Supplemental Data for Markovitz et al.

The diversity of the immune response to the A2 domain of human factor fVIII

Supplemental Figure Legends

Figure S1. Epitope mapping of anti-A2 MAbs using homolog scanning mutagenesis. Binding of anti-A2 MAbs to HP fVIII. FVIII was captured on a microtiter plate using immobilized anti-C2 antibody, followed by addition of biotinylated anti-A2 MAb and detection using streptavidin alkaline phosphatase. Bars represent the range of duplicate determinations.

Figure S2. Effect of group A, B and C A2 MAbs on thrombin – catalyzed proteolytic cleavage of full-length fVIII. Thrombin (0.5 nM) was incubated with 100 nM fVIII for 10 min at 37 ºC in the presence of increasing MAb concentrations. The reaction was stopped by the addition of 10 µl non-reducing SDS-PAGE Sample Buffer (Pierce Biotechnology) and heating at 98°C for 5 minutes. Samples (40 µl) were loaded onto a 10% SDS-PAGE Ready Gel (BioRad 161-1155) and electrophoresis was performed at 150 volts for 60 minutes. Molecular weight standards (“Stds”) are 250, 150, 100, 75, 50, 37, and 25 kDa. “Ctrl” represents fVIII not exposed to thrombin.

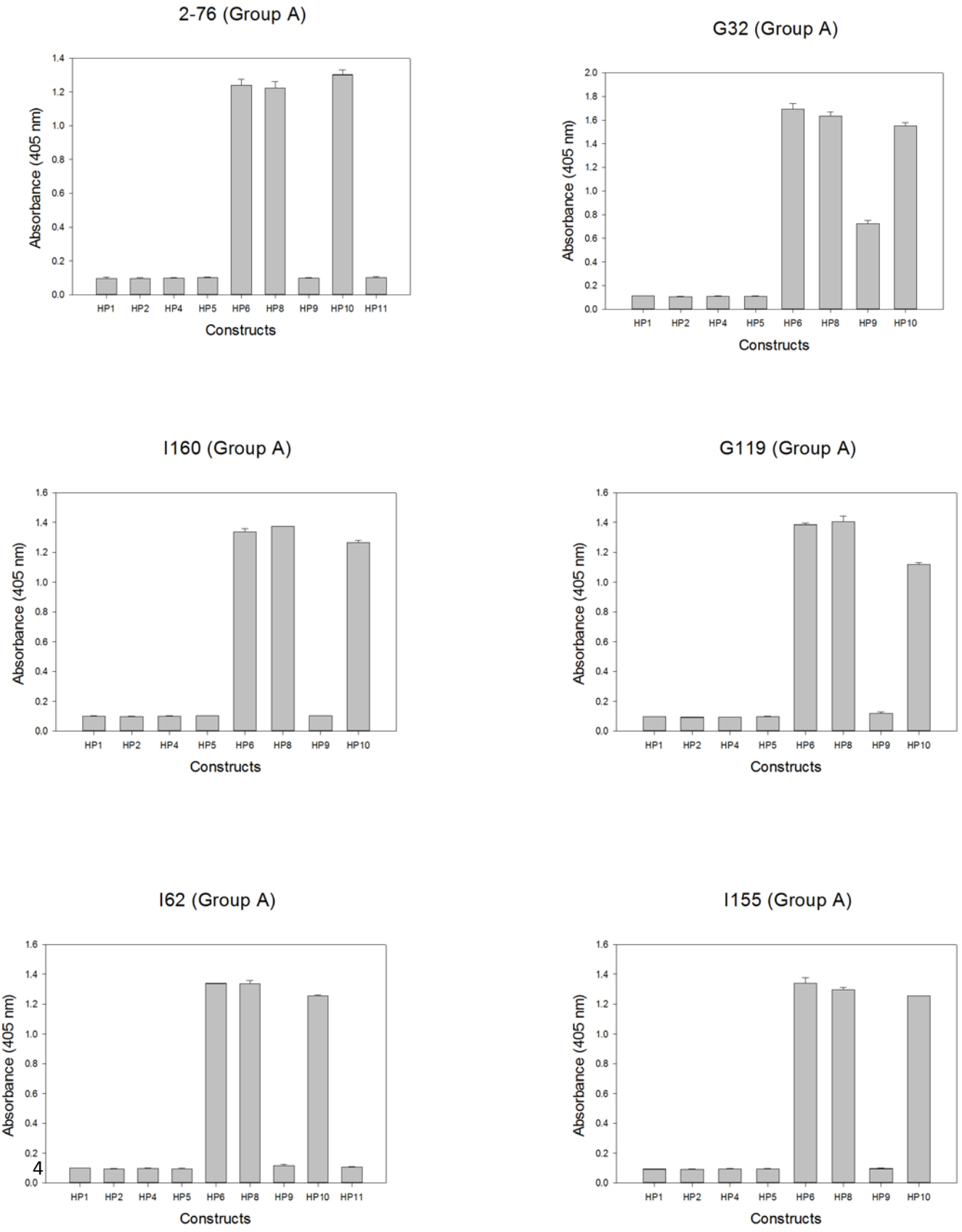
Figure S3. Effect of group D and E anti-A2 MAbs on thrombin – catalyzed proteolytic cleavage of B domain less fVIII. FVIII (100 nM) was incubated with thrombin (0.5 nM) for 10 min at 37 ºC in the presence of increasing MAb concentrations, followed by SDS-PAGE as described in Figure S2.

