Figure 1: Different VWF Constructs

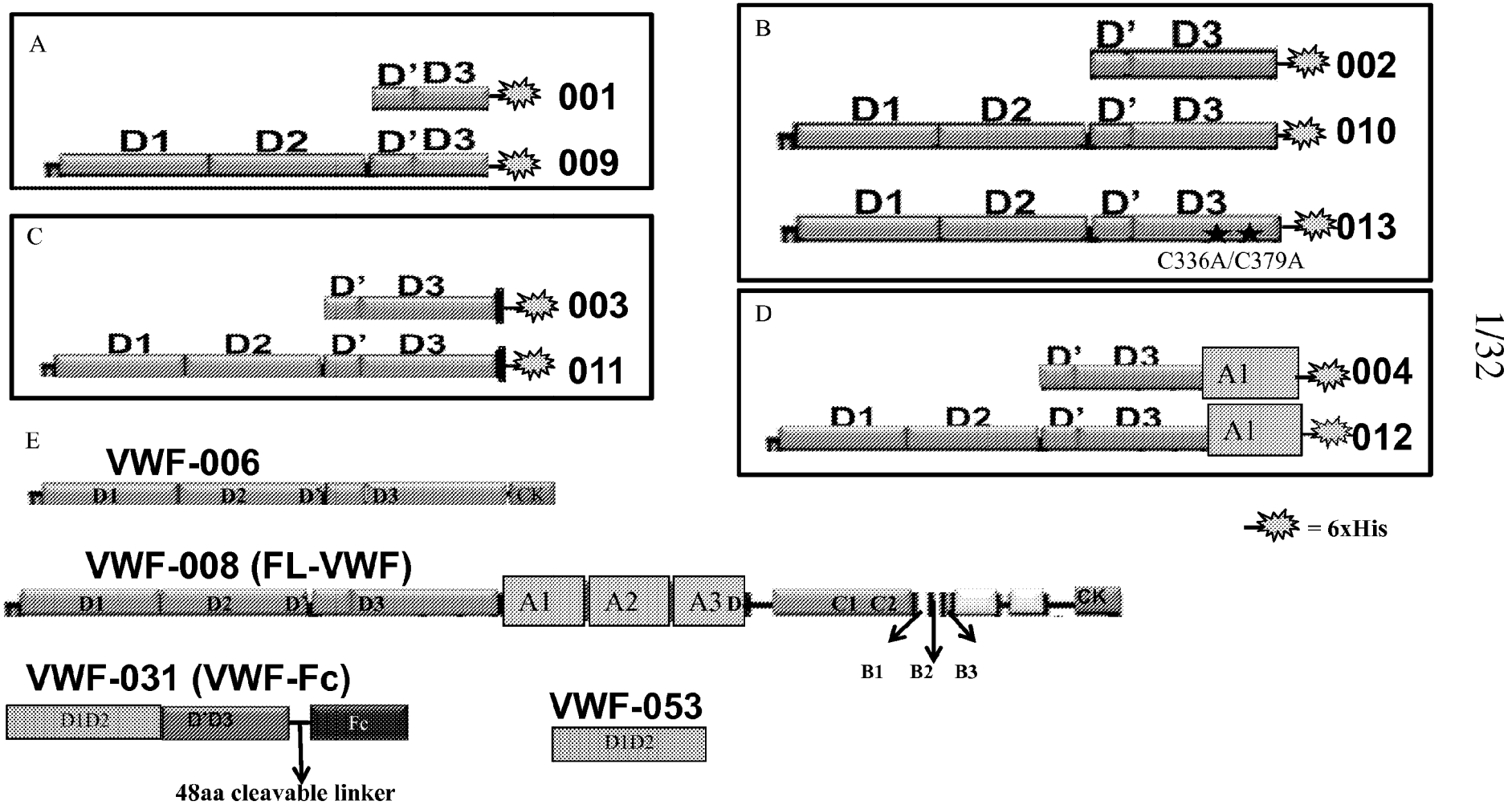
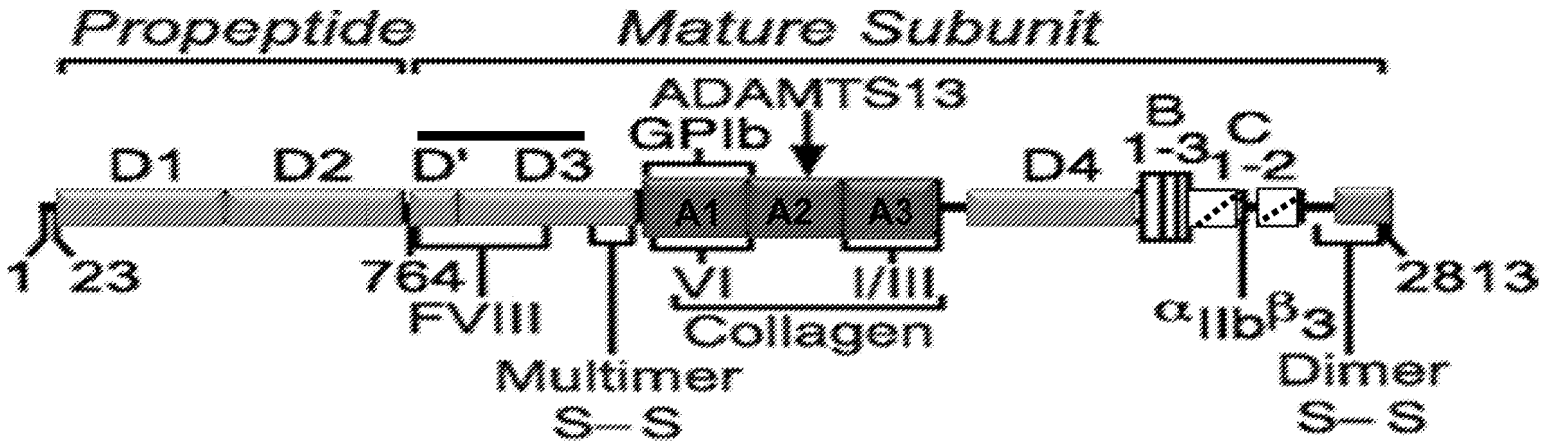


Figure 1F: Von Willebrand Factor



- ~ 250 kDa protein, forms multimers (> 20 MDa) by disulfide bonding

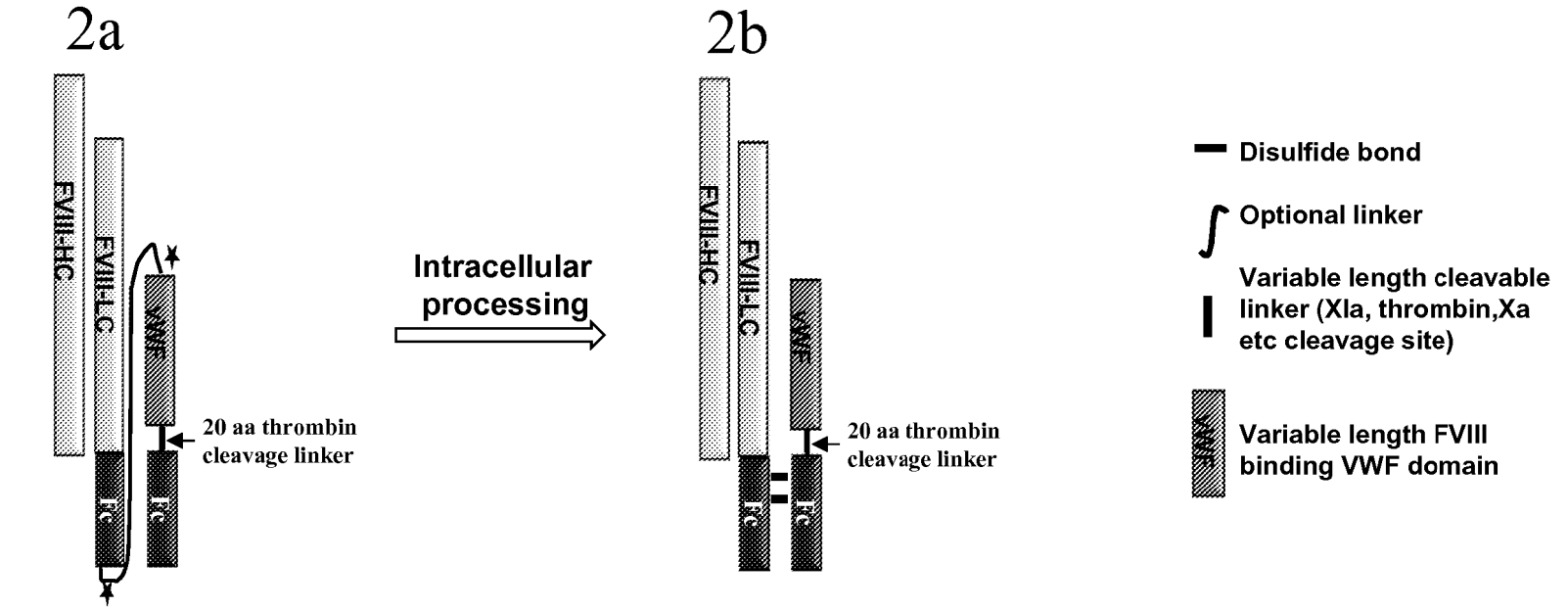
- Associates with FVIII (95-98%) in non-covalent complex
  - Protects FVIII from protease cleavage/activation
  - Stabilizes heavy & light chain
  - Prevents clearance of FVIII by scavenger receptors

  } **Extends half-life**

  - Clearance of FVIII-vWF complex through vWF-receptors
  - Prevents pinocytosis and recycling of rFVIIIFc?

  } **Limits half-life**

Figure 2: FVIII Constructs (FVIII/VWF heterodimer)

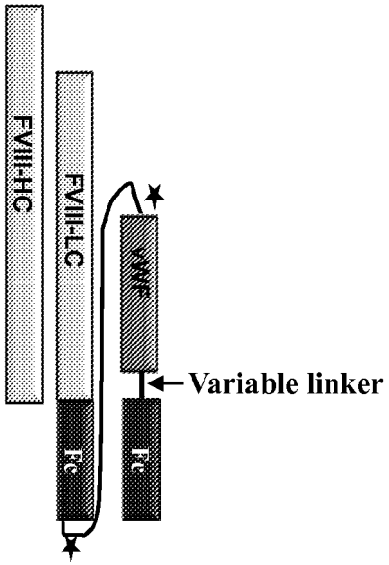


FVIII-064/065
DNA construct
Processable linker

FVIII-064/065
Protein product

FVIII-064: Contains VWF D'D3 1-477 aa with C336A/C379A
FVIII-065: Contains VWF D'D3 1-276 aa
FVIII-136: FVIII-064 in pCDNA backbone
FVIII-148: Dual chain FVIII molecule in FVIII-136 has been changed to a single chain FVIII by introducing R1645A/R1648A mutation into FVIII gene

Figure 3: FVIII Constructs (variable linker)



FVIII-064, 159, 160, 178, 179

In vivo processable linker

DNA construct	Linker between VWF and Fc
FVIII-064	20 aa= ID {2X(GGGGS)}LVPRGSGG
FVIII-159	35 aa= IS{5X(GGGGS)}LVPRGSGG
FVIII-160 and its derivatives	FVIII-160: 48 aa= IS{6X(GGGGS)}LVPRGSGGGGSGGGGS
	FVIII-180: FVIII-160 with K2092A mutation in FVIII C1 domain
	FVIII-181: FVIII-160 with F2093A mutation in FVIII C1 domain
	FVIII-182: FVIII-160 with K2092A/F2093A mutations in FVIII C1 domain
FVIII-178	73 aa= IS{11X(GGGGS)} LVPRGSGGGGSGGGGS
FVIII-179	98 aa= IS{16X(GGGGS)} LVPRGSGGGGSGGGGS
VWF= D'D3 (1-477aa with C336A/C379A)	

