAGGGAG CTCCTATGAGCTGATGCAGC CACCC
E1/E2	scFv	VL	VL Chicken hum rev scFv	CAAGTCCTCTTCAGAAATAAG CTTTTGTTCGGATCCTAGGAC GGTCAGCTTGGTCCCTC
E1/E2	Fab	VL	VL Chicken hum rev GGA	GCGCGCGGTCTCTGTCCTAG GACGGTCAGCTTGGTCCCTC
E2	Fab/scFv	VH	E2 VH FR1 CDR1 FR2 for	GCCTGGGGGGTCCCTGAGA CTCTCCTGTGCAGCCTCTGG ATTCACCTTTAGCGATAATGG CATGGCCTGGGTCCGCC
E2	Fab/scFv	VH	E2 VH CDR1 Fr2 CDR2 for	GATAATGGCATGGCCTGGGT CCGCCAGGCTCCAGGGAAG GGGCTGGAGTKSGTTRSCGG TATTAGTGATATCGGTAGTTA C
E2	Fab/scFv	VH	E2 VH CDR2 for	GTATTAGTGATATCGGTAGTT ACACAAGCTACGGGGCGTCG GTGAAGGGCC
E2	Fab/scFv	VH	E2 VH FR2 for	CGGGGCGTCGGTGAAGGGC CGGKYCACCATCTCCAGAGA CAATTCCMMAARCACGSTAT

				ATCTGCAAATGAACAGCCTG AGAGCCG
E2	Fab/scFv	VH	E2 VH Fr3 CDR3 for	CTGCAAATGAACAGCCTGAG AGCCGAGGACACGGCCGTAT ATTACTGTGCGAAACGTGTTT ATCGTGGTAGTTATGGTGC
E2	Fab/scFv	VH	E2 VH Fr3 CDR3 Fr4 rev	TGAGGAGACGGTGACCAGG GTTCCTTGGCCCCATGAGTC GATCTCACCAGCACCATAACT ACCACGATAAACACGTTTCG CACAGTAATATAC
E2	Fab/scFv	VL	E2 VL Fr1 CDR1 Fr2 for	GACGGCCAGGATCACCTGCT CCGGGGGTGGTAGCAGCAG CTACTATGGCTGGTACCAGC AGAAGCCAGGCCAGGCCC
E2	Fab/scFv	VL	E2 VL Fr2 CDR2 Fr3 for	GCAGAAGCCAGGCCAGGCC CCTGTGWYGGTGATATATGA TAGCACCAAGAGACCCTCGG GGATCCCTGAGCGATTCTC
E2	Fab/scFv	VL	E2 VL Fr3 CDR3 for	CAGTGGAGTCCAGGCAGAAG ATGAGGCTGACTATTACTGTG GTGGCTACGACACCAGTGGT ATATTCGGCGGA
E2	Fab/scFv	VL	E2 VL CDR3 Fr4 rev	TAGGACGGTCAGCTTGGTCC CTCCGCCGAATATACCACTG GTGTCGTAGCCACCAC

Permutated Vernier residue positions are indicated in red. Overhangs for subsequent Golden Gate cloning are marked in blue, while overhangs for the formation of a scFv construct are shown in violet. Homologous sequences to the pCT vector utilized for gap repair cloning are shown in green.

Table S2. Barcode primers utilized for NGS analysis.