Figure S4. Effect of VWF on thrombin-catalyzed proteolytic cleavage of full-length fVIII. Full-length fVIII (100nM) was incubated in the absence (Gel A) or presence (Gel B) of VWF (1000nM monomer equivalents) for 10 minutes at 37°C. Thrombin was added (0.5nM) and the reaction was stopped at varying times and analyzed by SDS-PAGE as described in Figure S2.

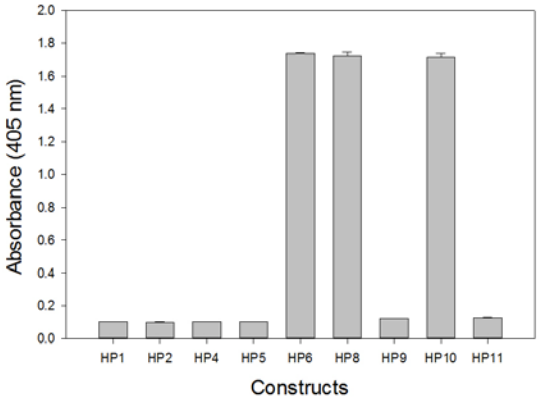
Figure S5. Effect of group E MAb 1D4 on thrombin – catalyzed proteolytic cleavage of full-length fVIII/VWF complex. Full-length fVIII (100nM) was incubated in the absence (Gel A) or presence (Gel B) of VWF (1000nM monomer equivalents) for 10 minutes at 37°C. MAb 1D4 (125nM) was added, followed by additional incubation for 10 minutes. Thrombin was added (0.5nM) and the reaction was stopped at varying times and analyzed by SDS-PAGE as described in Figure S2.

Figure S6. Effect of Group D and E MAbs on fVIII binding to VWF. Immulon-1B plates were coated overnight at 4ºC with 6µg/ml VWF in 0.15 M NaCl/20 mM HEPES/0.05% sodium azide, pH 7.4. FVIII (1 nM) was mixed with an equal volume of anti-A2 or anti-C2 MAb at concentrations ranging from 0 to 10 µg/ml, incubated for 2 hours at 37ºC, followed by the addition of the mixture to VWF-coated microtiter wells and incubation for 1hr at room temperature. Bound FVIII was measured using biotinylated anti-A1 MAb 2-116 as secondary antibody, streptavidin-alkaline phosphatase conjugate and p-nitrophenylphosphate. Error bars represent sample standard deviations of triplicate determinations.

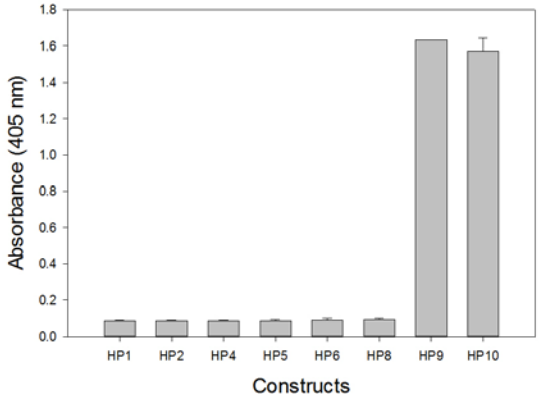
Figure S1


2-76 (Group A)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10 HP11
G32 (Group A)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10
I160 (Group A)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10
G119 (Group A)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10
I62 (Group A)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10 HP11
I155 (Group A)
Absorbance (405 nm)
Constructs
HP1 HP2 HP4 HP5 HP6 HP8 HP9 HP10