Figure 4: FVIII Constructs

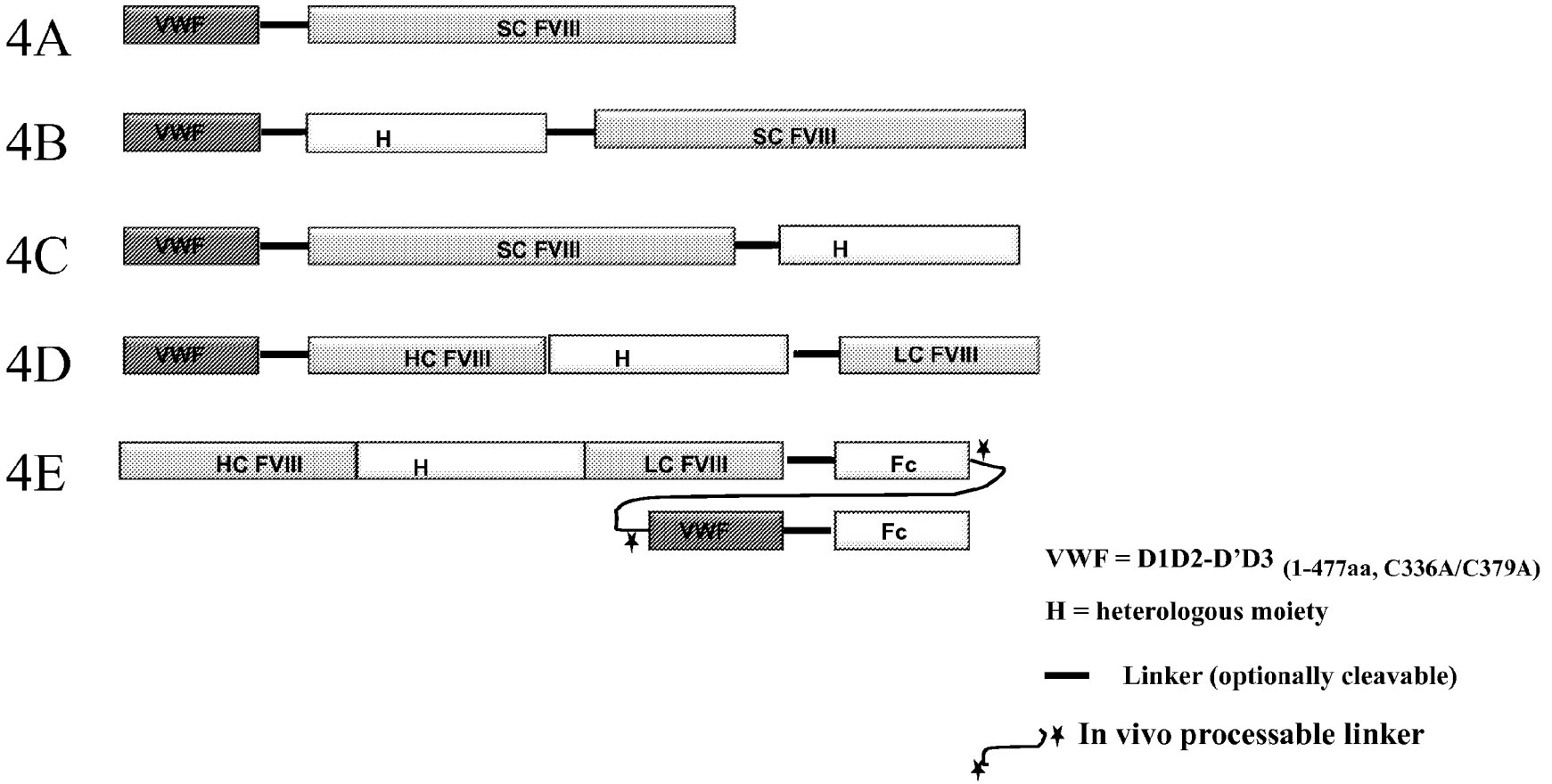
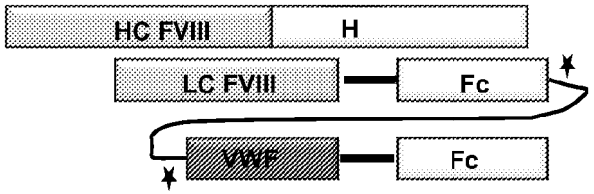
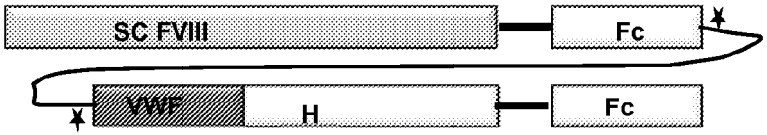


Figure 4: FVIII Constructs (continued)

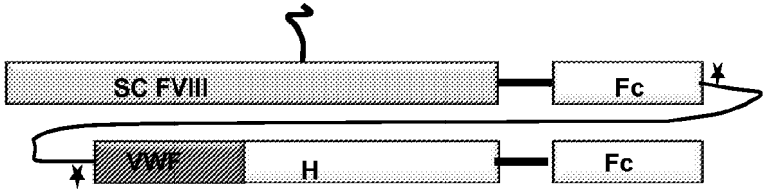
4F



4G



4H



= PEG or HES

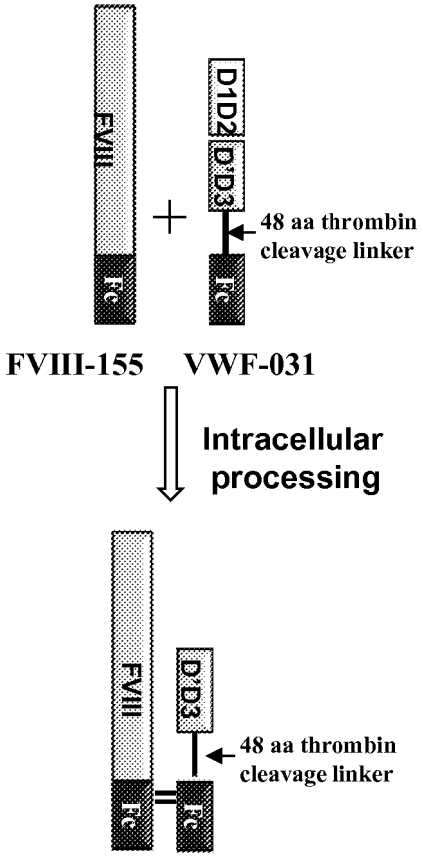
VWF = D1D2-D'D3 (1-477aa, C336A/C379A)

H = heterologous moiety

Linker (optionally cleavable)

In vivo processable linker

Figure 5: FVIII Constructs (Co-transfection system)



FVIII-155	Single chain (SC) - FVIII R1645A/R1648A directly fused to a single Fc
VWF-031	D1D2D'D3 (1-477aa with C336A/C379A) fragment linked to a single Fc with a 48aa thrombin cleavable linker

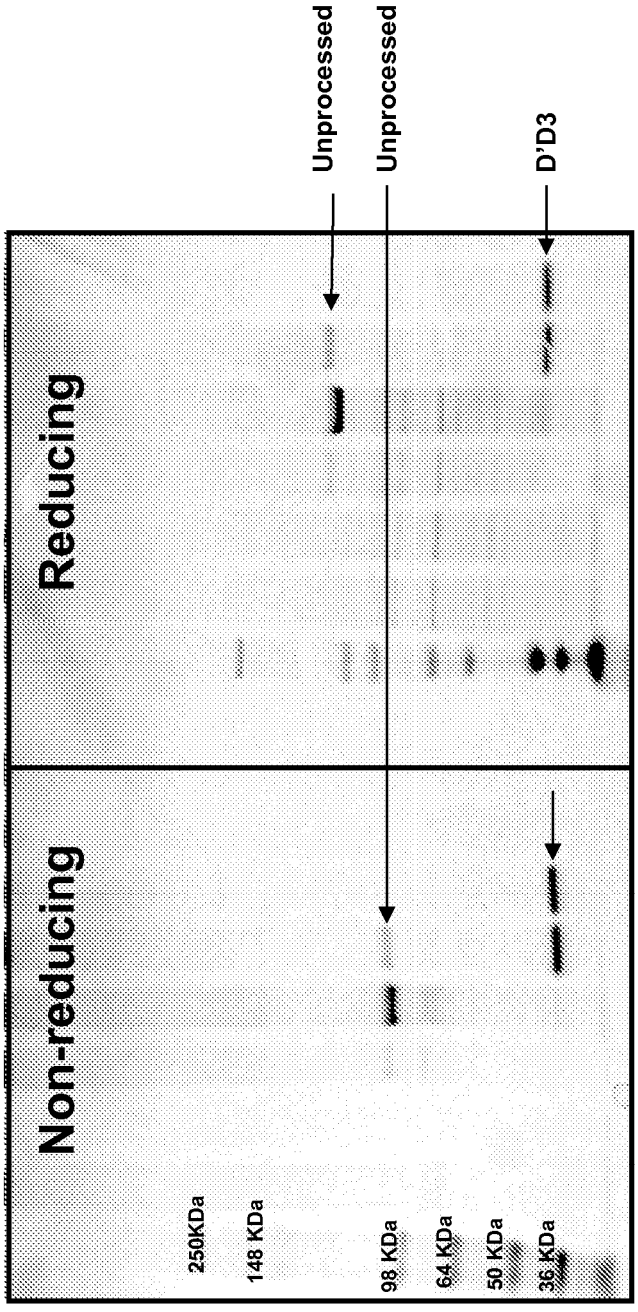
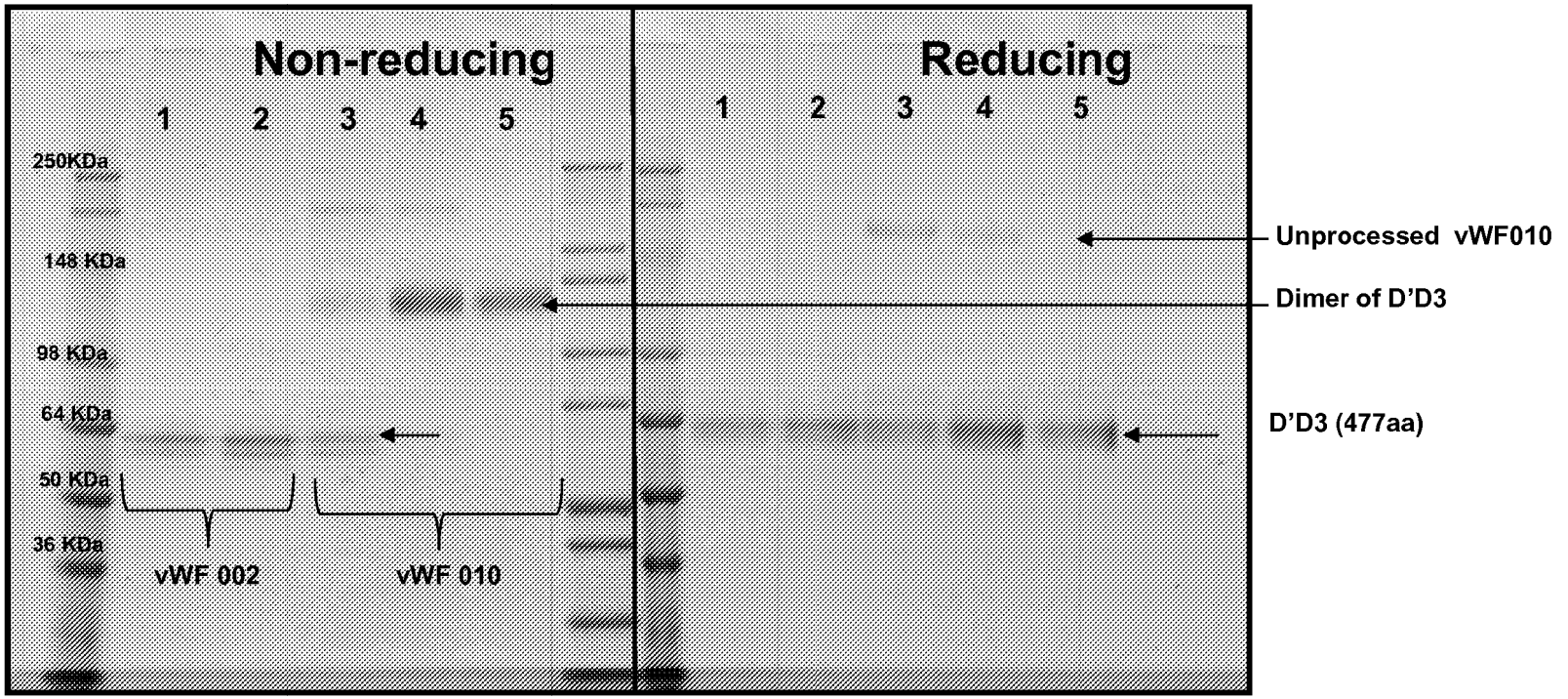


