Supplementary Material for

Transfer of engineered biophysical properties between different antibody formats and expression systems

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Construction of Expression Plasmids

Expression vectors for mammalian HEK293 cells were first designed for the independent expression of heavy or light chain, respectively, using the pMORPH® vector series (MorphoSys; Steidl *et al.*, 2008). The V_H and V_L genes of the previously analyzed scFv fragments (Ewert *et al.*, 2003) were amplified and inserted into pMORPH®2_h_IgG1f, pMORPH®2_h_Igκ or the pMORPH®2_h_Igλ2 vectors. These vectors led to the expression of correctly assembled full-length IgG into the supernatant of mammalian cells upon co-transfection. These proteins carry no tags. The creation of Fab expression vectors was performed likewise, in this case however, the V_H/C_H1 chain was fused to a myc-tag and $(his)_6$-tag at its C-terminus for detection and purification purposes.

For the establishment of stable cell lines using the Flp-In system (Invitrogen), the two expression cassettes for H and L, respectively, were combined in a single vector based on the pcDNA5/FRT series (Invitrogen). After removal of the intrinsic *Mfe*I cleavage site by site-directed mutagenesis (using primers MfeI_del_for: 5′ - GGC AAG GCT TGA CCG ACA GTT GCA TGA AGA ATC TGC - 3′ and MfeI_del_rev: 5′ - GCA GAT TCT TCA TGC AAC TGT CGG TCA AGC CTT GCC - 3′ to create the pcDNA5/FRT/-MfeI plasmid), the final vectors were constructed as follows. The heavy chain gene was amplified from the corresponding pMORPH® vector using the primers HC_NheI_for (5′ - CCC AAG CTG *GCT AGC* GCC ACC ATG AAA CAC C - 3′) and HC_XbaI_rev (5′ - GCG TCC *TCT AGA* GCG TAC CCA ATT CAA CAG GC - 3′). The resulting amplicon contained the complete coding sequence of the heavy chain as well as a poly A sequence derived from the human growth hormone, but no CMV promoter. The amplification of the light chain gene was performed using LC_XbaI_for (5′ - GCG TCC *TCT AGA* CGC GAT GTA CGG GCC AGA TAT ACG - 3′) and LC_ApaI_rev (5′ - GCC ACC CGT TTA AAC *GGG CCC* C - 3′), resulting in an amplicon containing a CMV promoter, the complete coding sequence of the light chain, but no poly A sequence. Regions complementary to the sequences found in the parental vectors are underlined, while nucleotides allowing for cleavage by *Nhe*I, *Xba*I or *Apa*I are printed in italics. After double-digesting the heavy chain amplicon with *Nhe*I-*Xba*I and the light chain amplicon with *Xba*I-*Apa*I, the resulting fragments were ligated into the *Nhe*I-*Apa*I digested pcDNA5/FRT/-MfeI plasmid. Since this vector already contained one intrinsic CMV promoter and a poly A sequence (derived from bovine growth hormone), both the sequences coding for the heavy as well as for the light chain were finally present as complete expression cassettes including CMV promoter and poly A sequences of their own. The final vectors were sequenced and tested by transient transfection for their functionality before being used for the establishment of stable cell lines.

The *Pichia* expression vectors are based on the vector pGAPZαB (Invitrogen). The sequences for the light chains and the different heavy chain variants were amplified from the pMORPH® vectors described above. Since for expression in *Pichia pastoris* a plasmid has to be integrated into the genome, also these genes were combined within one single vector, retaining them as independent expression cassettes, each with its own GAP promoter and termination sequence. To assemble the

final vectors, the cloning of intermediate vectors was necessary. First, two pGAPZαB derivatives were constructed for each construct: one containing the gene encoding the corresponding light chain, the other one holding the heavy chain gene as complete expression cassettes. All expression vectors were obtained by inserting a PCR-amplified fragment from the pMORPH® vectors containing the sequence coding for the mature heavy or light chain into the multiple cloning site (MCS) of pGAPZαB. In these vectors, the POI is placed behind the α-factor pre-pro (αMFpp) sequence and under control of the constitutive GAP promoter.