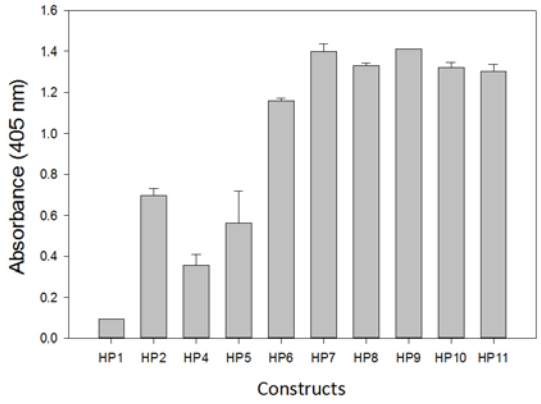
2-105 (Group A)



4A4 (Group A)



B157 (Group AB)



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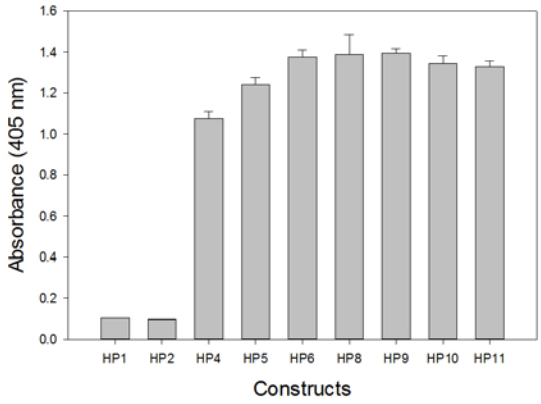
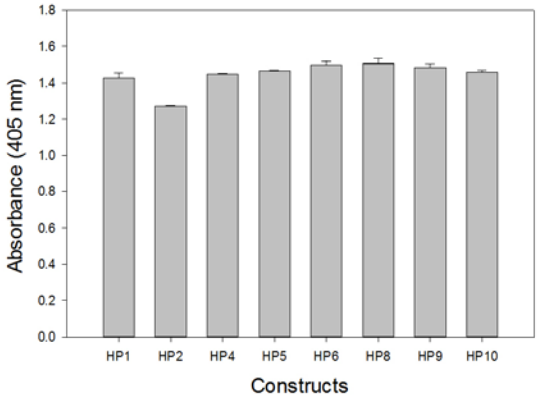
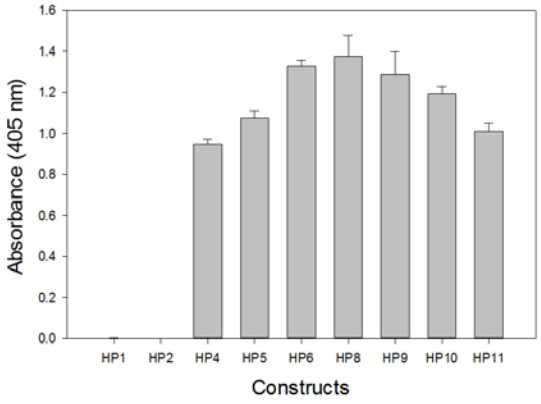


4F4



G139 (Group B)



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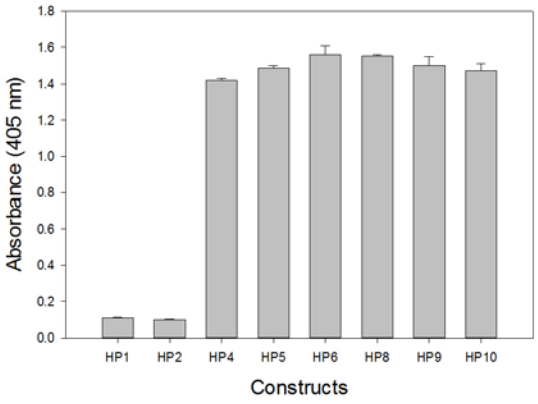
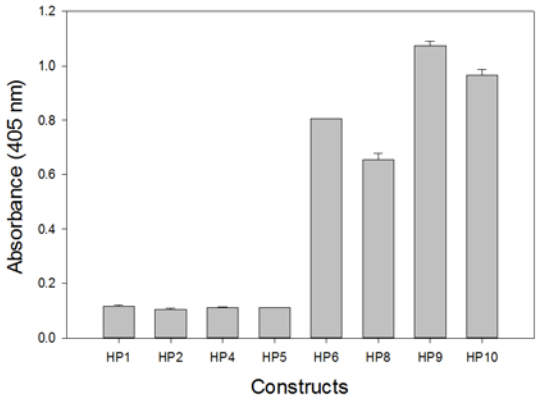
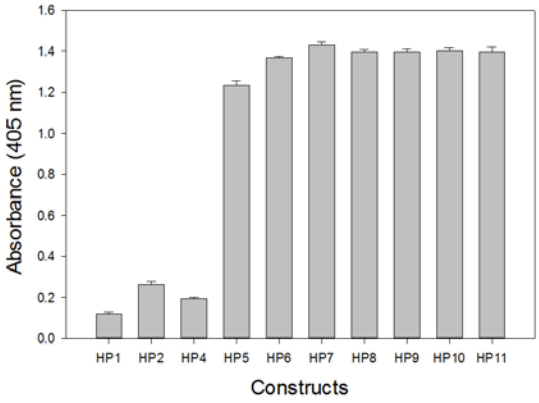