Figure 6: Purification of VWF 009 (D1D2D'D3 $_{1\text{-}276\ aa}$ x 6 his)

Purified VWF-009 exist as a monomer

Figure 7: Purified vWF 002 and 010 (D'D3 $_{1\text{-}477\ aa}$ x 6 his)



Doublet around 60kDa shows different glycosylation state

1- vWF -002 IMAC Fraction 1A3
2- vWF -002 IMAC Fraction 1B1
3- vWF -010 IMAC Fraction 1B3
4- vWF -010 IMAC Fraction 2A1
5- vWF -010 IMAC Fraction 2A2

Figure 8: Thrombin digestion of FVIII-VWF heterodimer

Thrombin digestion of FVIIIFC-VWF protein

1- Marker
2- rFVIII-Fc
3- rFVIII-Fc +Thrombin
4- Empty
5- FVIIIFc-VWF
6- FVIIIFc-VWF +Thrombin
A1= A1 domain of FVIII
A2= A2 domain of FVIII
Δa3 LC = Light chain of FVIII

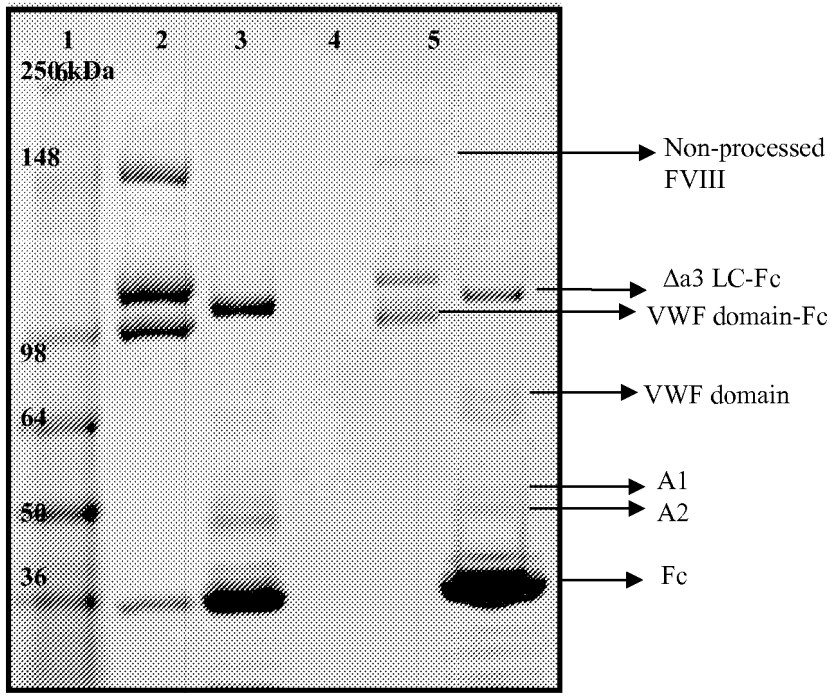
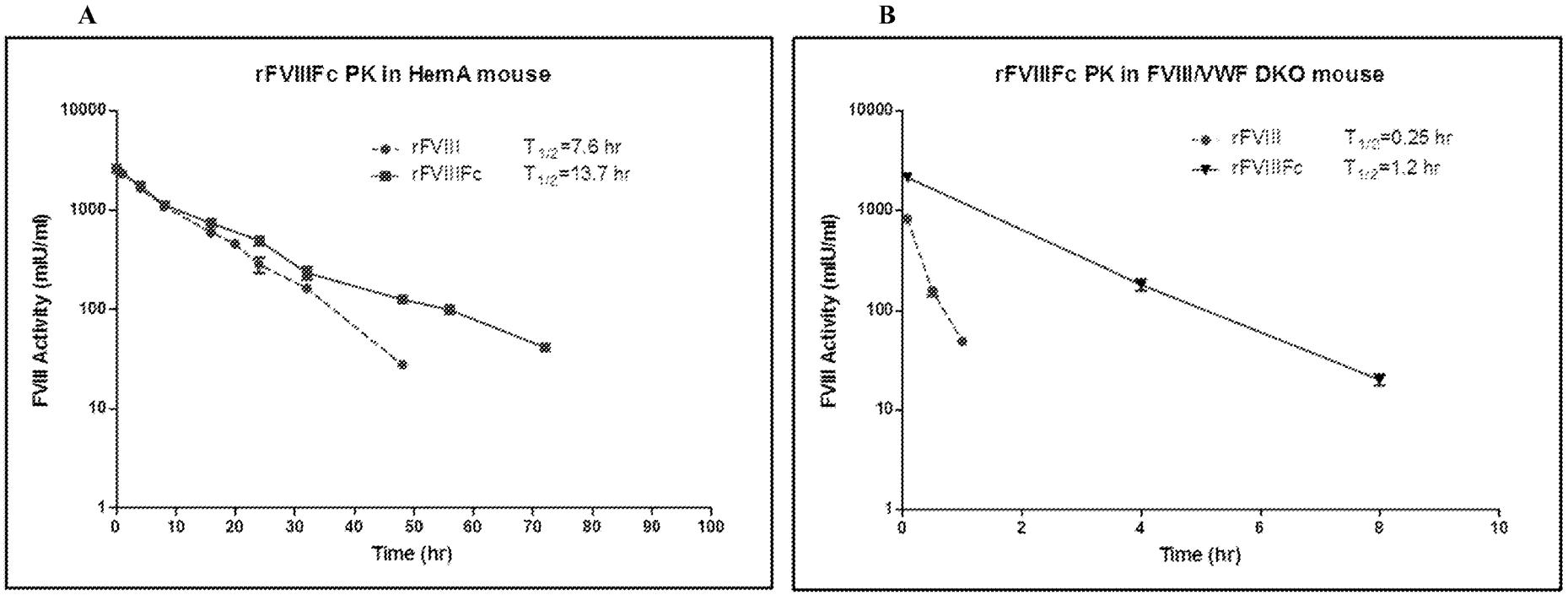


Figure 9: FVIII-VWF interaction is a limiting factor for FVIII half-life extension



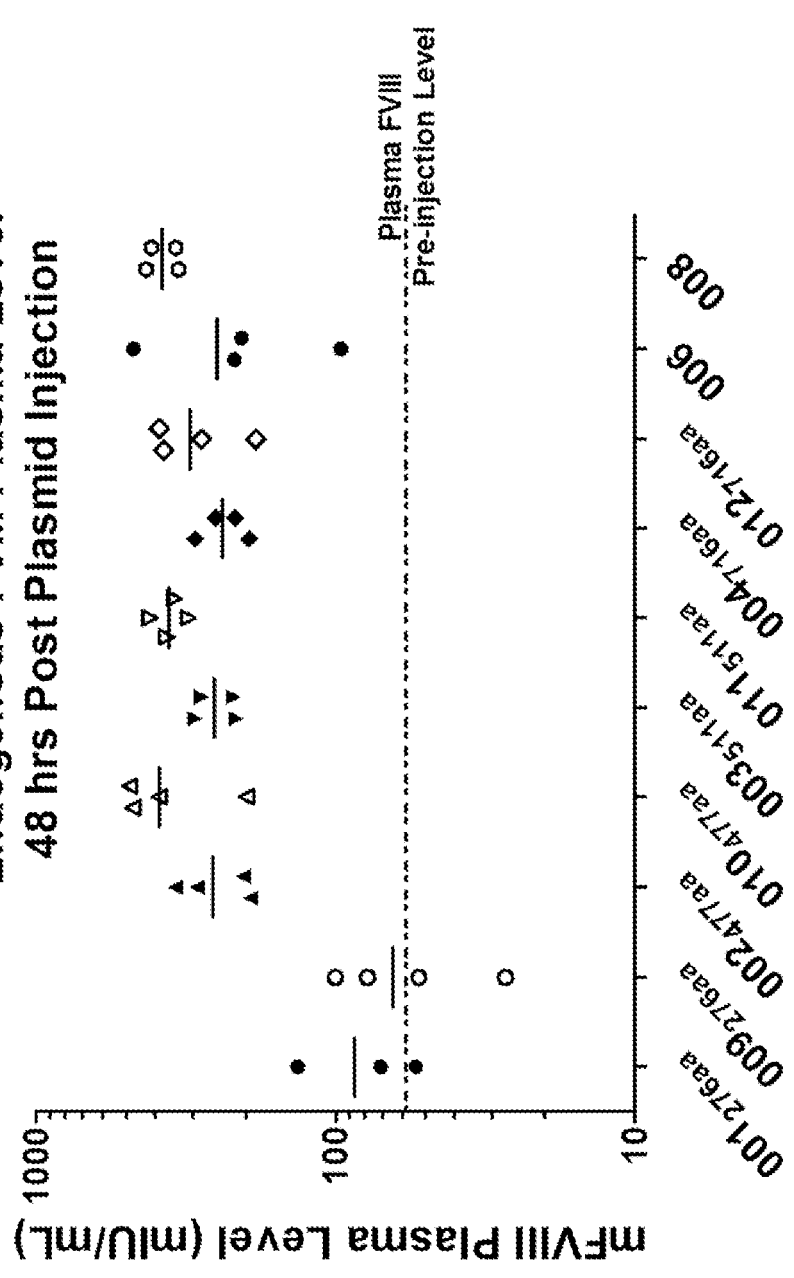


Figure 10A: Full length D'D3 dimer provides same FVIII protection as the Full length VWF molecule

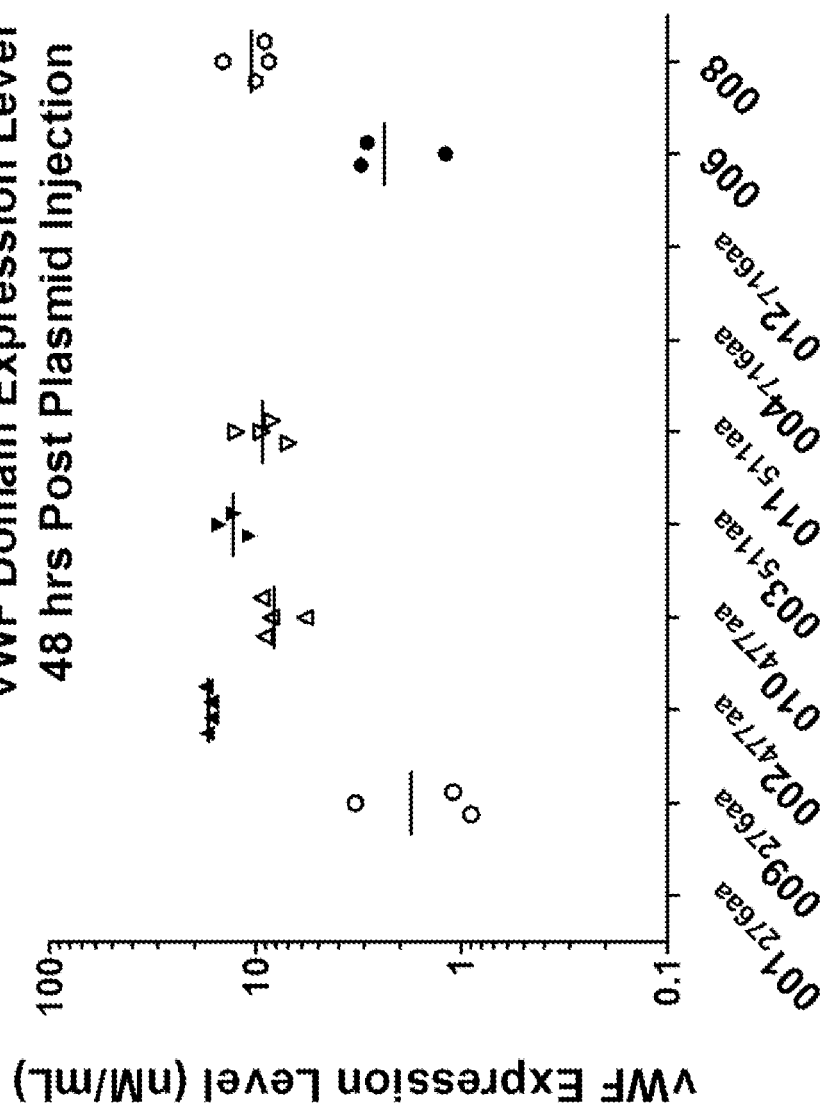


Figure 10B: Full length D'D3 dimer provides same FVIII protection as the Full length VWF molecule