As restriction enzymes, *EcoR*I introduced downstream of the N-termini and *Xba*I introduced upstream of the C-termini of the corresponding genes were used. The heavy chain fragment was amplified using either PP_WT_for (5′ - GCG TCC *GAA TTC* AA CAG GTG CAA TTG CAA CAG TC - 3′) or PP_M_for (5′ - GCG TCC *GAA TTC* AA CAG GTG CAA TTG GTA CAG TC - 3′) in combination with PP_HC_rev (5′ - GCG TCC *TCT AGA* TCA CTA TCA TTT ACC CGG AGA CAG GG - 3′). The light chain fragments were created in an analogous manner using the oligonucleotides PP_λ_for (5′ - GCG TCC *GAA TTC* AA GAT ATC GAA CTG ACC CAG CC - 3′) and PP_λ_rev (5′ - GCG TCC *TCT AGA* TCA CTA TGA ACA TTC TGT AGG GGC CAC - 3′) for the lambda light chain and PP_κ_for (5′ - GCG TCC *GAATTC* AA GAT ATC GTG CTG ACC CAG AG - 3′) and PP_κ_rev (5′ - GCG TCC *TCTAGA* TCA CTA AAC ACT CTC CCC TGT TGA AGC TC - 3′) for the kappa counterpart. Regions complementary to the N- or C-termini of the heavy or light chain, respectively, are underlined, while nucleotides allowing for cleavage by *EcoR*I or *Xba*I are printed in italics. Two consecutive additional stop codons were introduced into the construct by the dotted underlined triplets. The AA doublet in the forward primers encoded together with the original nucleotides from the MCS the four additional residues AGIQ upstream of the mature N-terminus of either the light or heavy chains, respectively. These additional amino acids were removed in a later step from the heavy chain construct by assembly PCR using the following primers: WT_AGIQdel_for (5′ - GA GAG GCT GAA GCT CAG GTG CAA TTG CAA CAG TCT GGT CCG GG - 3′) and WT_AGIQdel_rev (5′ - G CAA TTG CAC CTG AGC TTC AGC CTC TCT TTT CTC GAG AGA TAC CC - 3′) or M_AGIQdel_for (5′ - GA GAG GCT GAA GCT CAG GTG CAA TTG GTA CAG TCT GGT CCG GG - 3′) and M_AGIQdel_rev (5′ - C CAA TTG CAC CTG AGC TTC AGC CTC TCT TTT CTC GAG AGA TAC CC - 3′). The AGIQ-coding sequences of the light chains were removed by conventional cloning using the unique *Xho*I-*Nco*I cleavage sites, employing the oligonucleotides λ_AGIQdel_for (5′ - GCG TCG *CTC GAG* AAA AGA GAG GCT GAA GCT GAT ATC GAA CTG ACC CAG CC - 3′) or κ_AGIQdel_for (5′ - GCG TCG *CTC GAG* AAA AGA GAG GCT GAA GCT GAT ATC GTG CTG ACC CAG AGC - 3′) in combination with LC_AGIQdel_rev (5′ - CCG GAA CGG CAC TGG TCA ACT TGG C - 3′). Regions complementary to the N-termini of the light chains are underlined, while nucleotides allowing the cleavage by *Xho*I or *Nco*I are printed in italics.

Finally, the two vectors (each coding for either the heavy or light chain, respectively) were combined in a single vector for integration into the *Pichia* genome. This step was performed on the basis of the methods described by Ogunjimi and co-workers (Ogunjimi *et al.*, 1999). Briefly, the vectors containing the heavy or light chain gene was double-digested with either *Mlu*I-*BamH*I or *Mlu*I-*Bgl*II, respectively.

As digestion with *BamH*I-*Bgl*II resulted in compatible overhangs, the subsequent ligation eliminated the used *Bgl*II cleavage site, leading to only one remaining *Bgl*II cleavage site in the final plasmid, which was subsequently used for linearization of the plasmid for genomic integration. This vector contained both independent expression cassettes but only one origin of replication as well as only one Zeocin™ resistance marker.

Ewert S., Honegger A. and Plückthun A. (2003) *Biochemistry,* **42**, 1517-1528.
Ogunjimi A.A., Chandler J.M., Gooding C.M., Recinos I.A. and Choudary P.V. (1999) *Biotechnology Letters,* **21**, 561-567.
Steidl S., Ratsch O., Brocks B., Durr M. and Thomassen-Wolf E. (2008) *Mol. Immunol.,* **46**, 135-144.

