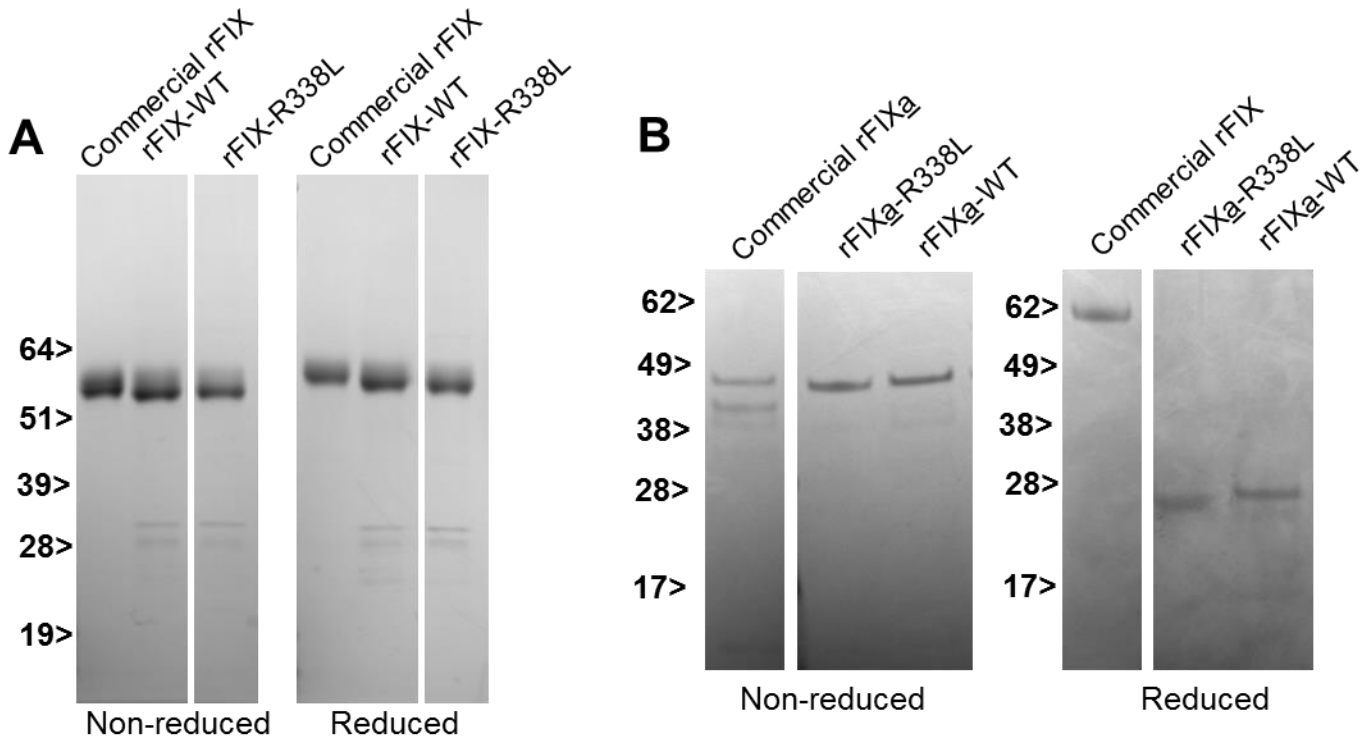
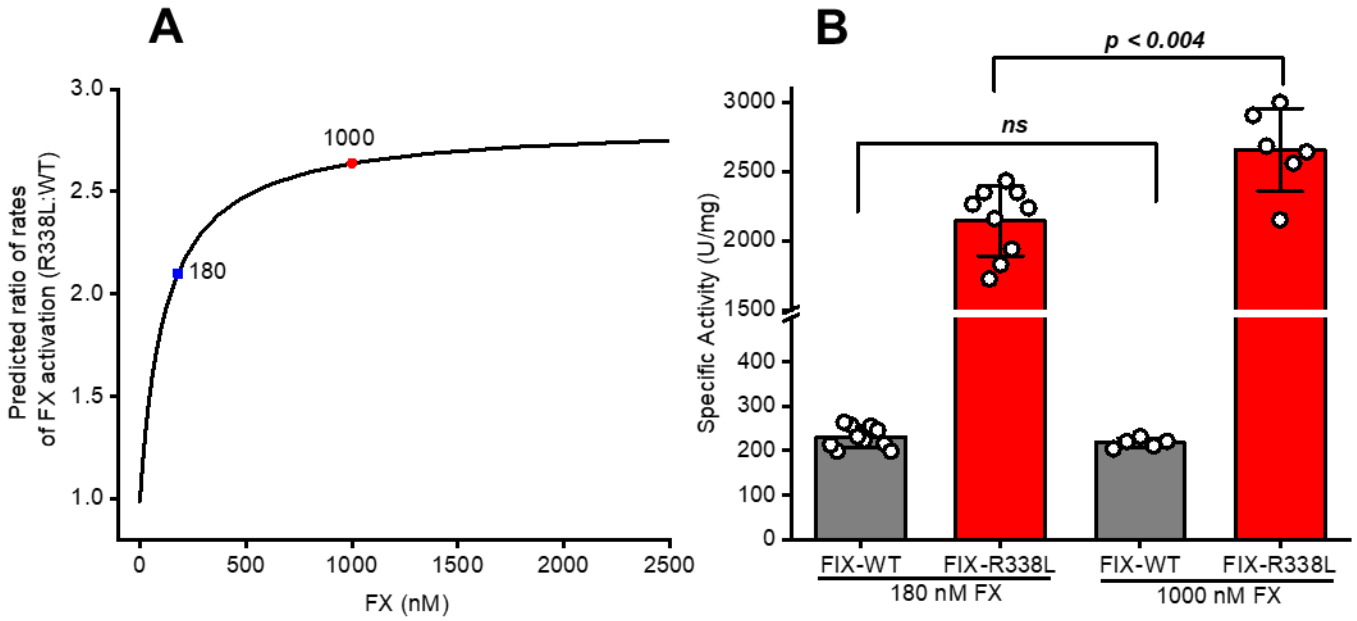
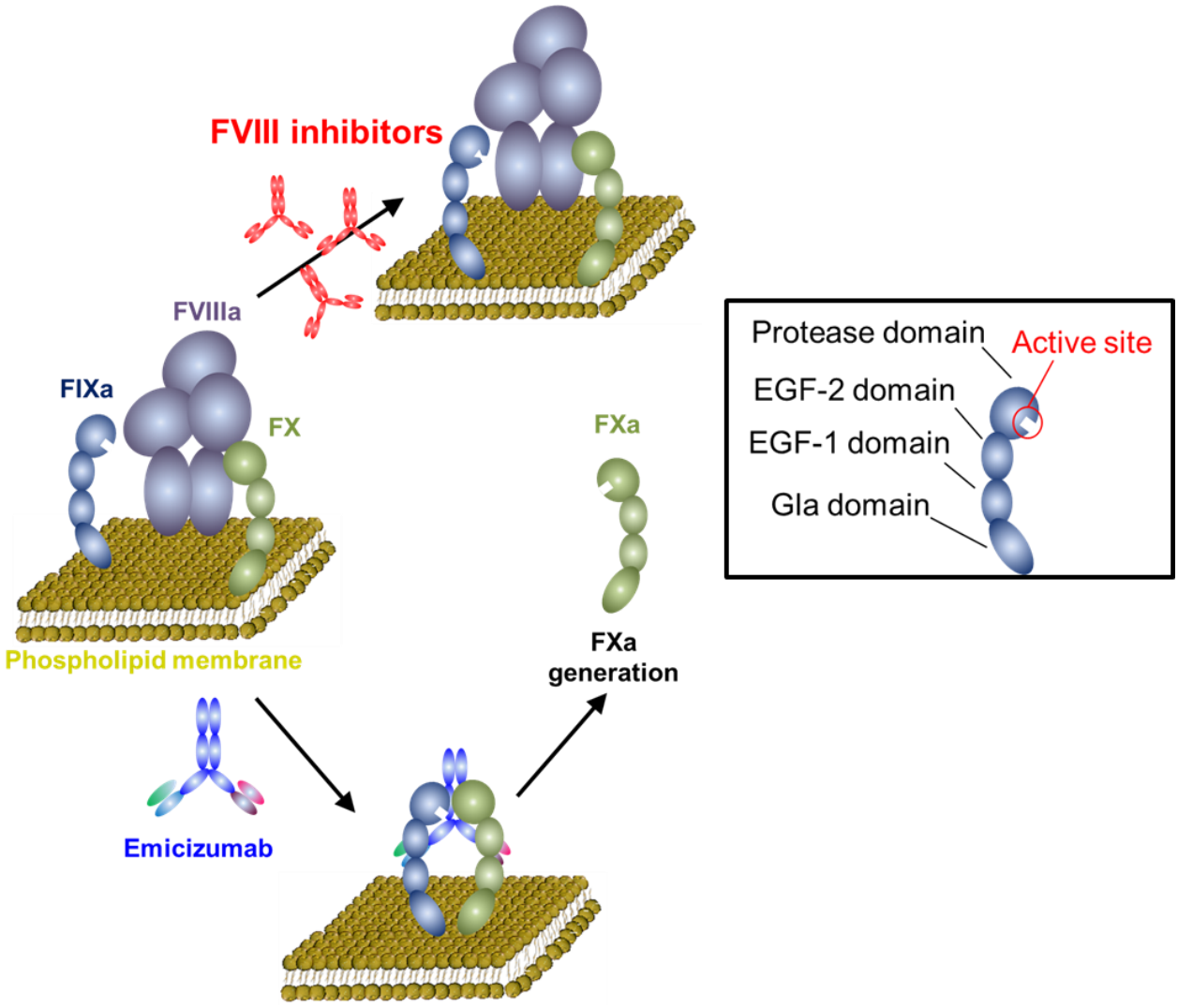


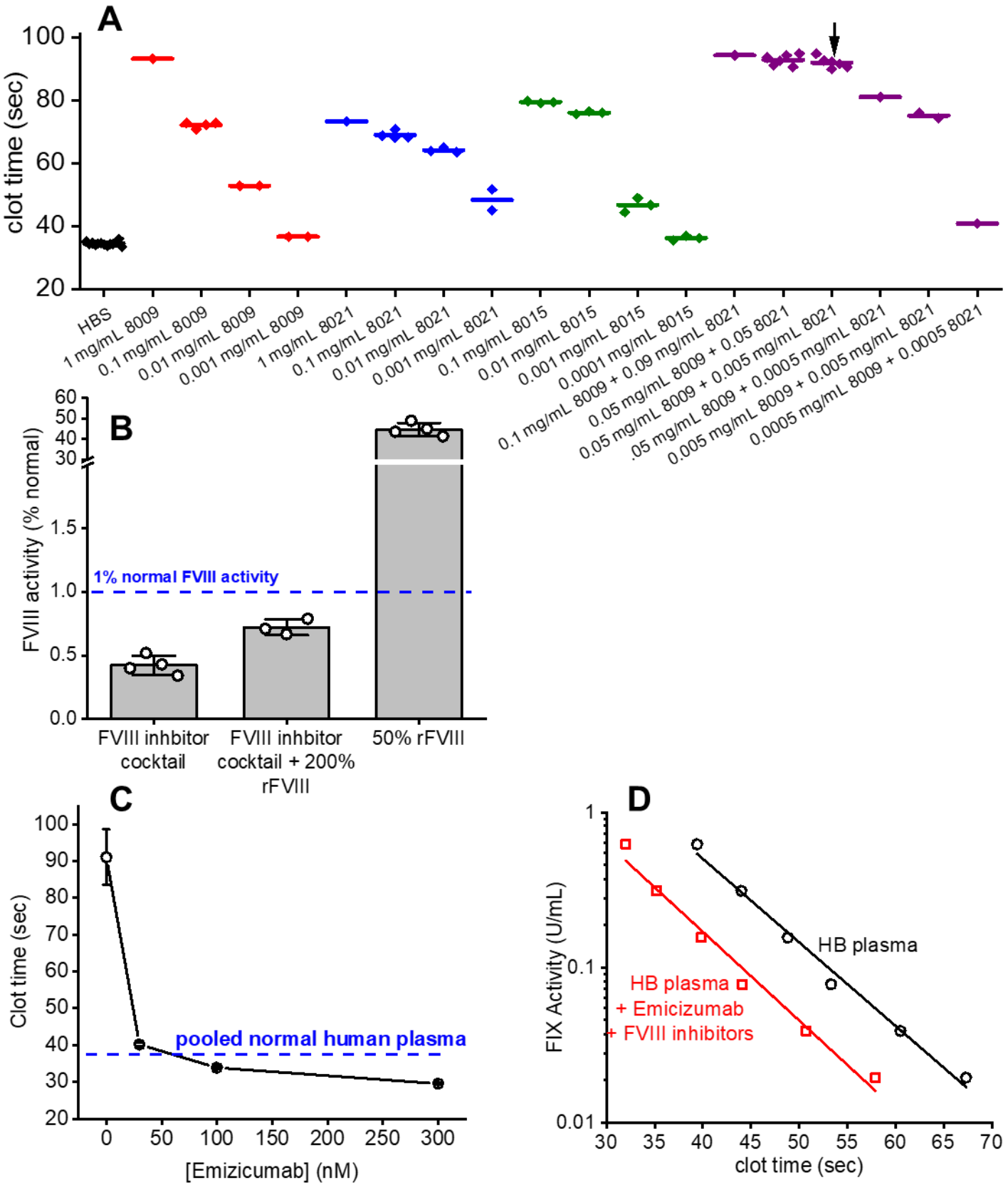
Supplementary Figure 1: Lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) analysis of rFIX and rFIXa variant proteins. Purified rFIX (**A**) or rFIXa (**B**) protein of -WT and -R338L as well as commercial rFIX/FIXa-WT in the range between 0.5 – 1.0 µg were subjected to LDS-PAGE with or without reducing conditions. Lanes were run on the same gel but in