Figure 11: BDD-FVIII PK in FVIII-VWF DKO mice with VWF-010 or VWF-002 co-injection

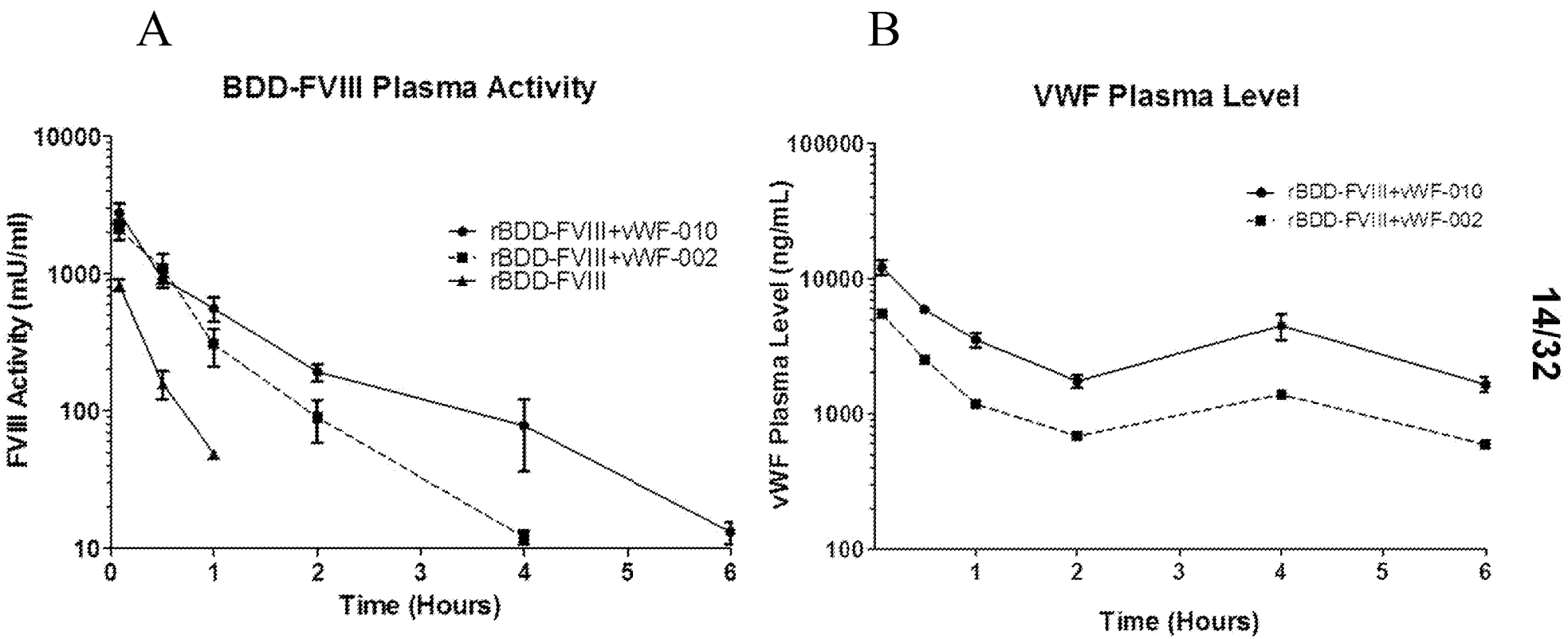
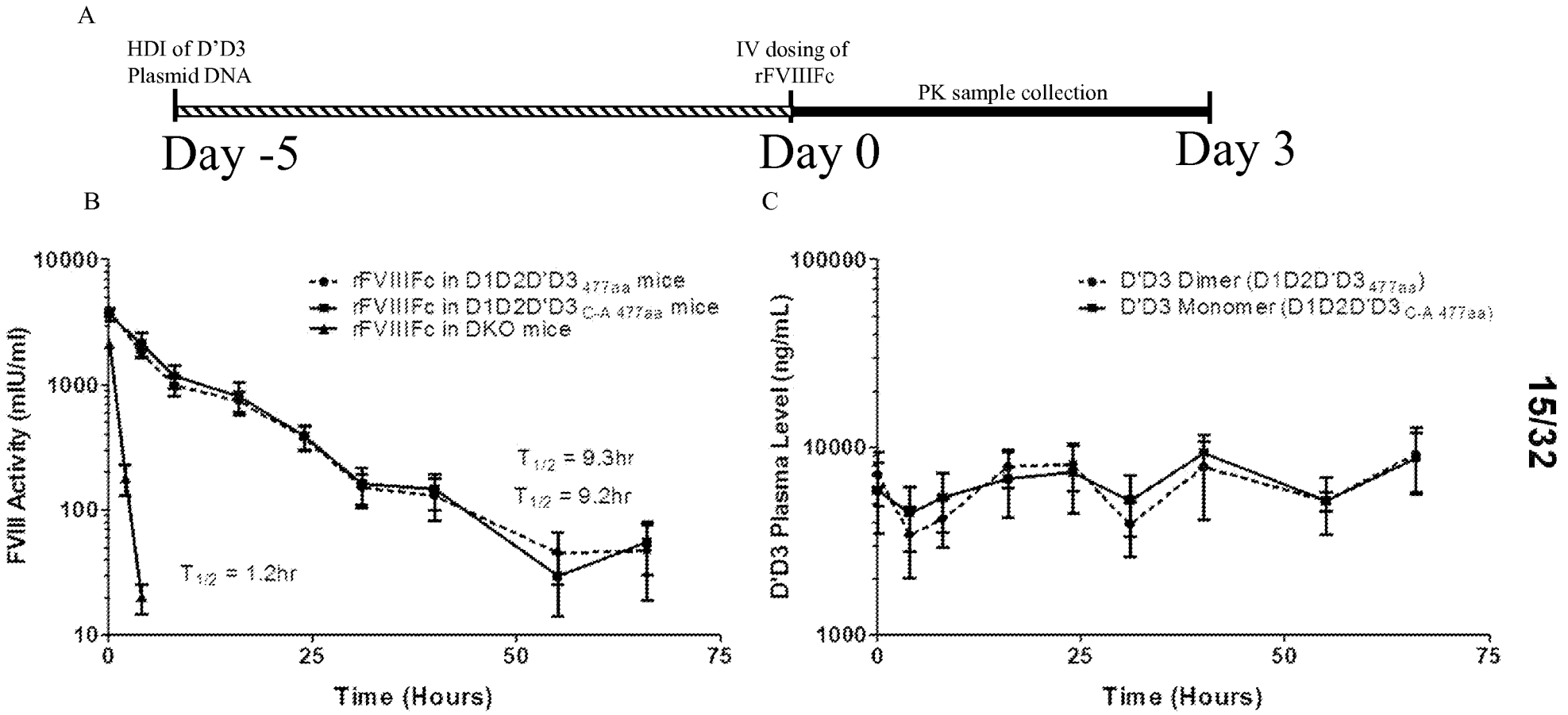
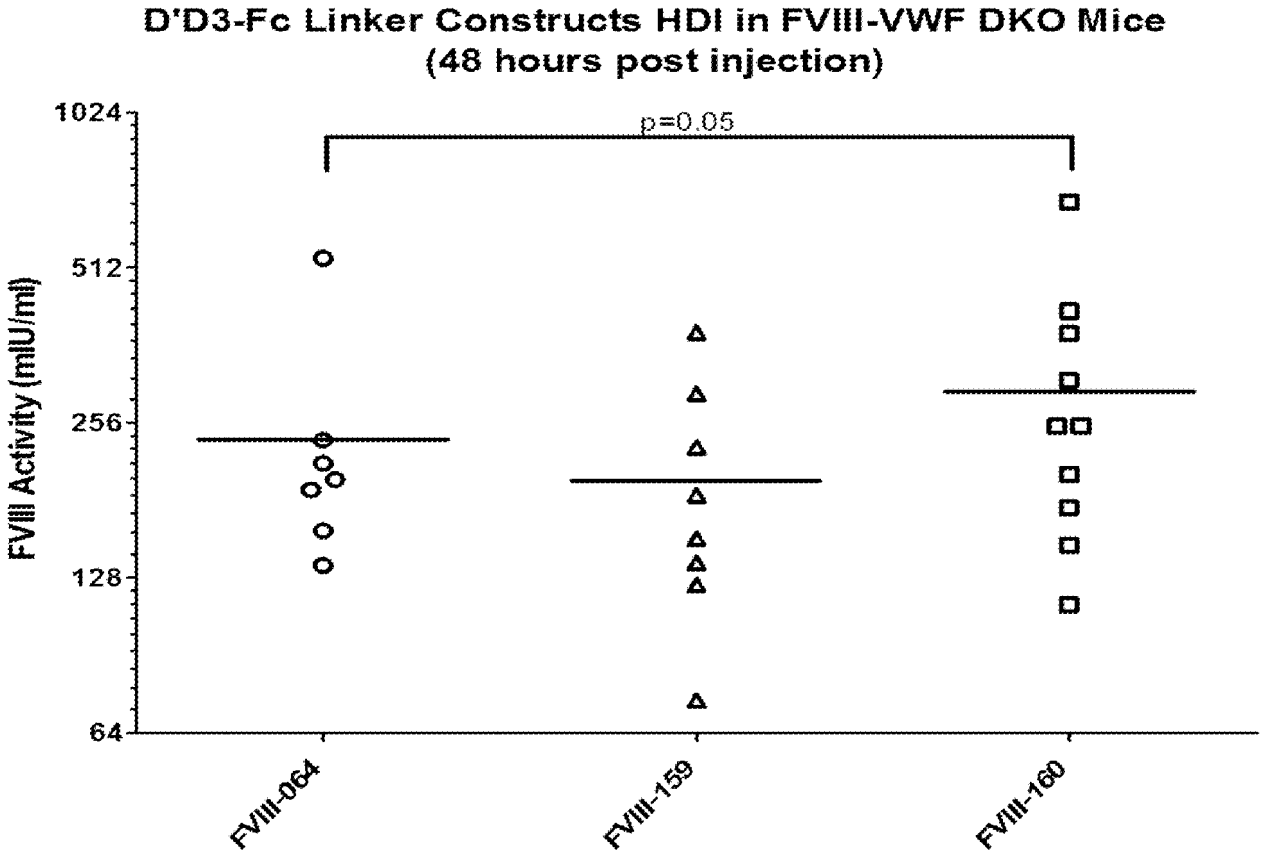


Figure 12: rFVIIIFc PK in VWF D'D3 Expressing Mice



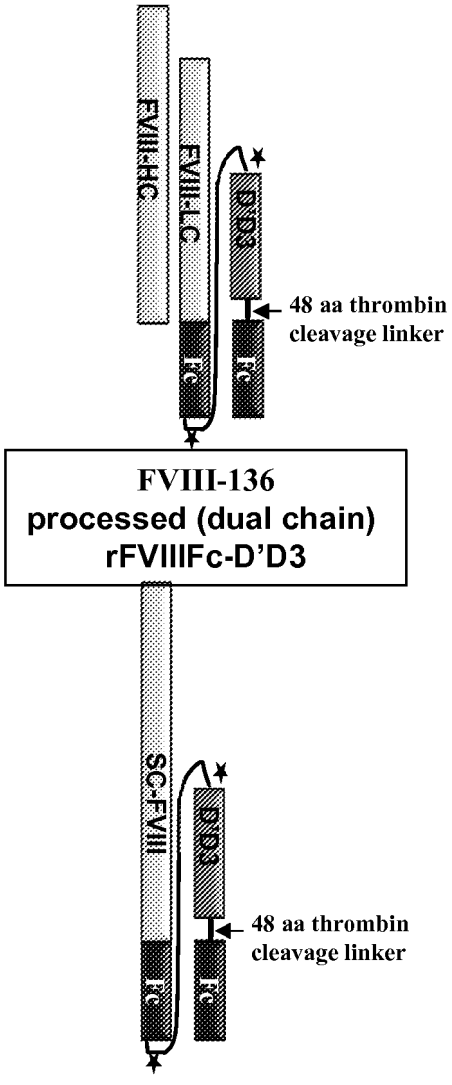
- D'D3 dimer and its monomer synthesized with D1D2 domain provide same FVIII protection
- Circulating VWF D'D3 fragments confer an additional 8 fold FVIII half-life extension
- rFVIIIFc recovery was increased from 40% to 70%