Table S1. Overview of measured stabilities

			ITF	GdnHCl	DSF	DSC
IgG 2C2	**WT**		70.4°C*	2.5 M	n.d.	86.0°C
	M		71.8°C*	3.8 M	n.d.	87.8°C
		Δ =	**1.4°C**	**1.3 M**	**-**	**1.8°C**
IgG 6B3	**WT**		67.6°C	2.0 M	74.5°C	72.1°C
	M		70.8°C	2.6 M	77.0°C	74.3°C
		Δ =	**3.2°C**	**0.6 M**	**2.5°C**	**2.2°C**
Fab 6B3	**WT**		69.7°C	2.0 M	76.5°C	72.6°C
	M		74.2°C	2.6 M	80.0°C	76.6°C
		Δ =	**4.5°C**	**0.6 M**	**3.5°C**	**4.0°C**

* determined in the presence of 1 M GdnHCl

n.d., not determined

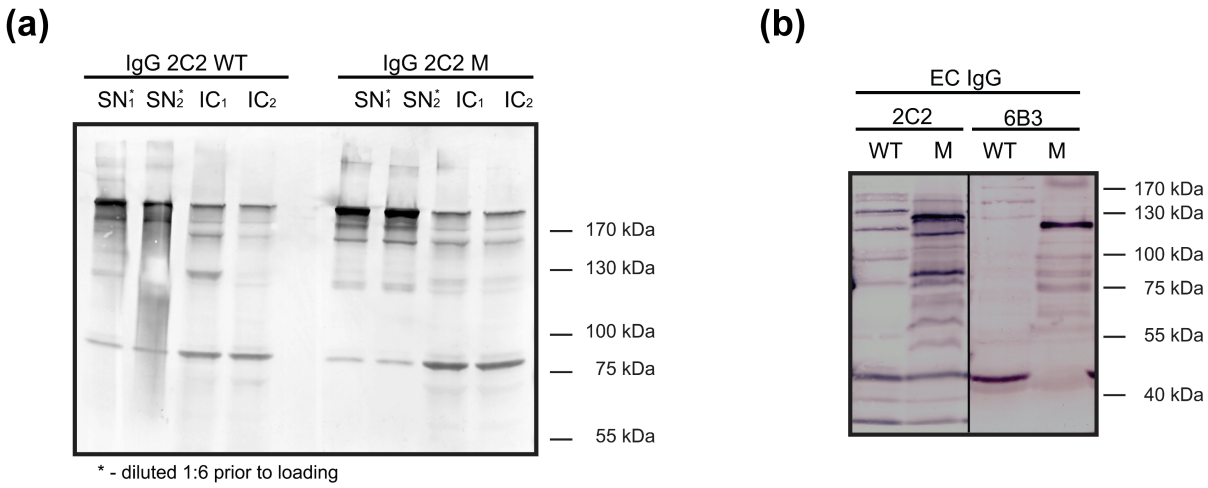

Fig. S1. Comparison of secreted IgG amounts with intracellular levels and IgG expression in *E. coli*. (a) Western blot analysis of IgG 2C2 WT and M variants found in the supernatant (SN) and intracellularly (IC) in stably transformed HEK cells. Samples were taken in duplicates and the cells were lysed with RIPA-lysis buffer containing 1% NP-40 and Na-deoxycholate. The supernatant was diluted 6-fold to allow the detection of the significantly lower intracellular IgG levels on the same blot. Samples were heated to 95°C for 5 min and the detection performed with antibodies specific for the respective light chain. **(b)** Western blot analysis of full-length IgGs expressed in *E. coli*. The OD-normalized periplasm was isolated and separated by non-reducing SDS-PAGE.