Primer Name	Sequence (5'-3')
E1 scFv VH Initial rev	AAATGACGTTCTCTGAAATGAGGAGACGGTGACCAGGGTTC CTTG
E1 scFv VH R1 rev	AAATGACGTTCTTCTAAATGAGGAGACGGTGACCAGGGTTC CTTG
E1 scFv VH R2 rev	AAATGACGTTCTGCGAAATGAGGAGACGGTGACCAGGGTTC CTTG
E1 scFv VL Initial rev	AAATGAATCTCTCTGAAATAGGACGGTCAGCTTGGTCCCTC
E1 scFv VL R1 rev	AAATGAATCTCTTCTAAATAGGACGGTCAGCTTGGTCCCTC
E1 scFv VL R2 rev	AAATGAATCTCTGCGAAATAGGACGGTCAGCTTGGTCCCTC
E2 scFv VH Initial rev	AAATGACGTGCGCTGAAATGAGGAGACGGTGACCAGGGTTC CTTG
E2 scFv VH R1 rev	AAATGACGTGCGTCTAAATGAGGAGACGGTGACCAGGGTTC CTTG
E2 scFv VH R2 rev	AAATGACGTGCGGCGAAATGAGGAGACGGTGACCAGGGTT CCTTG
E2 scFv VL Initial rev	AAATGAATCGCGCTGAAATAGGACGGTCAGCTTGGTCCCTC
E2 scFv VL R1 rev	AAATGAATCGCGTCTAAATAGGACGGTCAGCTTGGTCCCTC
E2 scFv VL R2 rev	AAATGAATCGCGGCGAAATAGGACGGTCAGCTTGGTCCCTC
E1 Fab VH Initial rev	AAAGCTCGTTCTCTGAAATGAGGAGACGGTGACCAGGGTTC CTTG
E1 Fab VH R1 rev	AAAGCTCGTTCTTCTAAATGAGGAGACGGTGACCAGGGTTC CTTG
E1 Fab VH R2 rev	AAAGCTCGTTCTGCGAAATGAGGAGACGGTGACCAGGGTTC CTTG
E1 Fab VL Initial rev	AAAGCTATCTCTCTGAAATAGGACGGTCAGCTTGGTCCCTC
E1 Fab VL R1 rev	AAAGCTATCTCTTCTAAATAGGACGGTCAGCTTGGTCCCTC
E1 Fab VL R2 rev	AAAGCTATCTCTGCGAAATAGGACGGTCAGCTTGGTCCCTC
E2 Fab VH Initial rev	AAAGCTCGTGCGCTGAAATGAGGAGACGGTGACCAGGGTT CCTTG
E2 Fab VH R1 rev	AAAGCTCGTGCGTCTAAATGAGGAGACGGTGACCAGGGTTC CTTG
E2 Fab VH R2 rev	AAAGCTCGTGCGGCGAAATGAGGAGACGGTGACCAGGGTT CCTTG
E2 Fab VL Initial rev	AAAGCTATCGCGCTGAAATAGGACGGTCAGCTTGGTCCCTC
E2 Fab VL R1 rev	AAAGCTATCGCGTCTAAATAGGACGGTCAGCTTGGTCCCTC
E2 Fab VL R2 rev	AAAGCTATCGCGGCGAAATAGGACGGTCAGCTTGGTCCCTC
E1 scFv VH Initial for	AAATGACGTTCTCTGAAAGAGGTGCAGCTGTTGGAGTCTGG GGG
E1 scFv VH R1 for	AAATGACGTTCTTCTAAAGAGGTGCAGCTGTTGGAGTCTGG GGG
E1 scFv VH R2 for	AAATGACGTTCTGCGAAAGAGGTGCAGCTGTTGGAGTCTGG GGG
E1 scFv VL Initial for	AAATGAATCTCTCTGAAATCCTATGAGCTGATGCAGCCACCC
E1 scFv VL R1 for	AAATGAATCTCTTCTAAATCCTATGAGCTGATGCAGCCACCC
E1 scFv VL R2 for	AAATGAATCTCTGCGAAATCCTATGAGCTGATGCAGCCACC C

E2 scFv VH Initial for	AAATGACGTGCGCTGAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E2 scFv VH R1 for	AAATGACGTGCGTCTAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E2 scFv VH R2 for	AAATGACGTGCGGCGAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E2 scFv VL Initial for	AAATGAATCGCGCTGAAATCCTATGAGCTGATGCAGCCACCC
E2 scFv VL R1 for	AAATGAATCGCGTCTAAATCCTATGAGCTGATGCAGCCACCC
E2 scFv VL R2 for	AAATGAATCGCGGCGAAATCCTATGAGCTGATGCAGCCACCC
E1 Fab VH Initial for	AAAGCTCGTTCTCTGAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E1 Fab VH R1 for	AAAGCTCGTTCTTCTAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E1 Fab VH R2 for	AAAGCTCGTTCTGCGAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E1 Fab VL Initial for	AAAGCTATCTCTCTGAAATCCTATGAGCTGATGCAGCCACCC
E1 Fab VL R1 for	AAAGCTATCTCTTCTAAATCCTATGAGCTGATGCAGCCACCC
E1 Fab VL R2 for	AAAGCTATCTCTGCGAAATCCTATGAGCTGATGCAGCCACCC
E2 Fab VH Initial for	AAAGCTCGTGCGCTGAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E2 Fab VH R1 for	AAAGCTCGTGCGTCTAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E2 Fab VH R2 for	AAAGCTCGTGCGGCGAAAGAGGTGCAGCTGTTGGAGTCTGGGGG
E2 Fab VL Initial for	AAAGCTATCGCGCTGAAATCCTATGAGCTGATGCAGCCACCC
E2 Fab VL R1 for	AAAGCTATCGCGTCTAAATCCTATGAGCTGATGCAGCCACCC
E2 Fab VL R2 for	AAAGCTATCGCGGCGAAATCCTATGAGCTGATGCAGCCACCC

Stuffer sequences are marked in green, barcodes are shown red, encoding for clonotype, antibody format, domain and sorting round.