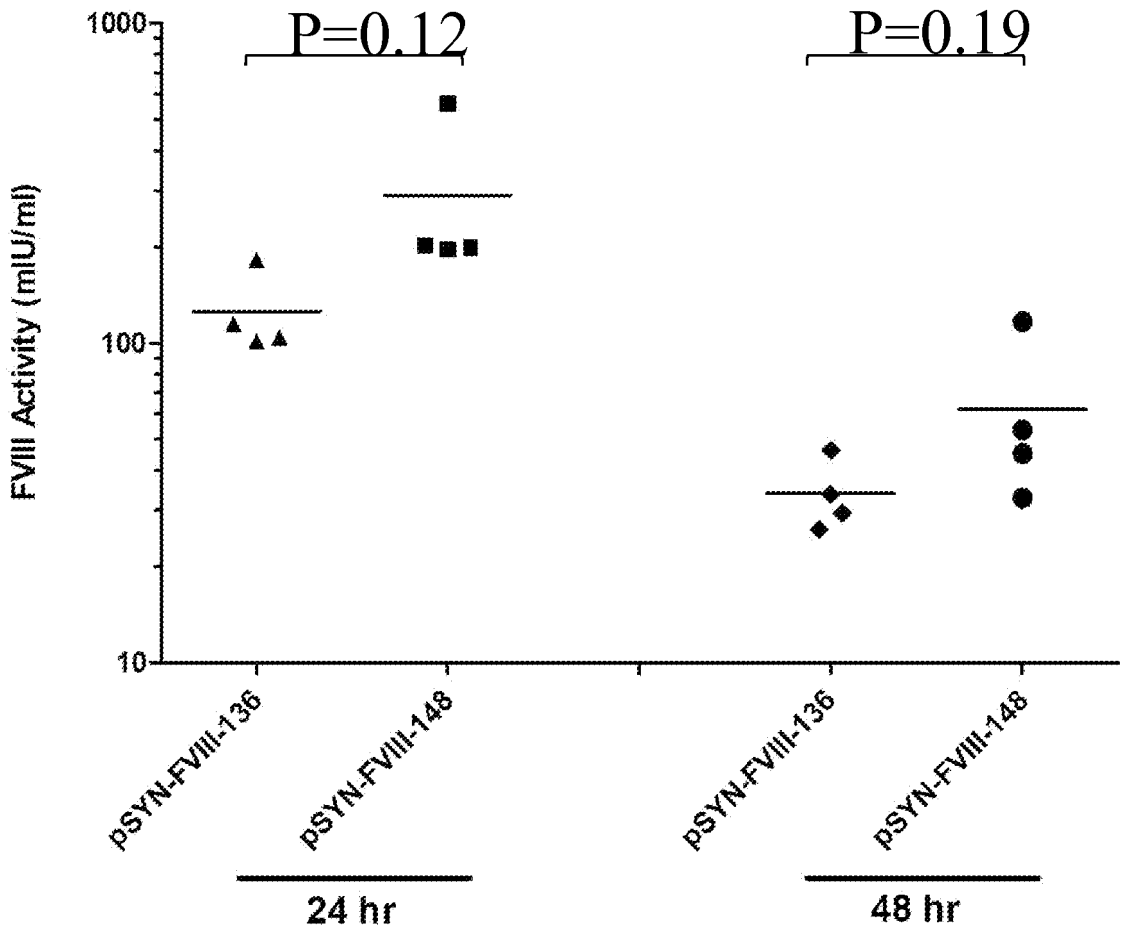
Figure 13: D'D3-Fc linker selection by HDI in FVIII/VWF DKO mice



FVIII-064: rFVIIIFc-VWF with 22aa Linker
FVIII-159: rFVIIIFc-VWF with 35aa Linker
FVIII-160: rFVIIIFc-VWF with 48aa Linker

Figure 14: HDI of Single Chain vs. Dual Chain FVIIIFc/D'D3 Heterodimer in FVIII/VWF DKO Mice





Processable linker

Figure 15: FVIII-155/VWF-031 binding to immobilized VWF by Octet assay

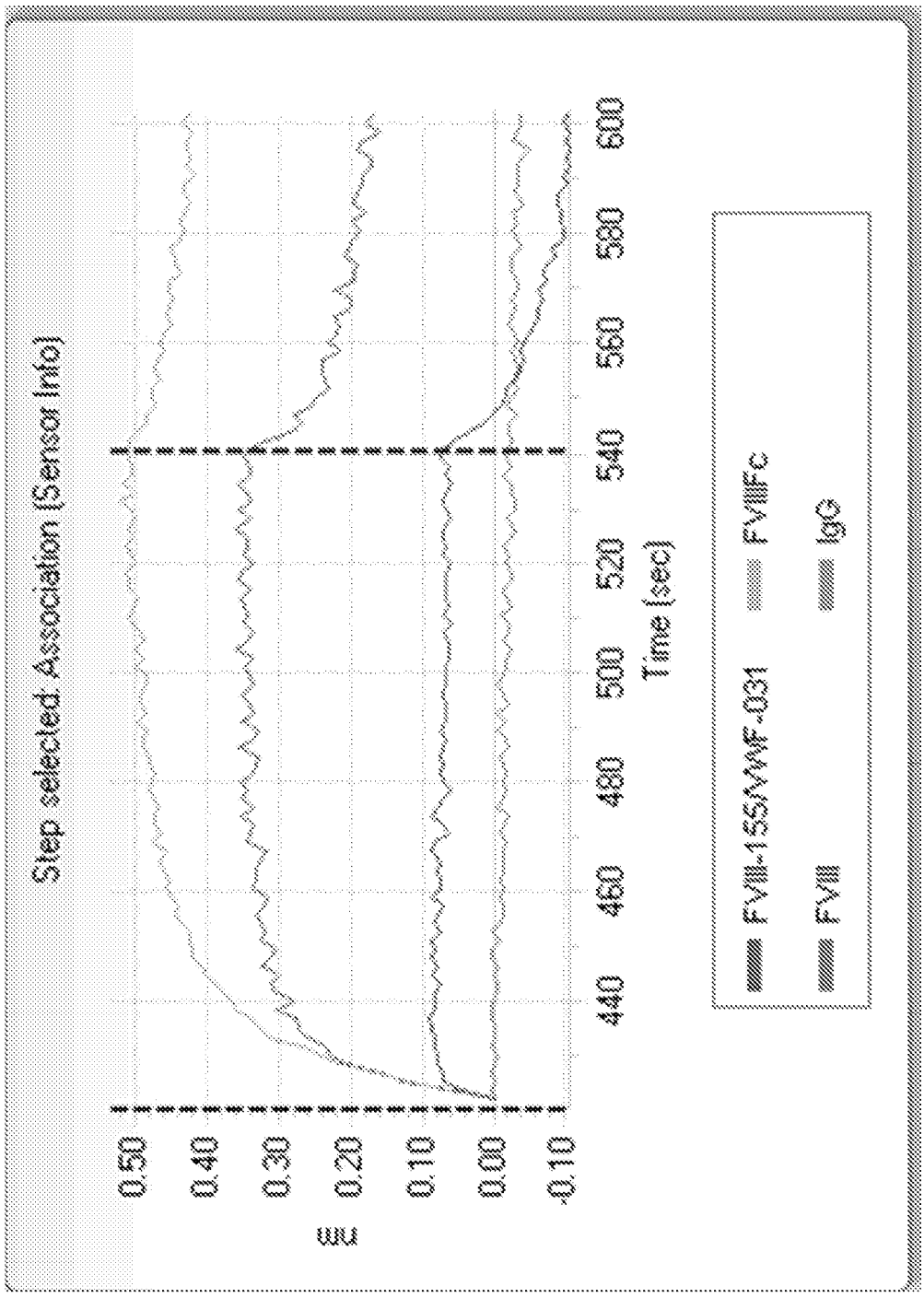


Figure 16: FVIII-155/VWF-031 and FVIIIFc PK in FVIII/VWF DKO mice

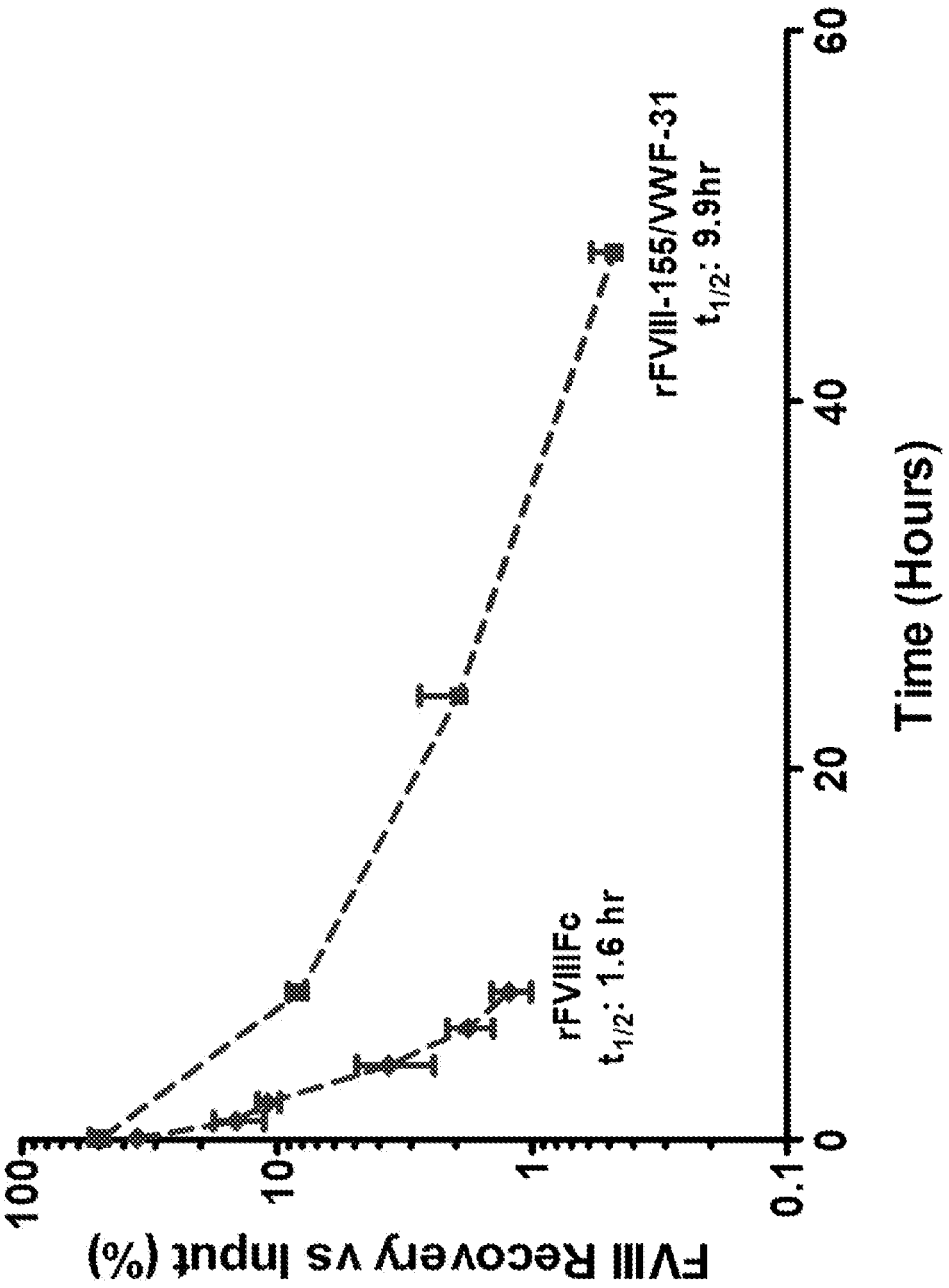
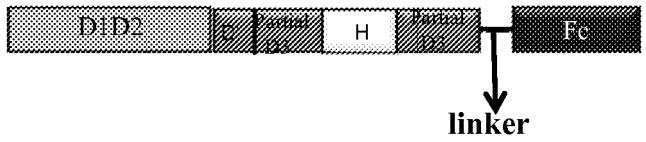
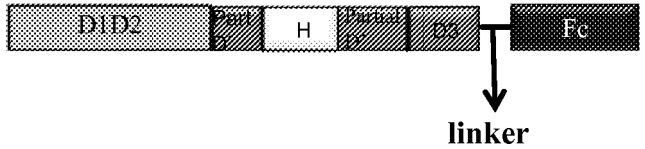


Figure 17: VWF Constructs

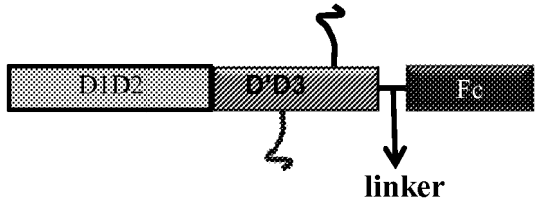
17A.