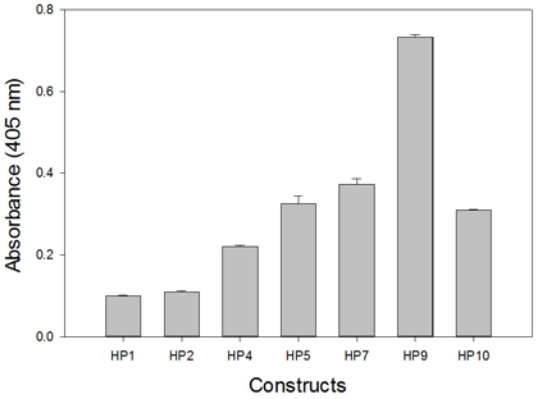
G6 (Group B)



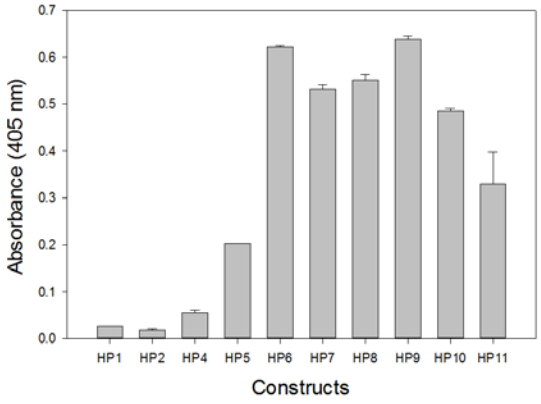
G4 (Group B)



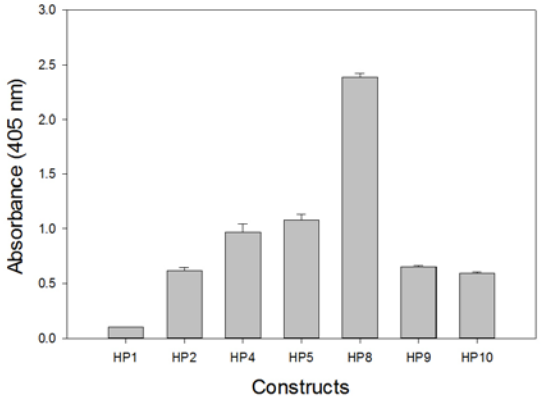
B99 (Group BCD)



B25 (Group C)



CLB-CAg 9 (Group D)



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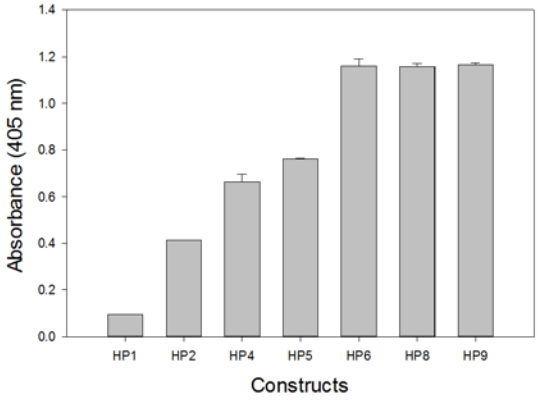
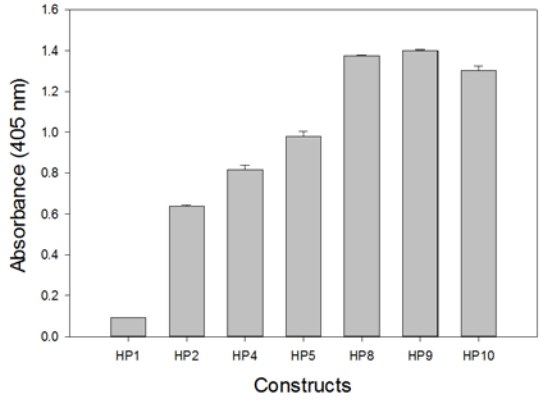


G74 (Group DE)



