Suppl. Table I. Design of mutations within the BIIB4 and BIIB5 scFvs for screening in a thermal challenge assay.

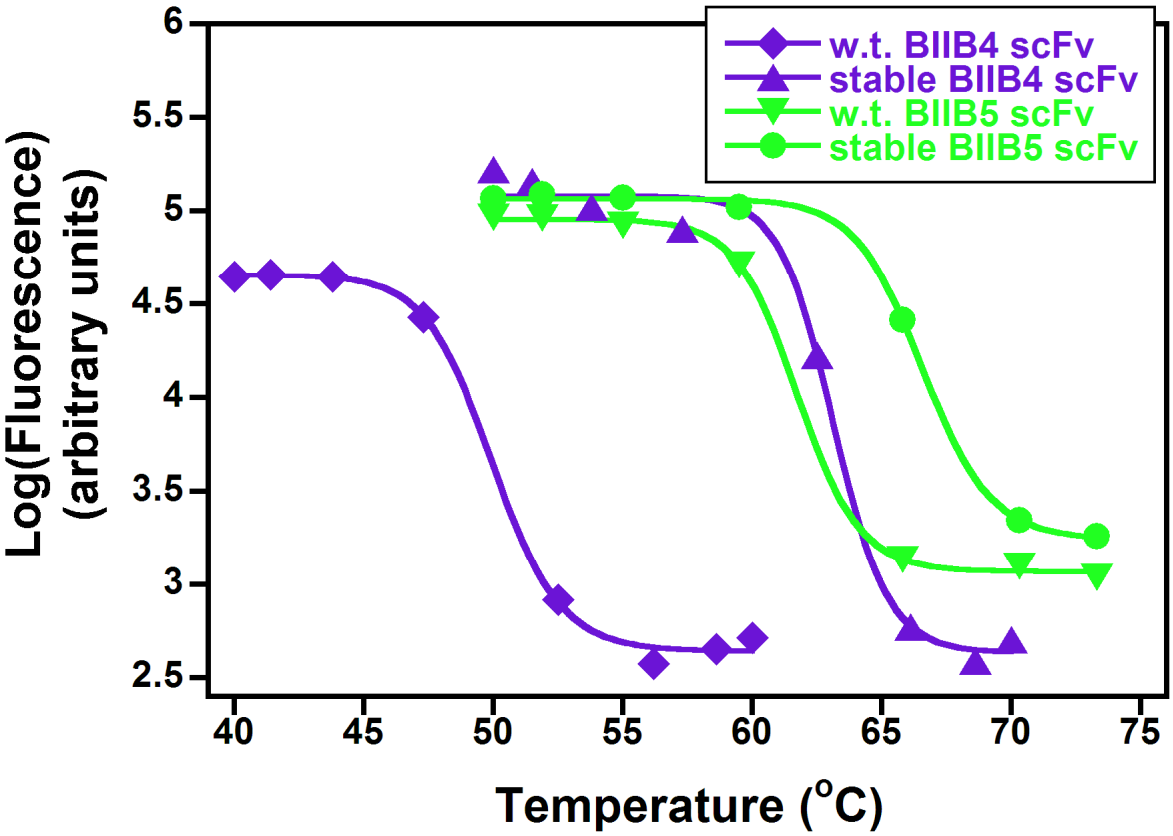
scFv position (Kabat numbering)	Residue Frequency[a]	Covar-iation[b]	DEEK or Rosetta[c]	scFv position (Kabat numbering)	Residue Frequency[a]	Covar-iation[b]	DEEK or Rosetta[c]
BIIB4 V_L				**BIIB5 V_L**			
Q3			V	24Q	R (0.0),K (0.2)	K,A	
M4		L		R30			T
T5			Y,A	D50	A (0.0),G (0.9)	I	
Q6			A,S	A51			G
D9	S (0.3)		S,G	G63	S (0.0)	T	
A12	S (0.3)		S	S72	T (0.2)	T	
V13	A (0.7)		A	F73	L (0.0)	L	
L15	V (0.5)		D,A,S,G	G76	S (0.0),N (0.0)	N,S	
A19	V(0.8)			I83	F (0.0),V (0.1)	E,T	
K24			R				
S25	A (0.5)	A,G					
N31			A				
P43	A (0.4)	A					
L50			G,A,W				
E55		P					
G57			T				
L58		I					
I75			F,L,A				
S77		G					
V83	F (0.3)	E					
A84			G,H,L				
V85		D					
BIIB4 V_H				**BIIB5 V_H**			
L5	Q (0.4),V (0.2)	Q,V	V	L5	V (0.5)		
L11			D,A,S,G	S21			E
Y35	S (0.1),H (0.2)			I31	S (0.0),N (0.0)		D,K
E46		G		R33	Y (0.0),A (0.0)		M
S49	A (0.5),G (0.6)	A,G		Q35	H (0.0),N (0.1)		S,Y,G
S52a		P	G	W47			F
V63			E,Q,A	T56	S (0.7)		
R83		K					
A84		P	D,S,G				