17B.



17C.



Variable length Linker (optionally cleavable by XIa, thrombin,Xa etc)

H = heterologous moiety

= PEG or HES

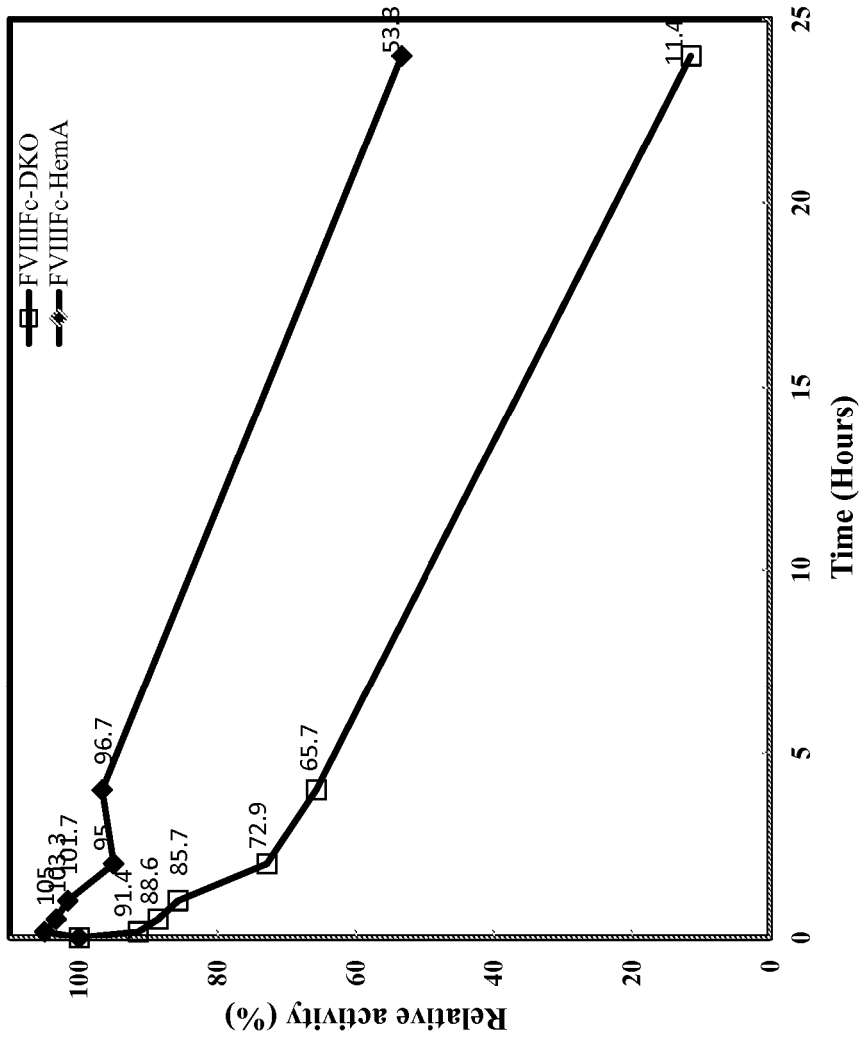
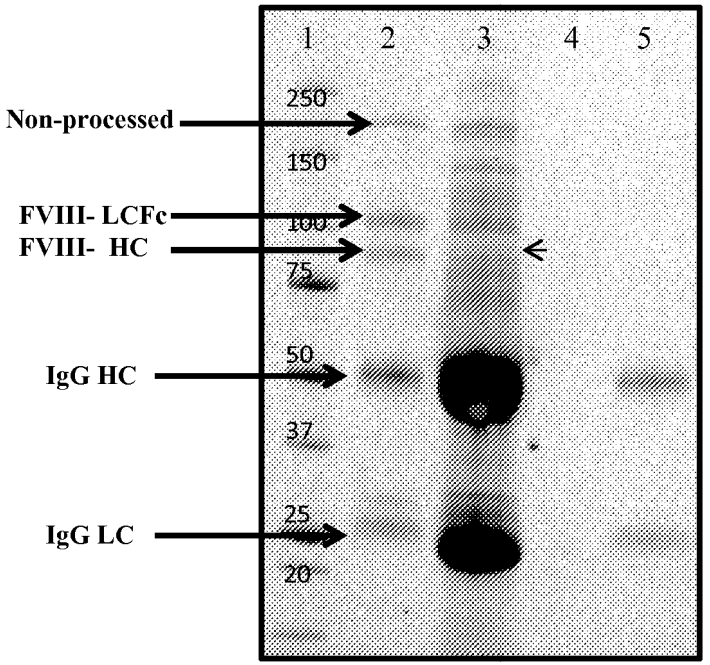


Figure 18A: Loss of FVIIIFc activity in both HemA and DKO plasma by Chromogenic Assay

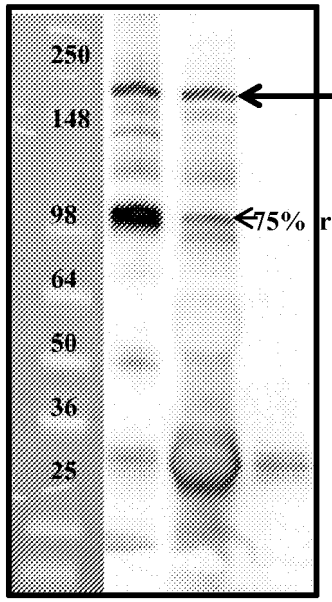
Figure 18B: Loss in FVIII activity is due to dissociation/ degradation of heavy chain (HC)

Reduced ; Bio-rad imager



Lane 1- Bio-rad unstain Marker
Lane 2- FVIIIFc+PBS
Lane 3-FVIIIFc+DKO plasma
Lane 4-Empty
Lane 5-Sheep anti-FVIII polyclonal alone

Western- GMA012 (anti-HC)



Lane 1- Bio-rad unstain Marker
Lane 2- FVIIIFc+PBS
Lane 3-FVIIIFc+DKO plasma
Lane 4-Sheep anti-FVIII polyclonal alone

Rxn- 5ug FVIIIFc was incubated with either 250ul of PBS or mouse DKO plasma for 24hrs at 37 deg. FVIIIFc was immuno-precipitated by adding 5ug sheep anti FVIII polyclonal (ab61370) for 1hr at RT and 100ul protein A beads. After 4x1ml PBS wash, beads were re-suspended in 50ul 1x reducing SDS-PAGE buffer. After boiling, 20ul was loaded on to 4-15% gel (i.e. ~ 1ug FVIIIFc).

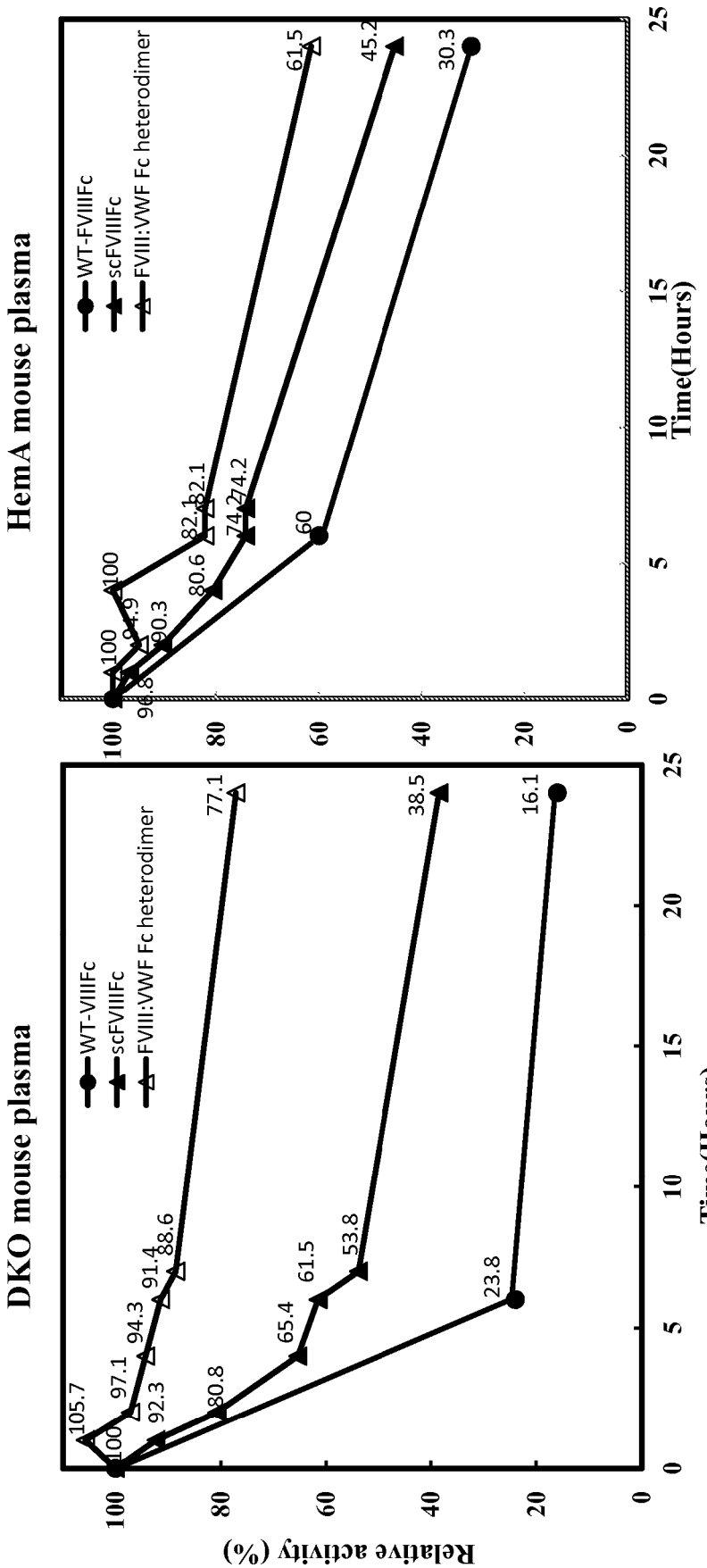
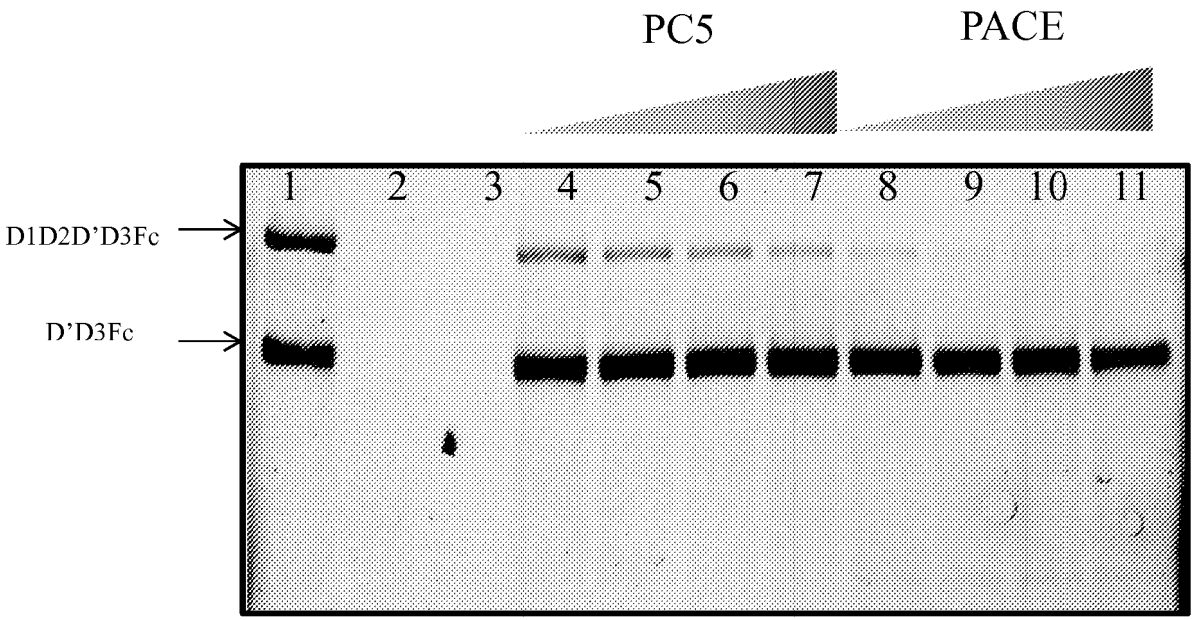