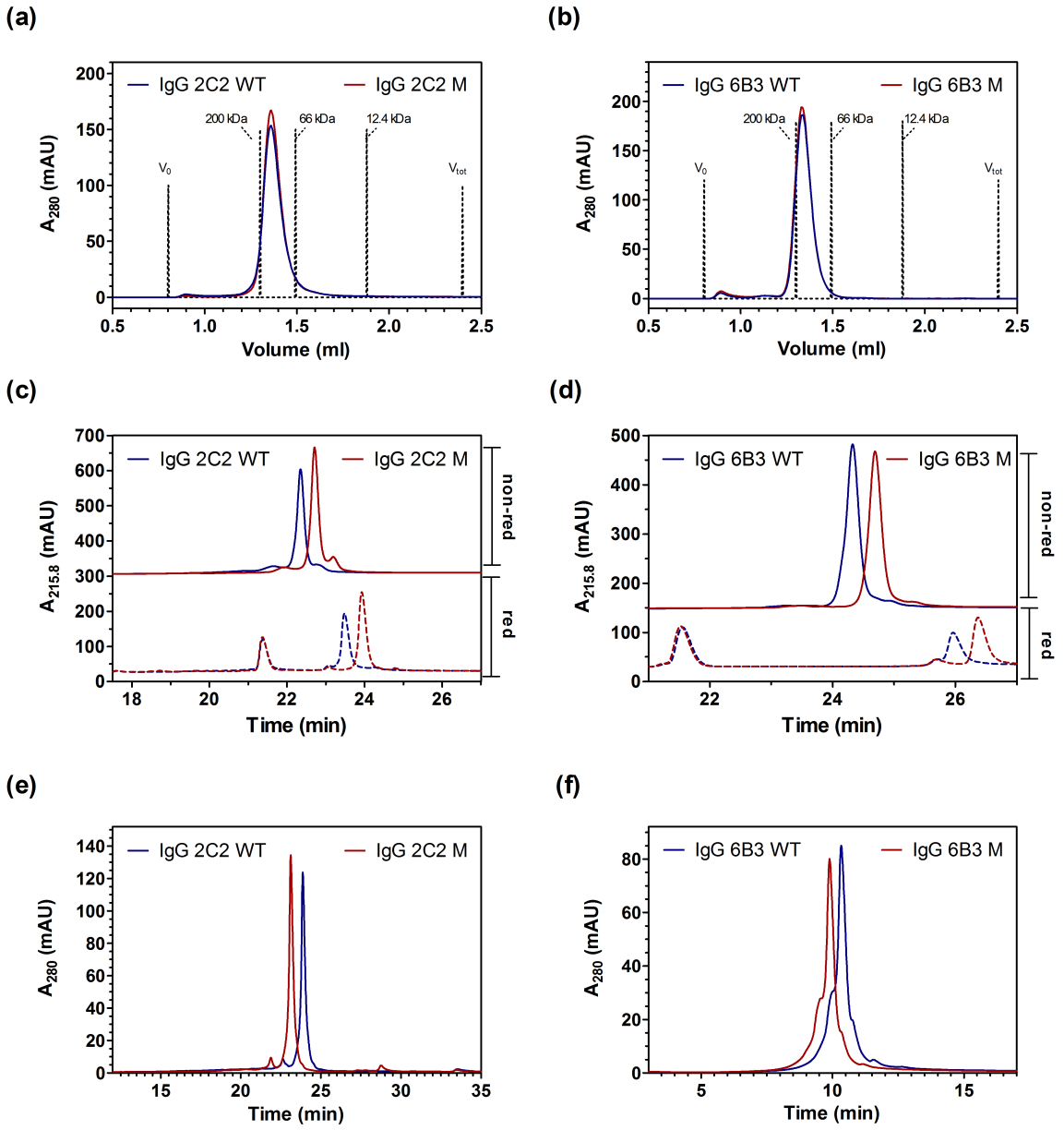

Fig. S2. Chromatographic analysis of HEK produced IgG variants. Signals derived from WT IgG are shown in blue, those of the mutant M in red. **(a)** Size exclusion chromatography (SEC) analysis of 2C2 variants. Dotted lines indicate elution volumes of molecular mass standards (from left to right): β-amylase (200 kDa), bovine serum albumin (66 kDa) and cytochrome c (12.4 kDa). Also shown are the void volume V_0 and the total volume V_{tot}. **(b)** SEC of IgG 6B3 variants analyzed analogous to panel (a). **(c)** Analysis of IgG 2C2 WT and M by reverse phase HPLC (RP-HPLC). Changes in the elution profiles are attributed to the increased hydrophobicity of the M variant. The upper panel shows the chromatogram recorded under non-reducing conditions, the lower one after reduction. **(d)** RP-HPLC of IgG 6B3 variants analogous to panel (c). **(e)** Cation exchange chromatography (CIEX) of IgG 2C2 variants. Differences in the elution profile of the M variant are caused by the additional negative charge introduced by the V72D mutation. **(f)** CIEX of IgG 6B3 variants analogous to panel (e).