Table S3. Clone annotations and their VH and VL domain combination corresponding to humanized full-length antibodies of clonotype E1. Most frequent clones (Vernier amino acids) are depicted in Supplementary Figure 4B.

Clone name	VH 1-E1 (Fab)	VH 2- E1 (Fab)	VH 3- E1 (Fab)	VL 1- E1 (Fab)	VL 2- E1 (Fab)	VL 3- E1 (Fab)
hF1-E1	x			x		
hF2-E1	x				x	
hF3-E1	x					x
hF4-E1		x		x		
hF5-E1		x			x	
hF6-E1		x				x
hF7-E1			x	x		
hF8-E1			x		x	
hF9-E1			x			x

Table S4. Clone annotations and their VH and VL domain combination corresponding to humanized scFv-Fc variants of clonotype E1. Most frequent clones (Vernier amino acids) are depicted in Supplementary Figure 4A.

Clone name	VH 1-E1 (scFv)	VH 2-E1 (scFv)	VH 3-E1 (scFv)	VL 1-E1 (scFv)	VL 2-E1 (scFv)	VL 3-E1 (scFv)
hS1-E1	x			x		
hS2-E1	x				x	
hS3-E1	x					x
hS4-E1		x		x		
hS5-E1		x			x	
hS6-E1		x				x
hS7-E1			x	x		
hS8-E1			x		x	
hS9-E1			x			x

Table S5. Clone annotations and their VH and VL domain combination corresponding to humanized full-length antibodies of clonotype E2. Most frequent clones (Vernier amino acids) are depicted in Figure 2B.

Clone name	VH 1-E2 (Fab)	VH 2-E2 (Fab)	VH 3-E2 (Fab)	VL 1-E2 (Fab)	VL 2-E2 (Fab)	VL 3-E2 (Fab)
hF1-E2	x			x		
hF2-E2	x				x	
hF3-E2	x					x
hF4-E2		x		x		
hF5-E2		x			x	
hF6-E2		x				x
hF7-E2			x	x		
hF8-E2			x		x	
hF9-E2			x			x

Table S6. Clone annotations and their VH and VL domain combination corresponding to humanized scFv-Fc variants of clonotype E2. Most frequent clones (Vernier amino acids) are depicted in Figure 2A.

Clone name	VH 1-E2 (scFv)	VH 2-E2 (scFv)	VH 3-E2 (scFv)	VL 1-E2 (scFv)	VL 2-E2 (scFv)	VL 3-E2 (scFv)
hS1-E2	x			x		
hS2-E2	x				x	
hS3-E2	x					x
hS4-E2		x		x		
hS5-E2		x			x	
hS6-E2		x				x
hS7-E2			x	x		
hS8-E2			x		x	
hS9-E2			x			x

Table S7. Clone annotations of humanized full-length antibodies (clonotype E1) and amino acid variations in Vernier positions located in VH domain and VL domain.

Clone	Vernier amino acid variation in VH domain	Vernier amino acid variation in VL domain
hF1-E1	L47, G49, F67, T75, S76, V78	T46, D66, S69, V71
hF2-E1	L47, G49, F67, T75, S76, V78	T46, Y66, N69, A71
hF3-E1	L47, G49, F67, T75, S76, V78	T46, Y66, S69, T71
hF4-E1	F47, G49, F67, T75, S76, V78	T46, D66, S69, V71
hF5-E1	F47, G49, F67, T75, S76, V78	T46, Y66, N69, A71
hF6-E1	F47, G49, F67, T75, S76, V78	T46, Y66, S69, T71
hF7-E1	L47, A49, V67, T75, S76, V78	T46, D66, S69, V71
hF8-E1	L47, A49, V67, T75, S76, V78	T46, Y66, N69, A71
hF9-E1	L47, A49, V67, T75, S76, V78	T46, Y66, S69, T71

Table S8. Clone annotations of humanized scFv-Fc variants (clonotype E1) and amino acid variations in Vernier positions located in VH domain and VL domain.