Figure 19: FVIIIFc activity by chromogenic assay

Figure 20: PC5 vs PACE Processing Of VWF-031(D1D2D'D3Fc)



Lane 1- VWF031 alone
Lane 2- PC5 alone
Lane 3-PACE alone
Lane 4- VWF031+PC5-2.5 %
Lane 5- VWF031+PC5-5 %
Lane 6- VWF031+PC5-7.5 %
Lane 7- VWF031+PC5-10 %
Lane 8- VWF031+PACE-2.5 %
Lane 9- VWF031+PACE-5 %
Lane 10- VWF031+PACE-7.5 %
Lane 11- VWF031+PACE-10 %

Figure 21A: No Binding Of FVIII-155/VWF-031 To VWF

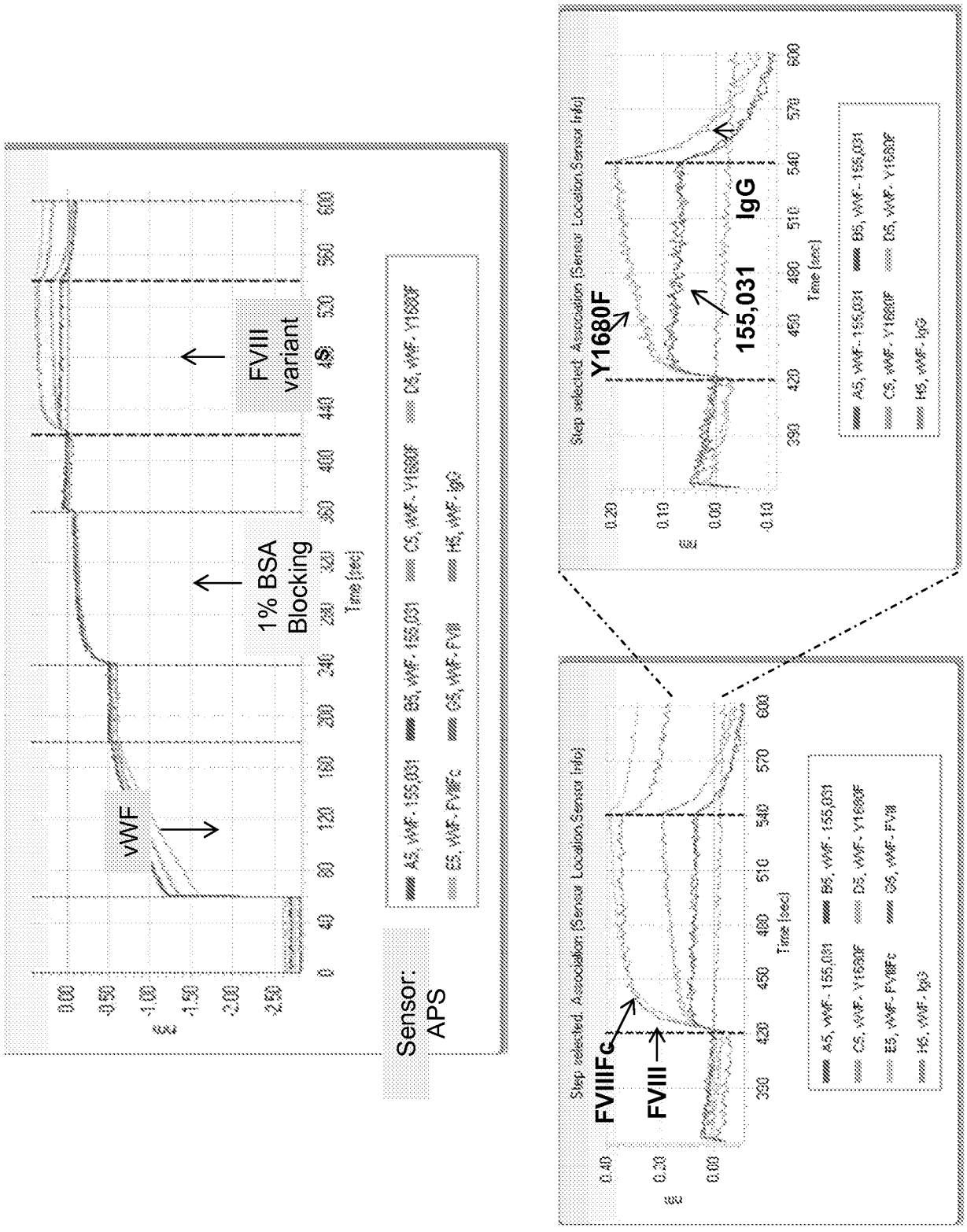
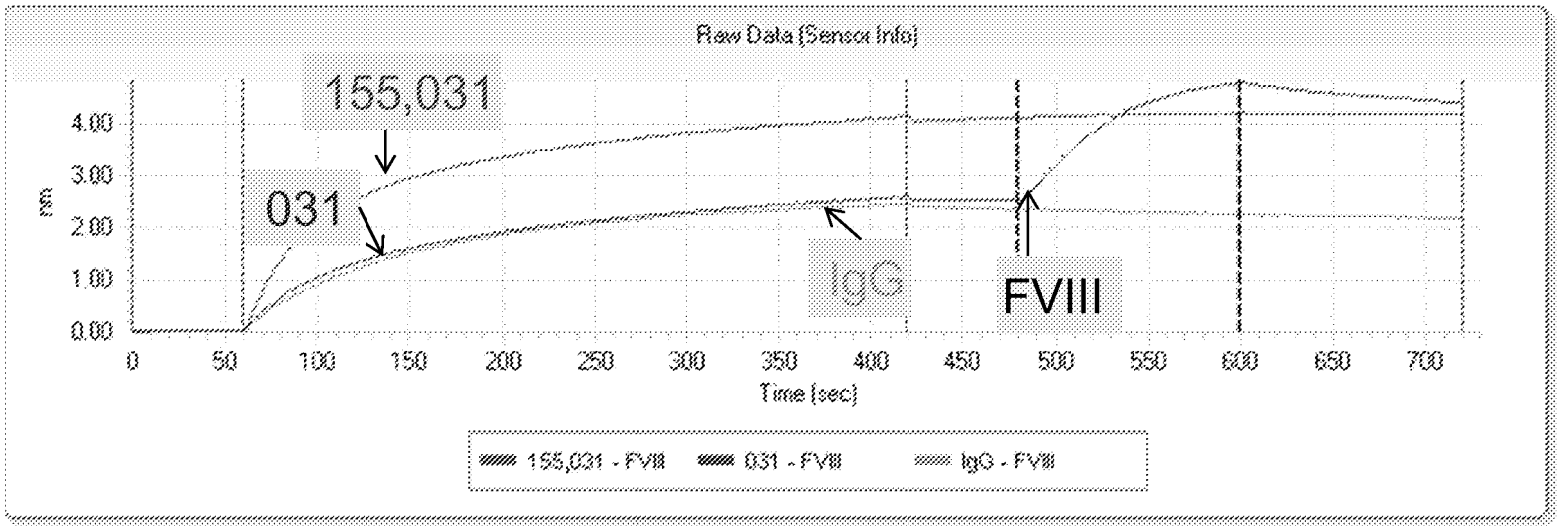


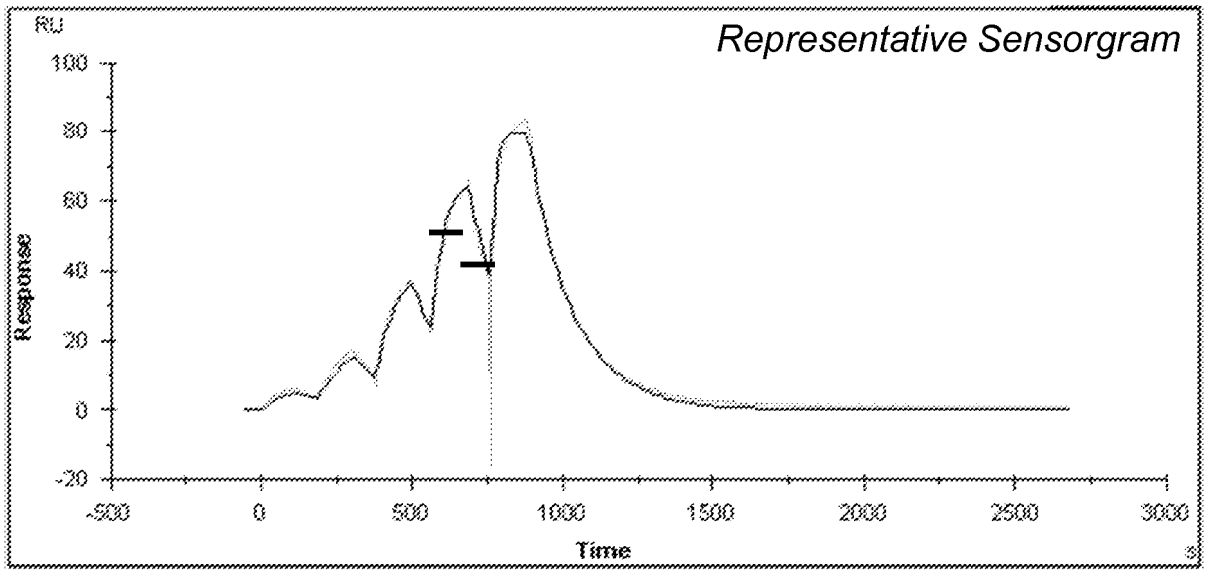
Figure 21B: FVIII-155/VWF-031 Does Not Bind To FVIII