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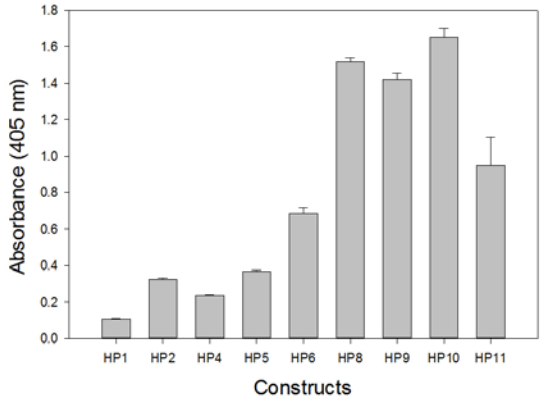

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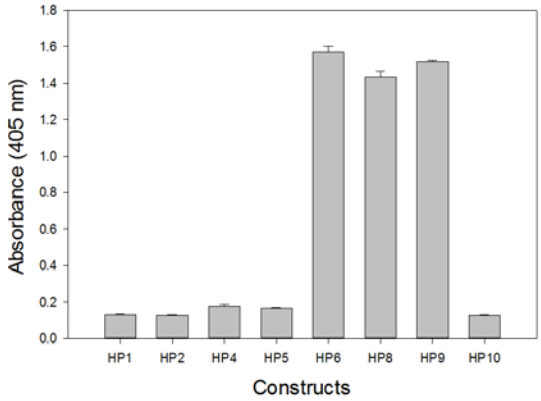
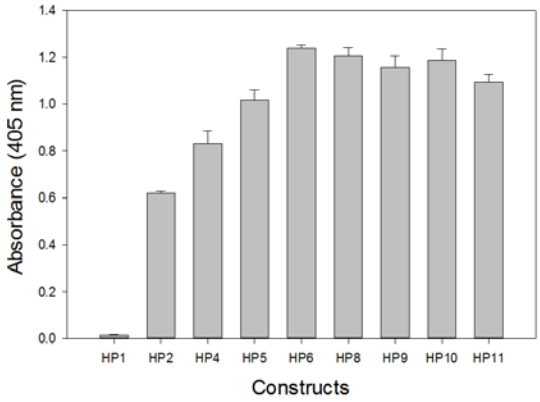


B66 (Group E)



1D4 (Group E)