Clone	Vernier amino acid variation in VH domain	Vernier amino acid variation in VL domain
hS1-E1	L47, A49, V67, T75, S76, V78	S46, T66, S69, A71
hS2-E1	L47, A49, V67, T75, S76, V78	T46, Y66, N69, V71
hS3-E1	L47, A49, V67, T75, S76, V78	S46, S66, N69, A71
hS4-E1	L47, G49, F67, P75, S76, L78	S46, T66, S69, A71
hS5-E1	L47, G49, F67, P75, S76, L78	T46, Y66, N69, V71
hS6-E1	L47, G49, F67, P75, S76, L78	S46, S66, N69, A71
hS7-E1	L47, G49, A67, Q75, S76, V78	S46, T66, S69, A71
hS8-E1	L47, G49, A67, Q75, S76, V78	T46, Y66, N69, V71
hS9-E1	L47, G49, A67, Q75, S76, V78	S46, S66, N69, A71

Table S9. Clone annotations of humanized full-lenght antibodies (clonotype E1) and amino acid variations in Vernier positions located in VH domain and VL domain.

Clone	Vernier amino acid variation in VH domain	Vernier amino acid variation in VL domain
hF1-E2	W47, A49, F67, Q75, S76, V78	T46, A66, N69, A71
hF2-E2	W47, A49, F67, Q75, S76, V78	S46, S66, S69, A71
hF3-E2	W47, A49, F67, Q75, S76, V78	T46, S66, N69, A71
hF4-E2	W47, A49, V67, K75, S76, V78	T46, A66, N69, A71
hF5-E2	W47, A49, V67, K75, S76, V78	S46, S66, S69, A71
hF6-E2	W47, A49, V67, K75, S76, V78	T46, S66, N69, A71
hF7-E2	W47, A49, F67, Q75, N76, V78	T46, A66, N69, A71
hF8-E2	W47, A49, F67, Q75, N76, V78	S46, S66, S69, A71
hF9-E2	W47, A49, F67, Q75, N76, V78	T46, S66, N69, A71

Table S10. Clone annotations of humanized scFv-Fc variants (clonotype E2) and amino acid variations in Vernier positions located in VH domain and VL domain.

Clone	Vernier amino acid variation in VH domain	Vernier amino acid variation in VL domain
hS1-E2	W47, A49, V67, Q75, S76, V78	S46, A66, S69, A71
hS2-E2	W47, A49, V67, Q75, S76, V78	S46, S66, S69, A71
hS3-E2	W47, A49, V67, Q75, S76, V78	S46, S66, N69, A71
hS4-E2	W47, A49, V67, K75, S76, V78	S46, A66, S69, A71
hS5-E2	W47, A49, V67, K75, S76, V78	S46, S66, S69, A71
hS6-E2	W47, A49, V67, K75, S76, V78	S46, S66, N69, A71
hS7-E2	W47, A49, F67, Q75, S76, V78	S46, A66, S69, A71
hS8-E2	W47, A49, F67, Q75, S76, V78	S46, S66, S69, A71
hS9-E2	W47, A49, F67, Q75, S76, V78	S46, S66, N69, A71

Table S11. Clone annotations of humanized scFv-Fc variants (clonotype E1 and E2) and amino acid variations in Vernier positions in VH domain and VL domain. All clones contain all amino acids corresponding to the human frameworks VH3-23 (JH4) and VL3 (JL2) only differ in antibody format and clonotype (CDR variability).

Clone	Vernier amino acid variation in V_H domain	Vernier amino acid variation in V_L domain
gS-E1 (grafted chicken scFv E1)	W47, S49, F67, K75, N76, L78	L46, S66, T69, V71
gS-E2 (grafted chicken scFv E2)	W47, S49, F67, K75, N76, L78	L46, S66, T69, V71
gF-E1 (grafted chimeric chicken Fab E1)	W47, S49, F67, K75, N76, L78	L46, S66, T69, V71
gF-E2 (grafted chimeric chicken Fab E2)	W47, S49, F67, K75, N76, L78	L46, S66, T69, V71

Table S12. Primer sequences used for reformatting. Overhangs incorporated for subsequent Golden Gate cloning are shown in red. In blue, overhangs encoding the linker between VH and VLs in later scFvs, are marked.