Sensor: Protein G

Figure 22: Interaction Of FVIII With VWF-031

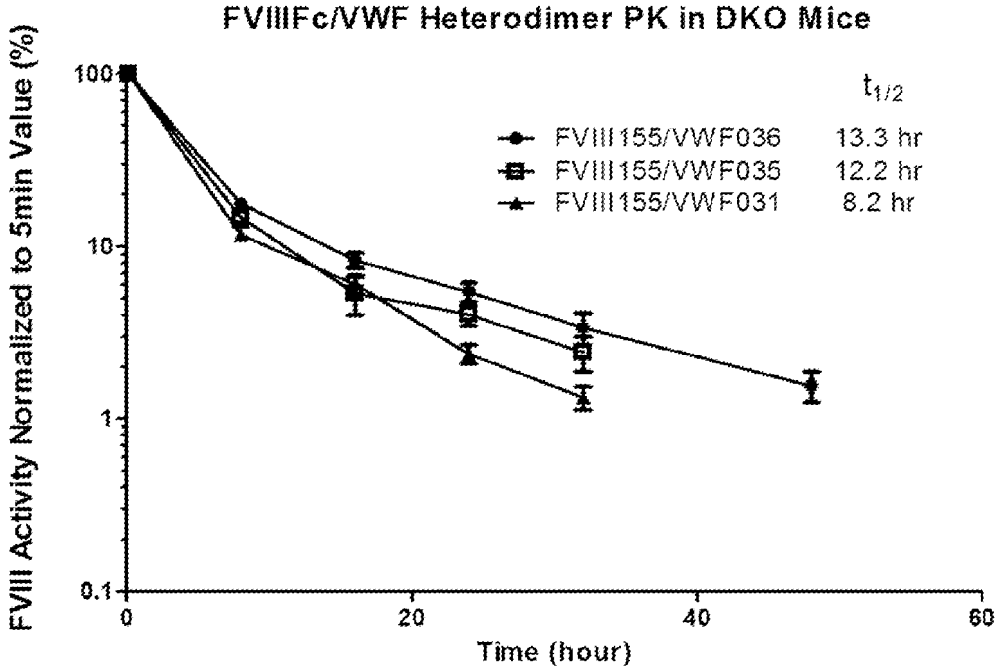
- 1000 RU of goat anti-human IgG immobilized
- 100 RU of VWF-031
- FVIII (B-domain deleted) applied in single-cycle kinetics mode
- 1:1 fit ; n=4



K_D = 10 ± 1 nM

(25-fold weaker than FL-VWF as ligand)

Figure 23: Effect of different linker length of FVIIIFc/VWF heterodimer on PK in FVIII/VWF DKO mice



FVIII-155	Single chain (SC) - FVIII R1645A/R1648A directly fused to a single Fc
VWF-031	D1D2D'D3 (1-477aa with C336A/C379A) fragment linked to a single Fc with a 48aa thrombin cleavable linker
VWF-035	D1D2D'D3 (1-477aa with C336A/C379A) fragment linked to a single Fc with a 73aa thrombin cleavable linker
VWF-036	D1D2D'D3 (1-477aa with C336A/C379A) fragment linked to a single Fc with a 98aa thrombin cleavable linker

Figure 24: Example of sortase ligation

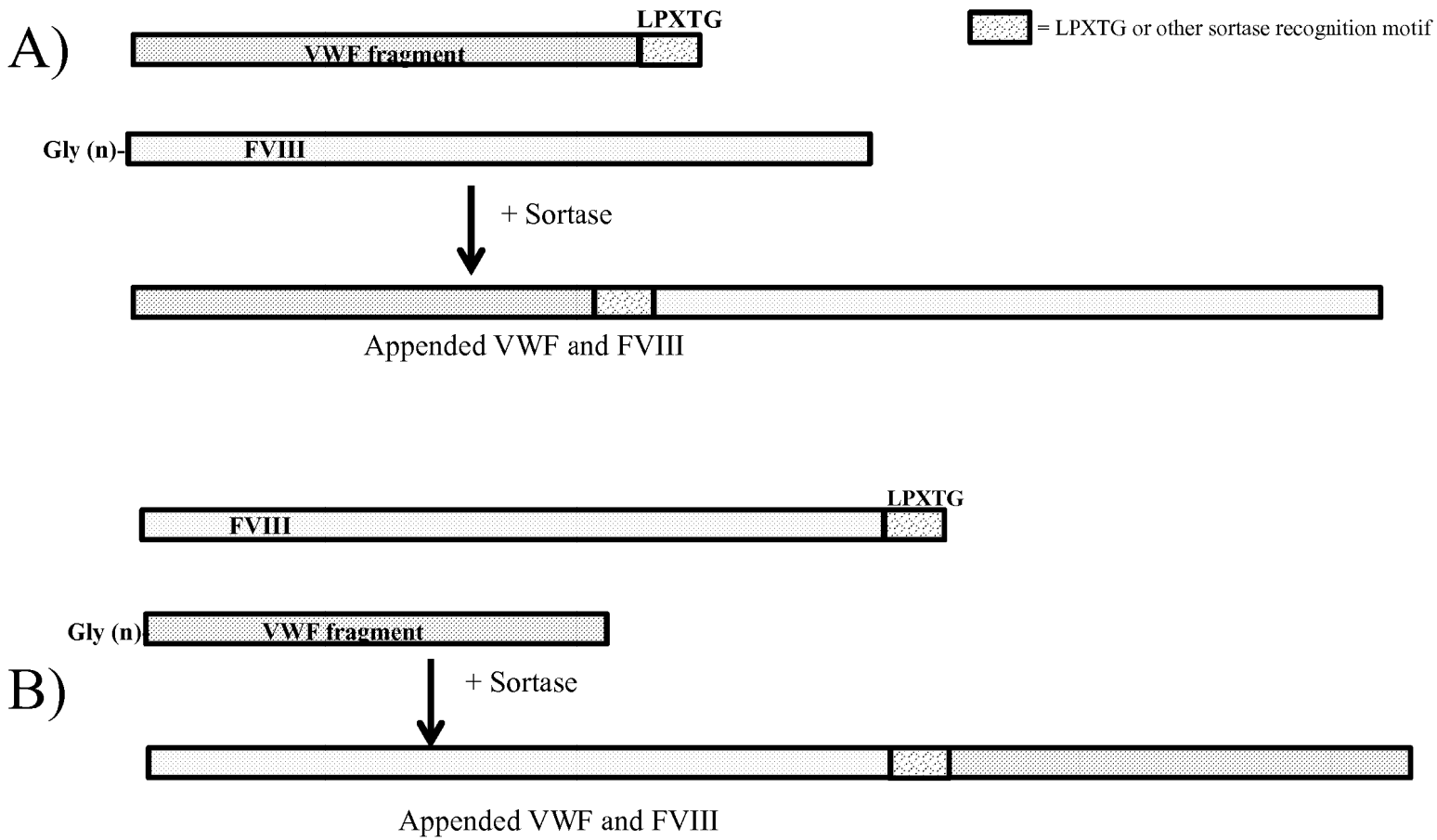


Figure 24: Example of sortase ligation

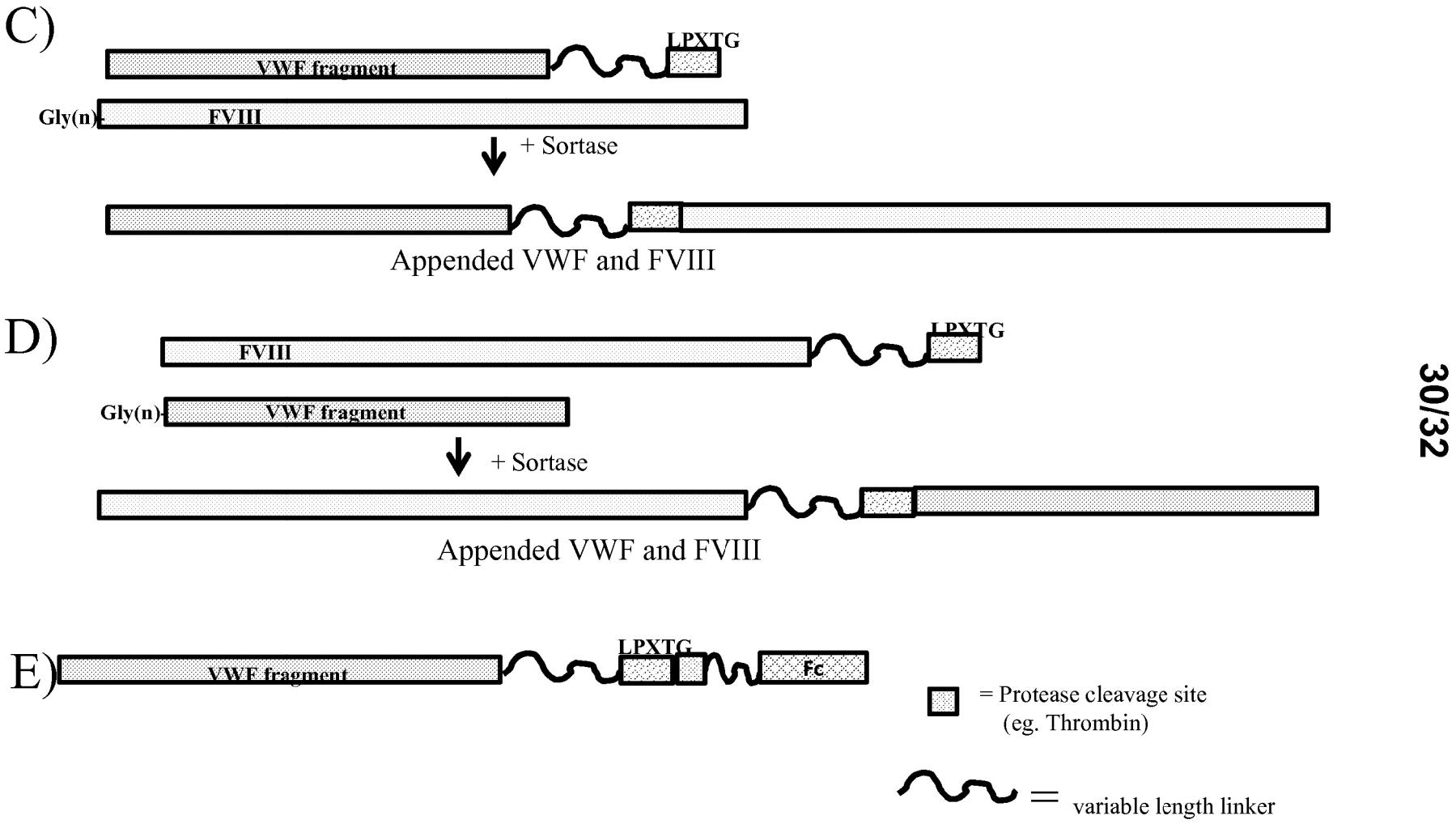
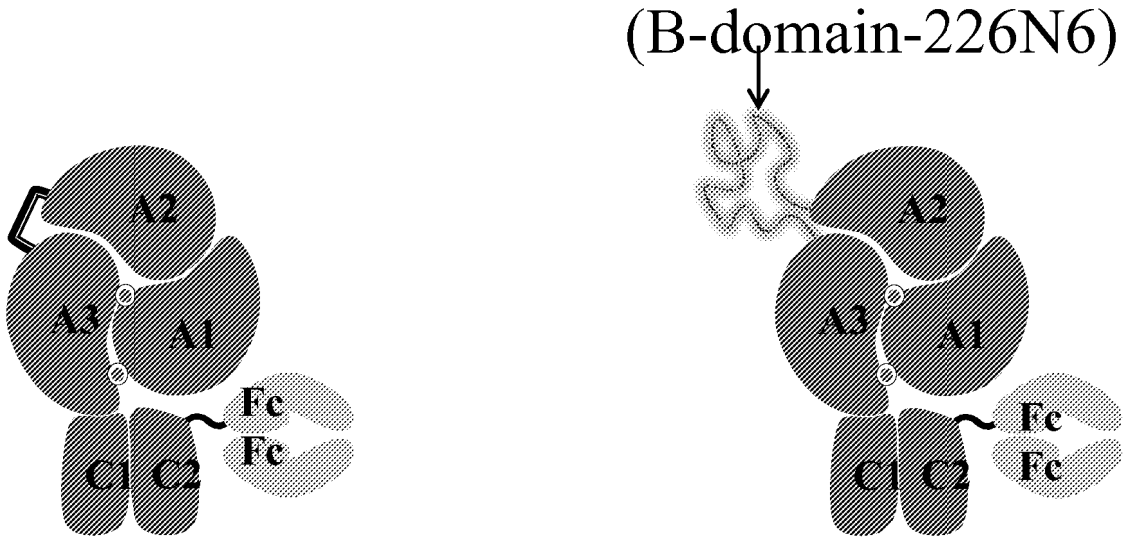


Figure 25:Schematic comparison of single chain FVIIIFc (FVIII155) and partial B-domain containing single chain FVIIIFc (FVIII198)



FVIII 155:Fc
(single chain FVIIIFc)

FVIII 198:Fc
(single chain FVIIIFc with 226N6)

Figure 26: The partial B-Domain increases the half-life of FVIII198 1.5 fold in comparison to FVIII155