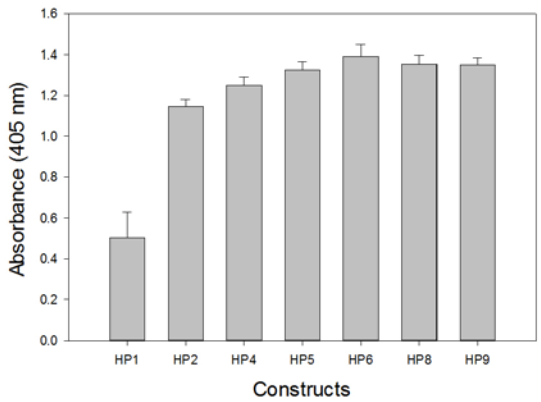


Figure S2

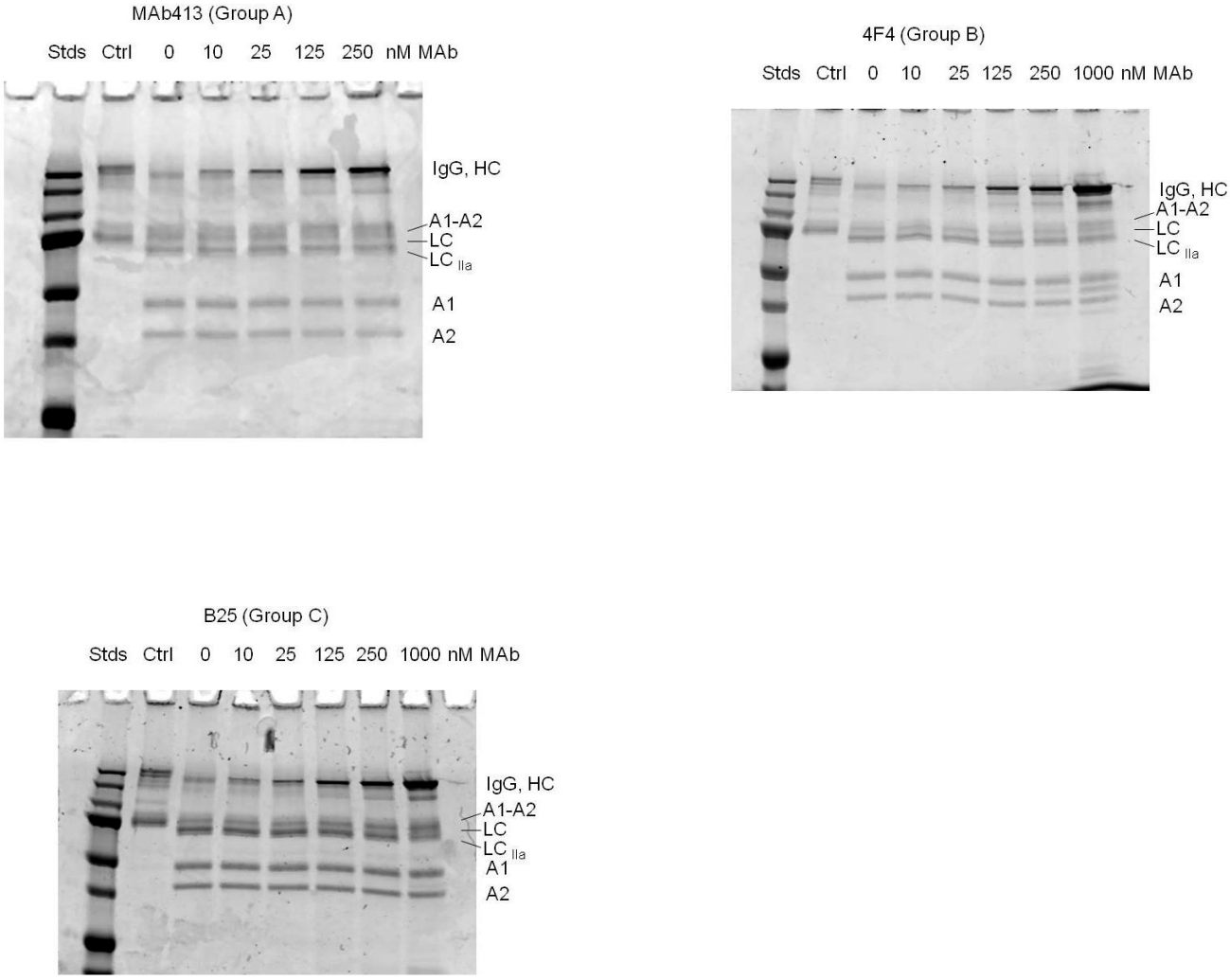

MAb413 (Group A)
Stds Ctrl 0 10 25 125 250 nM MAb
IgG, HC
A1-A2
LC
LC IIa
A1
A2
4F4 (Group B)
Stds Ctrl 0 10 25 125 250 1000 nM MAb
IgG, HC
A1-A2
LC
LC IIa
A1
A2
B25 (Group C)
Stds Ctrl 0 10 25 125 250 1000 nM MAb
IgG, HC
A1-A2
LC
LC IIa
A1
A2