[a]Residue frequency of the native residue at a particular position divided by the most common residue(s) frequency (exact values in parentheses) using a database of human variable domain sequences (Garber, E., Demarest, S.J. (2007) *Biochem. Biophys. Res. Commun.* **335**, 751-757).

[b]Mutation of native residue results in a net gain of correlation coefficients with ϕ-values > 0.25 (Wang, N., Smith, W.F., Miller, B.R., Aivazian, D., Lugovskoy, A.A., et al. (2009) *Proteins*, **76**, 99-114).

[c]Mutation of native residue results in a net lowering of the free energy of the system using the dead end elimination (DEEK) and Rosetta forcefields as described previously (Jordan, J.L., Arndt, J.W., Hanf, K., Li, G., Hall, J., et al. (2009) *Proteins*, **77**, 832-841).

Suppl. Fig. 1

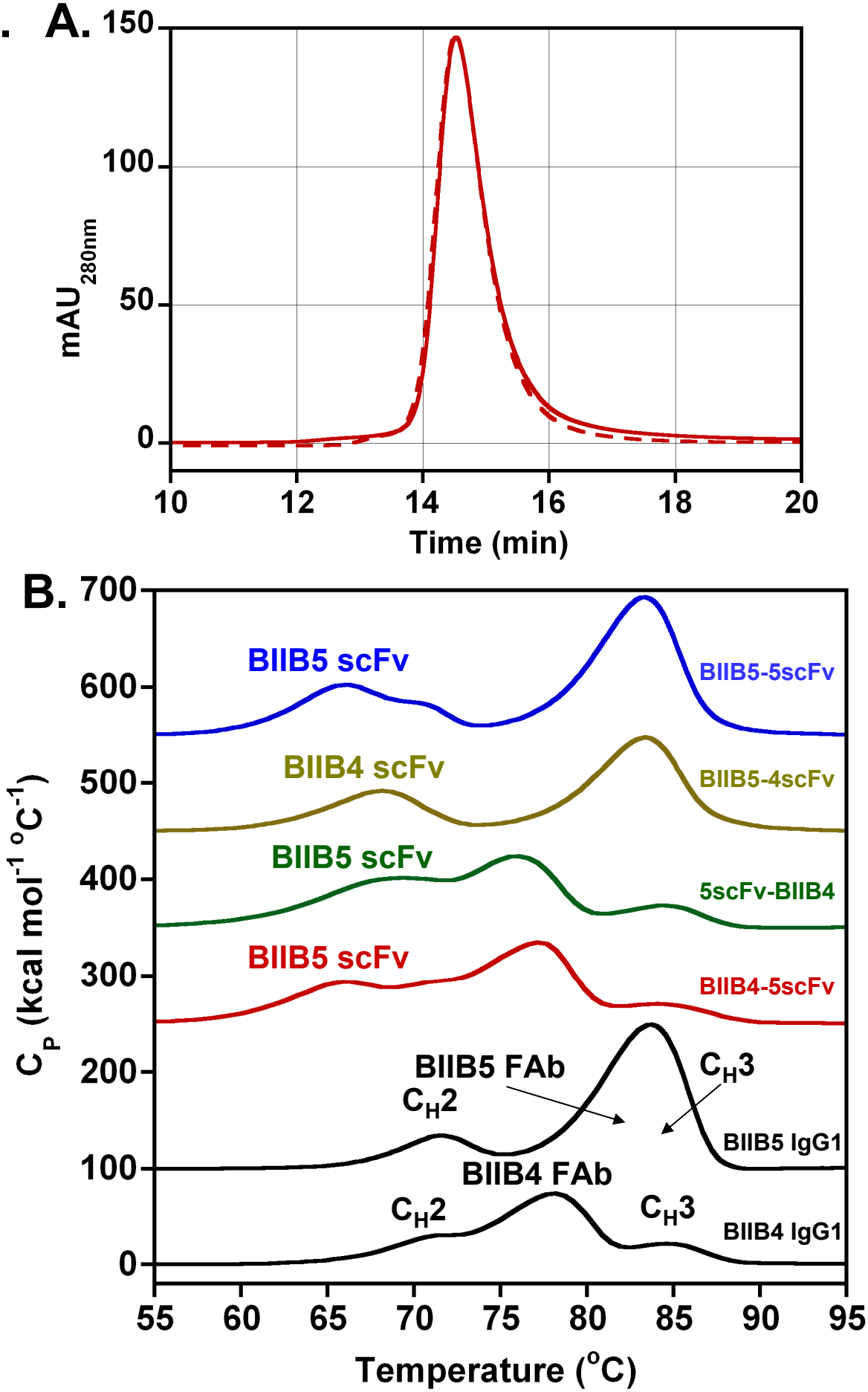


T_{50} assay demonstrating the stabilization of the BIIB4 and BIIB5 scFvs. The wild-type BIIB4 (♦) and BIIB5 (▼) scFvs and stabilized BIIB4 (▲) and BIIB5 (●) scFvs were assayed for their