Primer Name	Sequence (5'-3')
HumRe Fr4 scFv rev	TTTTTTGCTCTTCTTTCTAGGACGGTCAGCTTGGTCCCTC
HumRe VH Fr1 for	AAAAAGCTCTTCAAGTGAGGTGCAGCTGTTGGAGTCTGGGGG
HumRe VH Fr4 Fab rev	TTTTTTGCTCTTCTGGCTGAGGAGACGGTGACCAGGGTTCC
HumRe VH Fr4 scFv rev	TCCGCCCCCCGAACCGCCGCCGCCTGAGCCGCCTCCCCCTGAGGAGACGGTGACCAGGGTTCC
HumRe VL Fr1 Fab for	AAAAAGCTCTTCAAGTTCCTATGAGCTGATGCAGCCACCC
HumRe VL Fr1 scFv for	GGCGGCTCAGGCGGCGGCGGTTCGGGGGGCGGAGGGAGCTCCTATGAGCTGATGCAGCCACCC
HumRe VL Fr4 Fab Lam rev	TTTTTTGCTCTTCACCCTAGGACGGTCAGCTTGGTCCCTC

Table S13. Comparison of approved humanized antibodies with humanized E1 and E2 variants. Germlines and sequence identities of antibodies approved in the United States between 2018 and March 2020 were taken from IMGT. Germline identity of humanized E1 and E2 variants were determent utilizing the IgBlast online tool. The number of predicted of VH- and VL-derived peptides binding to MHCII were determined using the NetMHCIIpan 4.0 Server.

	Antibody	VH				VL				Overall Fv Germline Identity [%]	Format
		Germline Identity (human) [%]	No. of MHCII Binding Peptides		Germline (IMGT)	Germline Identity (human) [%]	No. of MHCII Binding Peptides		Germline (IMGT)		
			Strong Binding	Weak Binding			Strong Binding	Weak Binding			
Approved antibodies	Eptinezumab	81.4	4	13	IGHV3-66*01	86.2	4	22	IGKV1-27*01	83.7	IgG1
	Trastuzumab deruxtecan	81.6	4	8	IGHV3-66*01	86.3	2	4	IGKV1-39*01	83.9	IgG1 ADC
	Crizanlizumab	81.6	4	5	IGHV1-8*01	86.9	0	12	IGKV1-39*01	84.3	IgG2
	Brolucizumab	80.4	4	12	IGHV3-66*01	87.6	3	13	IGKV1-5*01	83.3	scFv
	Romosozumab	87.8	4	6	IGHV1-2*02	89.5	1	11	IGKV1-33*01	88.6	IgG2
	Polatuzumab vedotin	76.5	4	15	IGHV3-23*04	85.9	0	15	IGKV1-39*01	80.7	IgG1 ADC
	Risankizumab	79.4	3	14	IGHV1-69*02	80	5	9	IGKV1-27*01	79.7	IgG1
	Ibalizumab	76.5	3	6	IGHV1-46*01	92.9	0	8	IGKV4-1*01	84.8	IgG4
	Ravulizumab	81.6	2	10	IGHV1-46*01	84.2	0	11	IGKV1-39*01	82.9	IgG2/4
	Fremanezumab	85.7	4	11	IGHV3-7*01	85.3	5	8	IGKV3-11*01	85.5	IgG2
	Tildrakizumab	81.6	4	9	IGHV1-18*01	85.3	3	6	IGKV1-39*01	83.4	IgG1
	Mogamulizumab	83.7	4	9	IGHV3-21*01	81	0	13	IGKV2-29*02	82.3	IgG1
	Galcanezumab	82.7	3	17	IGHV1-69*02	87.4	0	13	IGKV1-39*01	83.9	IgG4
humanized E1 and E2 variants	hF8-E1	83.7	4	16	IGHV3-23*05	80.9	0	12	IGLV3-25*01	82.3	IgG1
	hF9-E1	83.7	4	16	IGHV3-23*05	80.9	0	10	IGLV3-25*01	82.3	IgG1
	hS6-E1	85.7	4	14	IGHV3-23*05	81.9	0	9	IGLV3-25*01	82.8	scFv

hS7-E1	83.7	4	16	IGHV3-23*05	80.9	0	9	IGLV3-25*01	82.3	scFv
hF1-E2	84.7	4	8	IGHV3-23*02	77.1	0	7	IGLV3-25*01	82	IgG1
hF8-E2	85.7	4	8	IGHV3-23*02	77.1	0	7	IGLV3-25*01	82.5	IgG1
hS2-E2	83.7	4	8	IGHV3-23*02	78.1	0	7	IGLV3-25*01	80.9	scFv
hS9-E2	84.7	4	8	IGHV3-23*02	78.1	0	7	IGLV3-25*01	81.4	scFv