Figure S3

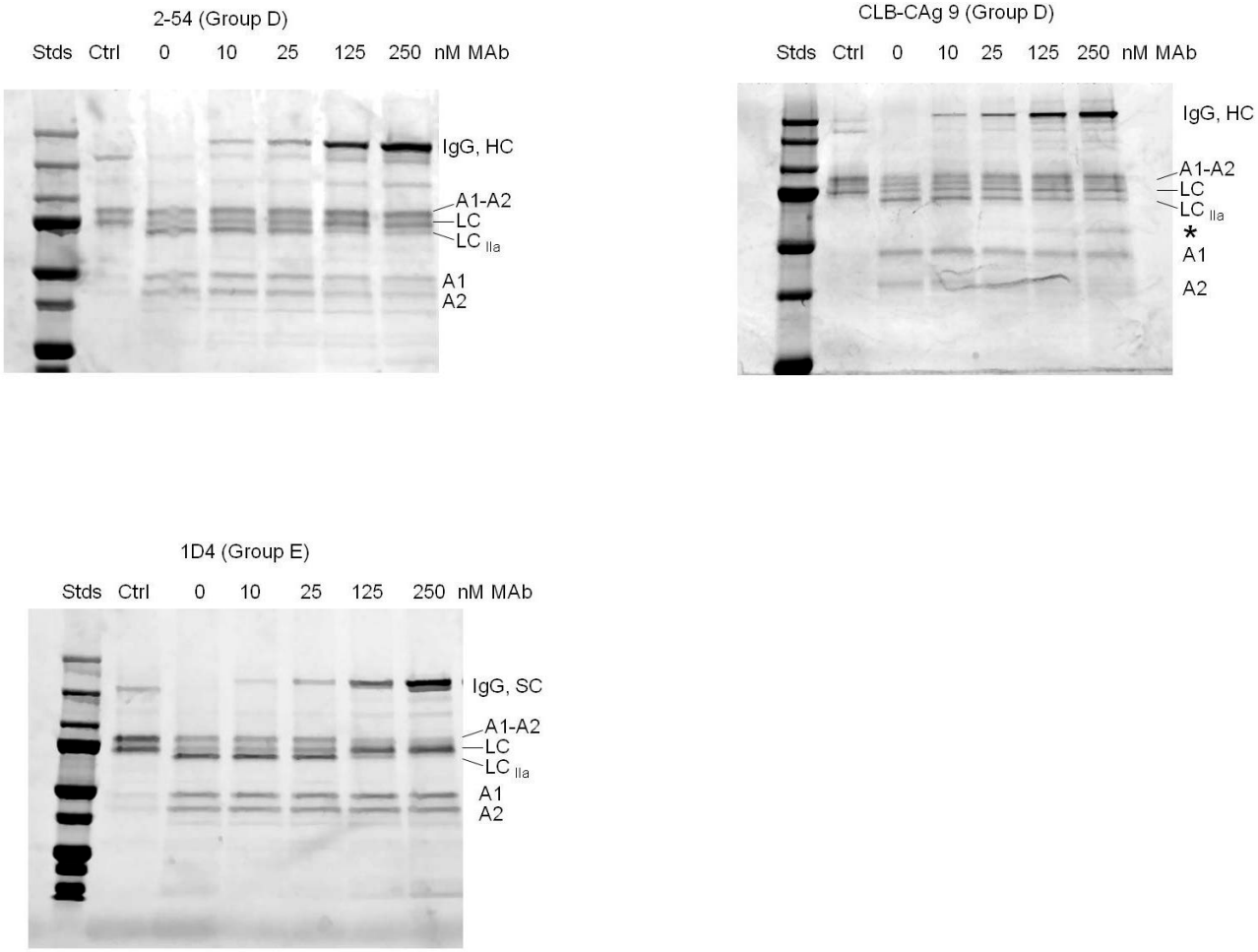

2-54 (Group D)
Stds Ctrl 0 10 25 125 250 nM MAb
IgG, HC
A1-A2
LC
LC IIa
A1
A2
CLB-CAg 9 (Group D)
Stds Ctrl 0 10 25 125 250 nM MAb
IgG, HC
A1-A2
LC
LC IIa
*
A1
A2
1D4 (Group E)
Stds Ctrl 0 10 25 125 250 nM MAb
IgG, SC
A1-A2
LC
LC IIa
A1
A2

Figure S4

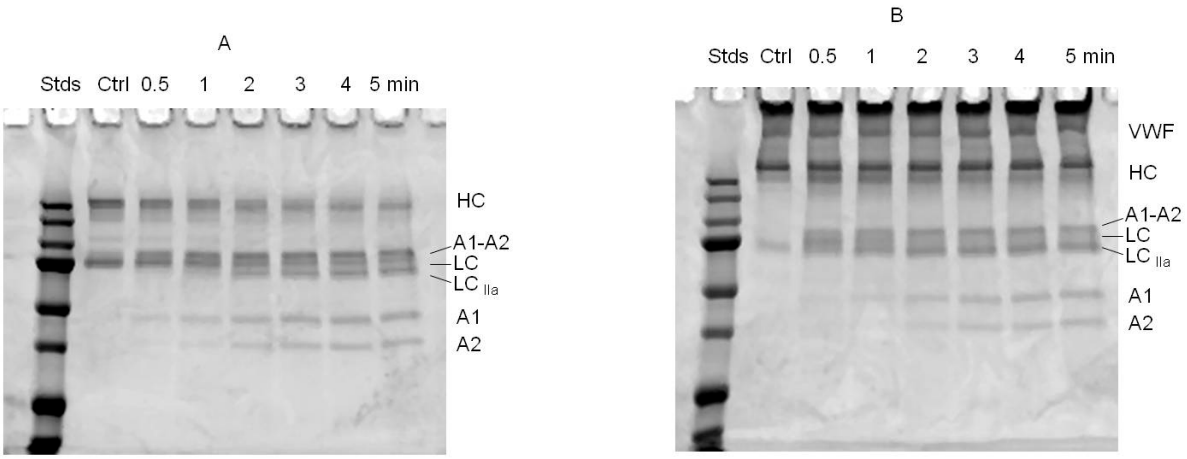

A
Stds Ctrl 0.5 1 2 3 4 5 min
HC
A1-A2
LC
LC IIa
A1
A2
B
Stds Ctrl 0.5 1 2 3 4 5 min
VWF
HC
A1-A2
LC
LC IIa
A1
A2