ability to bind antigen following thermal challenge for 1 hour at the temperatures specified in the plot. The midpoint where each scFv begins to unfold and aggregate with components of the culture media is denoted the 'T_{50}'. The BIIB4 and BIIB5 scFvs were found to have T_{50}s lower than our empirical cutoff of 65 °C (derived from FAb data). The scFvs were stabilized as described in the Results. The stabilized BIIB4 and BIIB5 scFvs both had T_{50}s above the empirical limits. The BIIB4 scFv had 5 stabilizing mutations while the BIIB5 scFv contained a single stabilizing mutation. The stabilized BIIB4 scFv had a T_{50} 16 °C higher than the wild-type scFv generated directly from the Fv of the BIIB4 antibody. The stabilized BIIB5 scFv had a T_{50} 4 °C higher than the wild-type scFv generated from the BIIB5 antibody.

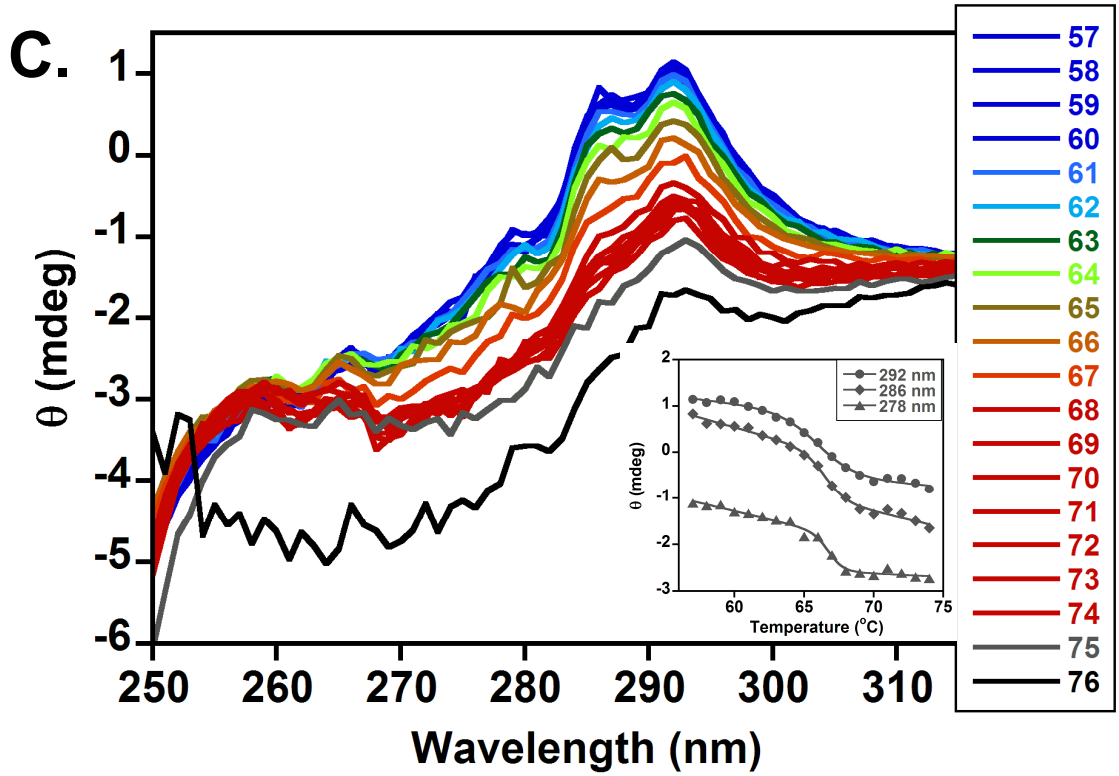
T_{50} Method: The T_{50} assay has been described (Miller et al., 2009) Reference). The antigen used for coating the DELPHIA plates was hIGF-1R(1-903)-Fc.