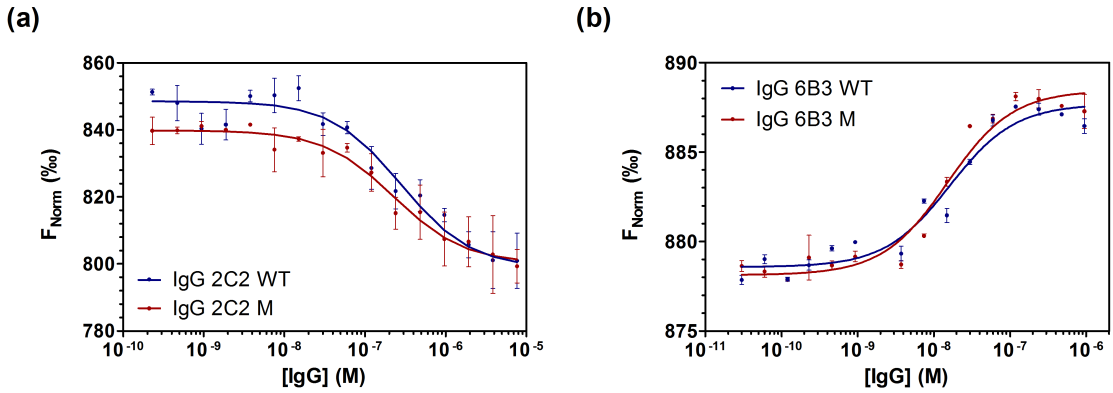

Fig. S3. Binding affinity of IgG mutants obtained from mammalian expression. Direct interaction studies between purified IgGs and their fluorescently labeled antigens (M18-transferrin for IgG 2C2 and myoglobin for IgG 6B3, respectively) analyzed using microscale thermophoresis technology. Normalized fluorescence (‰) is plotted against the IgG concentration titrated from 0.03 nM to 10,000 nM. The fitted K_D values lie within the experimental error range for WT and M and indicate that the binding is fully retained for **(a)** the IgG 2C2 variants and **(b)** IgG 6B3 WT and M, respectively.