Figure S5

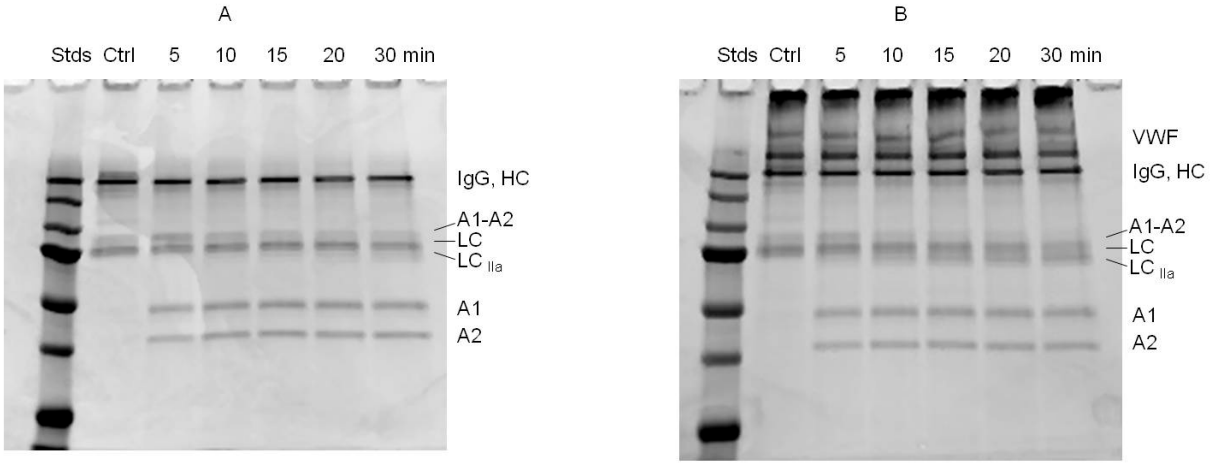
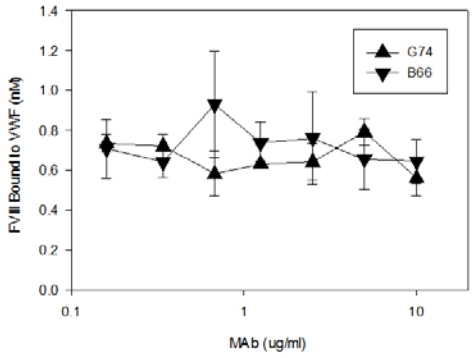
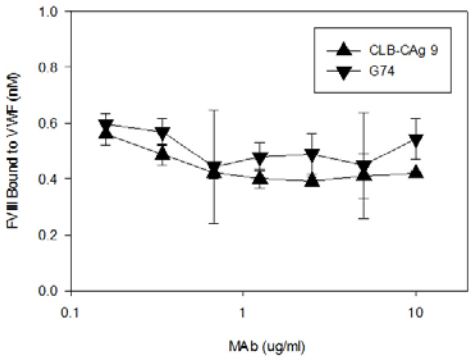
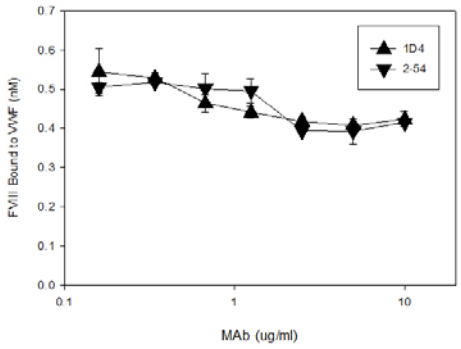
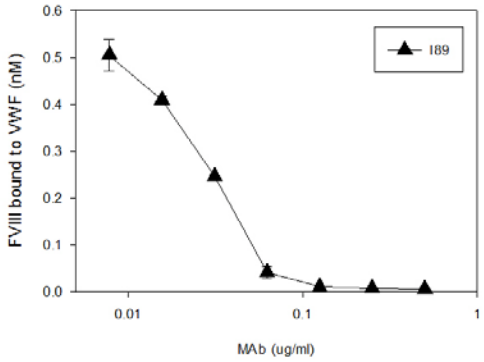

A
Stds Ctrl 5 10 15 20 30 min
IgG, HC
A1-A2
LC
LC IIa
A1
A2
B
Stds Ctrl 5 10 15 20 30 min
VWF
IgG, HC
A1-A2
LC
LC IIa
A1
A2

Figure S6.


G74
B66
FVIII Bound to VWF (nM)
MAb (ug/ml)


CLB-CAg 9
G74
FVIII Bound to VWF (nM)
MAb (ug/ml)


1D4
2-54
FVIII Bound to VWF (nM)
MAb (ug/ml)


I89
FVIII bound to VWF (nM)
MAb (ug/ml)