Miller, B.R., Glaser, S.M., Demarest, S.J. (2009) Rapid screening platform for scFvs in *Escherichia coli*. *Methods Mol. Biol.* **525**, 279-9.

Suppl. Fig. 2


A.
150
100
50
0
mAU280nm
10
12
14
16
18
20
Time (min)
B.
700
600
500
400
300
200
100
0
BIIB5 scFv
BIIB5-5scFv
BIIB4 scFv
BIIB5-4scFv
BIIB5 scFv
5scFv-BIIB4
BIIB5 scFv
BIIB4-5scFv
BIIB5 FAb
CH2
CH3
BIIB5 IgG1
BIIB4 FAb
CH2
CH3
BIIB4 IgG1
Cp (kcal mol-1 °C-1)
55
60
65
70
75
80
85
90
95
Temperature (°C)

Suppl. Fig. 2

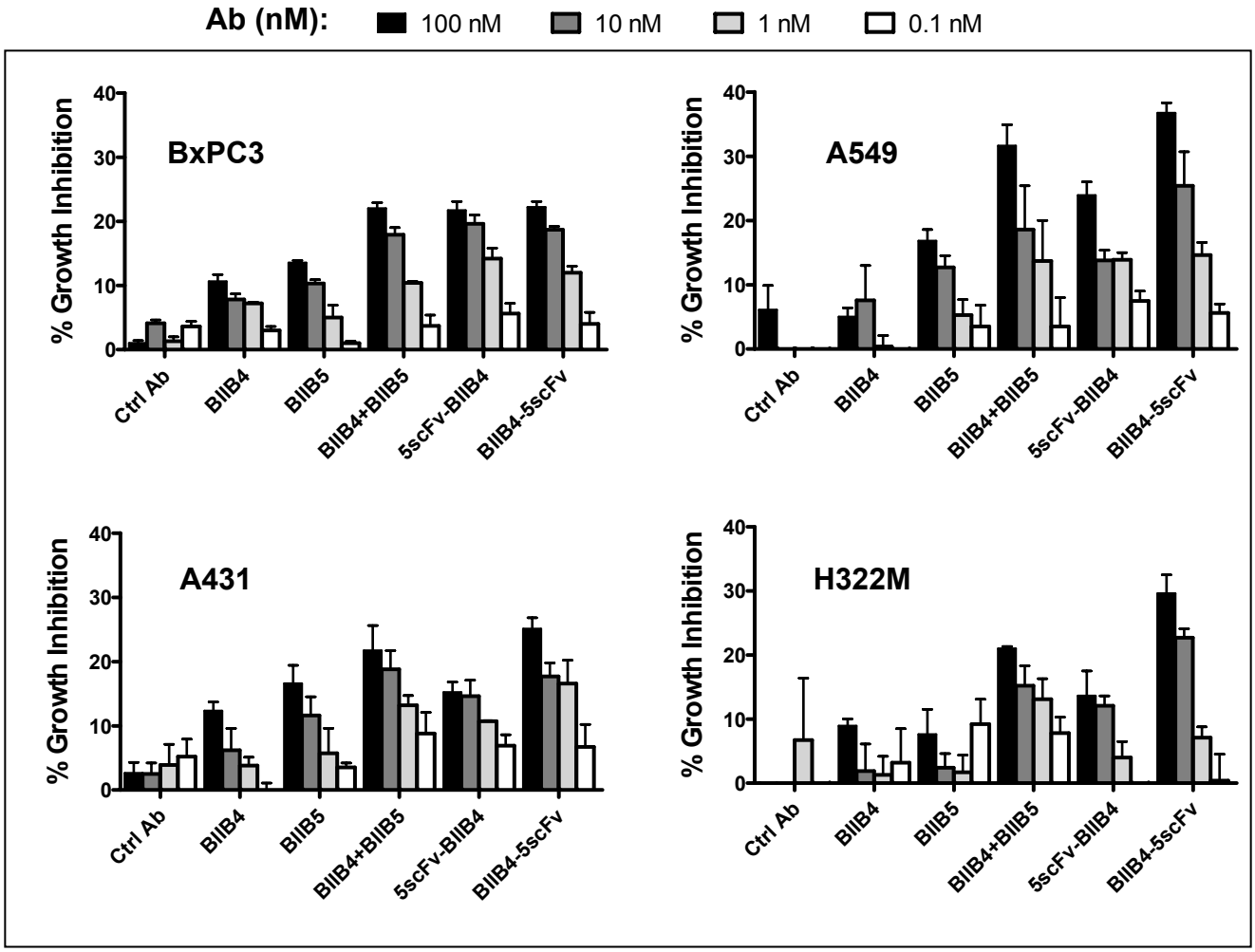


Suppl. Fig. 2: Stability of the BsAbs assessed by SEC/LS, DSC, and CD. All the BsAbs demonstrated very little aggregation in a PBS buffer over time periods of at least 2 months by SEC/LS. BIIB4-5scFv, *A.*, demonstrated <2% aggregate formation over 5 months in PBS. B. DSC curves (from bottom to top) of BIIB4 IgG1, BIIB5 IgG1, BIIB4-5scFv, 5scFv-BIIB4, BIIB5-4scFv, and BIIB5-5scFv. The thermal unfolding transitions of the C_H2, FAb, and C_H3 peaks of the BIIB4 and BIIB5 antibody IgG1 backbones are labeled on the curves of the parental IgG1 MAbs. The thermal unfolding transitions of the BIIB5 and BIIB4 stabilized scFvs are labeled on the DSC curves of the BsAbs. Generally, it is clear that the T_Ms of the stabilized BIIB5 and BIIB4 scFvs were above the empirical cutoff of 65 °C. *C.