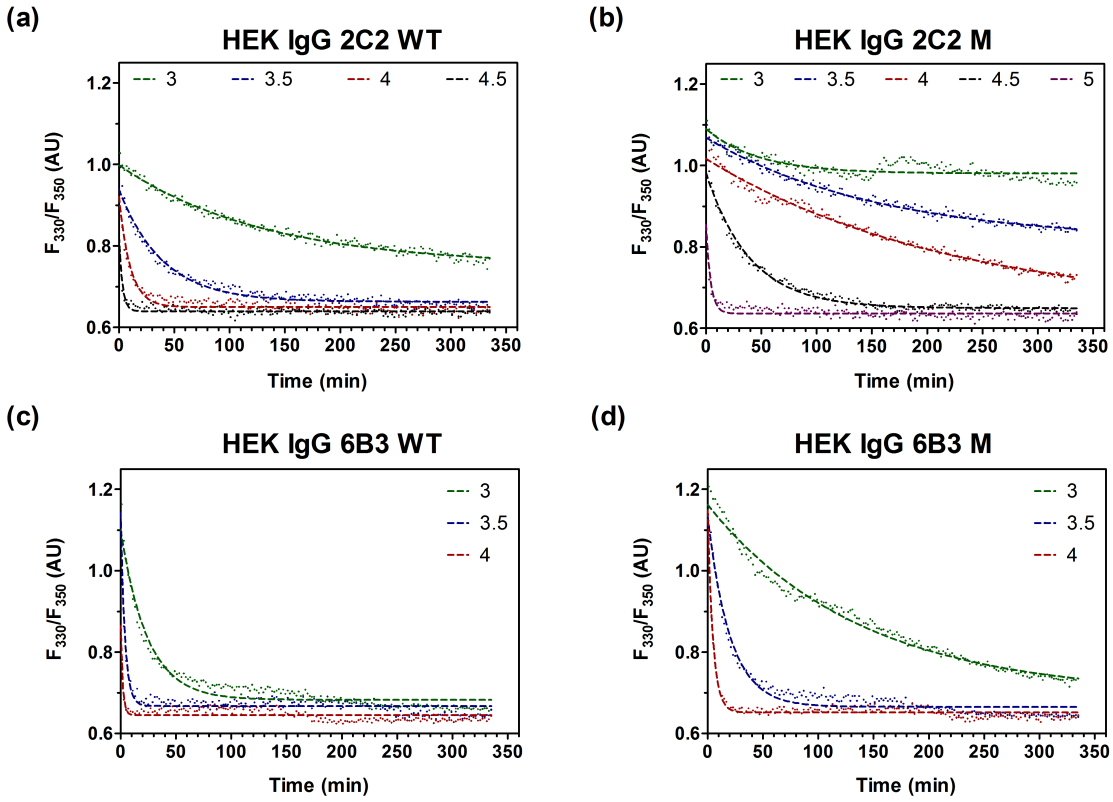

Fig. S4. Time-course of GdnHCl-induced denaturation of IgGs. The graphs were obtained from the F_{330}/F_{350} ratio plotted as a function of time. Shown are the signals derived from IgG sample in the presence of various GdnHCl concentrations. Unfolding was recorded over a period of 5.5 h for **(a)** HEK IgG 2C2 WT, **(b)** HEK IgG 2C2 M, **(c)** HEK IgG 6B3 WT and **(d)** HEK IgG 6B3 M.

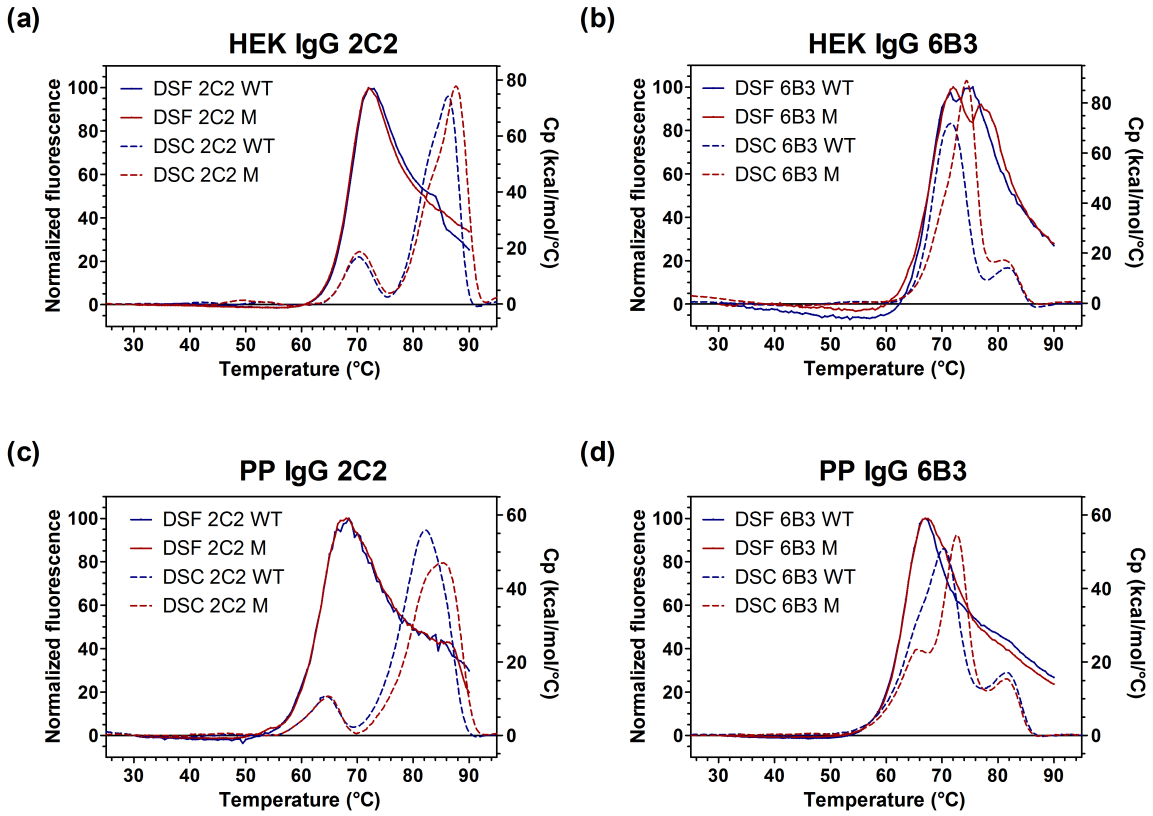

Fig. S5. Comparison of DSF and DSC data from Figures 7 and 8. To allow an easier comparison of domain stabilities, data recorded by DSF (displayed as continuous lines) and DSC (dotted lines) are combined within one single graph. Comparative graphs were generated for **(a)** HEK IgG 2C2, **(b)** HEK IgG 6B3, **(c)** PP IgG 2C2 and **(d)** PP IgG 6B3.