* Temperature-dependent near-UV circular dichroism (CD) spectra of the BIIB4-5scFv BsAb. The majority of the near UV CD signal was found to be absent in the BIIB4 MAb, thus originating from the BIIB5 scFv. Shown in the inset are the fits of BIIB5 scFv unfolding profile to a two-state thermal unfolding transition at multiple wavelengths.

Methods: Size exclusion chromatographs were generated by injecting 30 uL BsAb onto a TSKgel G3000SWXL, 5 mm, 250-Å Analytical SEC column (Tosoh Biosciences) equilibrated in 10 mM phosphate, 150 mM NaCl, 0.02% NaN_3 at pH 6.8 using an Agilent 1100 HPLC system. Light scattering data was collected using a miniDAWN static light scattering detector coupled to an in-line refractive index meter (Wyatt Technologies) and analyzed using the software provided by the manufacturer. DSC data were collected on a capDSC (MicroCal LLC) as described previously (Garber and Demarest, 2007). Near UV CD spectra of BIIB4-5scFv and 5scFv-BIIB4 at 5 μM were obtained using a Chirascan-plus spectrophotometer (Applied Photophysics Ltd.). Temperature ramps of the BsAbs were performed using a 1-cm UV cell and a 2-nm bandwidth. Temperature ramp rates were 1 °C/min and signal averaging times were 0.65 s/λ using a 1.0-nm step size.

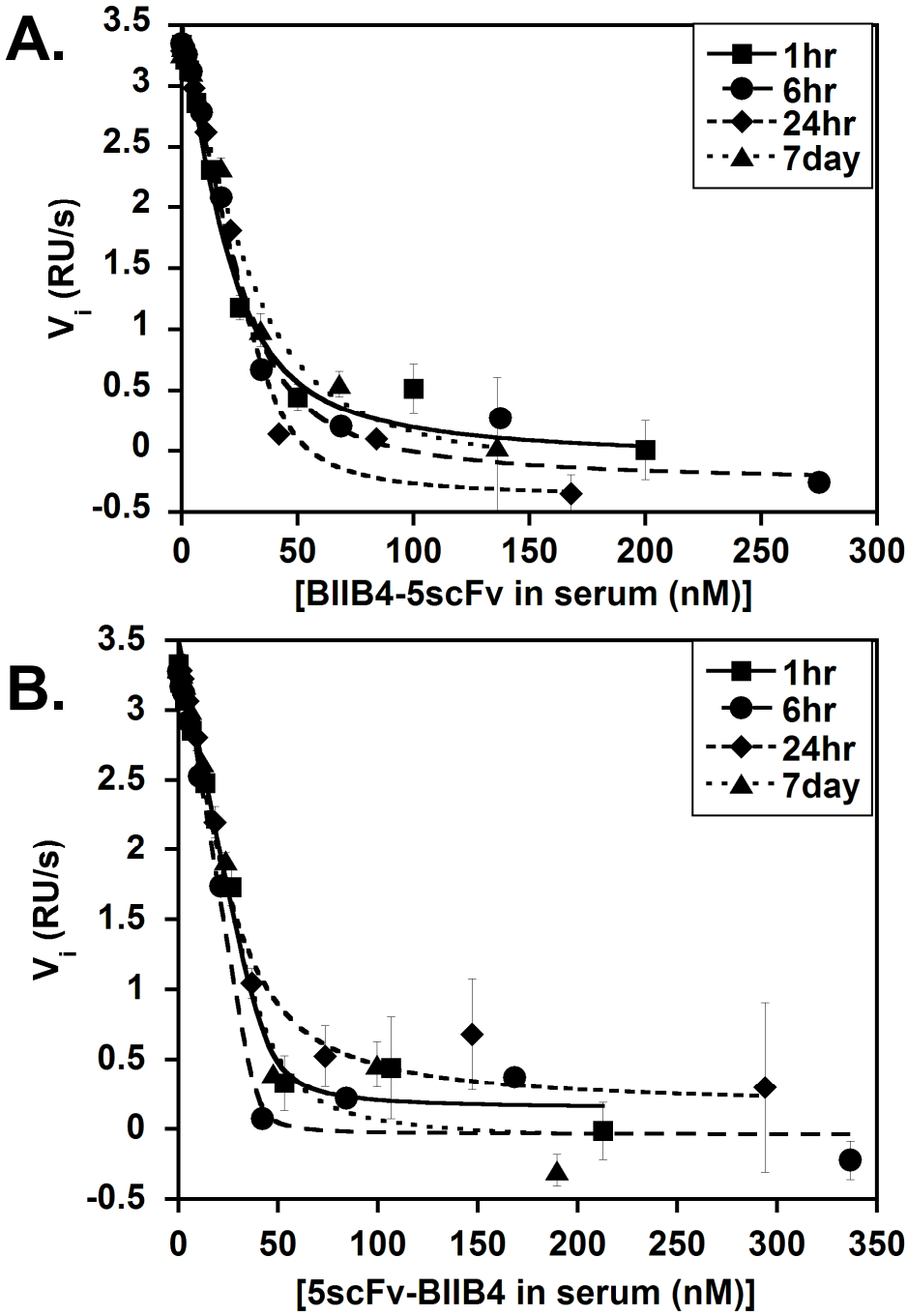
Garber, E., Demarest, S.J. (2007) A broad range of Fab stabilities within a host of therapeutic IgGs. *Biochem. Biophys Res. Commun.* **355,** 751-757

Suppl. Fig. 3



Suppl. Fig. 3: Inhibition of IGF-driven tumor cell growth in multiple tumor cell lines. Cells were stimulated by a mixture of IGF-1/IGF-2 (200 ng/mL each) in the presence of 10% FBS and treated with the indicated MAbs or BsAbs at 0.1, 1, 10, or 100 nM for three days before cell viability determination. Growth inhibition was normalized relative to no Ab treatment cultures.

Suppl. Fig. 4



Suppl. Fig. 4: Determination of the stoichiometric inhibition of hIGF-1R(1-903) binding to a Biacore CM5 chip surface labeled with BIIB4 (A.) and BIIB5 (B.) using mouse sera 1 hr, 6 hrs, 24 hrs, and 7 days post dosing with BIIB4-5scFv and 5scFv-BIIB4.

Methods: The equilbrium Biacore experiment was performed and analyzed as described for the purified proteins in the Methods. BsAb concentrations in sera were determined using an anti-human IgG-Fc ELISA as described in the Methods.