noncontiguous lanes. The bands were visualized with Coomasie blue staining. There is no evidence of FIXa contamination in the FIX samples and no evidence of FIX contamination in the FIXa samples.



Supplementary Figure 2: Dependence of specific activity of rFIX-WT and rFIX-R338L on FX concentration. (**A**) The enzyme kinetics of FIXa-WT and FIXa-R338L with FVIIIa (Figure 3) indicate that the ratio (FIXa-R338L:FIXa-WT) of rates of FX activation increase with increasing FX concentration; plot based on $ratio = \frac{k_{R338L}}{k_{WT}} (\frac{\frac{K_{M,WT}}{[FX]}+1}{\frac{K_{M,R338L}}{[FX]}+1})$. The FX concentration in normal human plasma (180 nM) is highlighted. (**B**) To assess the relevance of the determined enzyme parameters in the clotting of plasma, we measured the FIX activity for FIX-WT and FIX-R338L in a clotting assay with HB plasma (180 nM FX) and HB plasma augmented with FX concentrate to final concentration of 1000 nM. Each circle is a distinct clotting time measurement, bars represent mean of data, and error bars are ±SD. Means (n=5-10) were compared with a 2-tailed t-test with p-values ≥0.05 considered not significant (*ns*). There was no significant difference between the activities of FIX-WT, as is expected since the standard curve relating clot time with FIX activity was generated with dilutions of commercial rFIX-WT. However, there is a significant increase in the activity of FIX-R338L in the presence of 1000 nM FX compared to 180 nM FX in HB plasma, consistent with the measured enzyme kinetic parameters impacting the clotting assay.

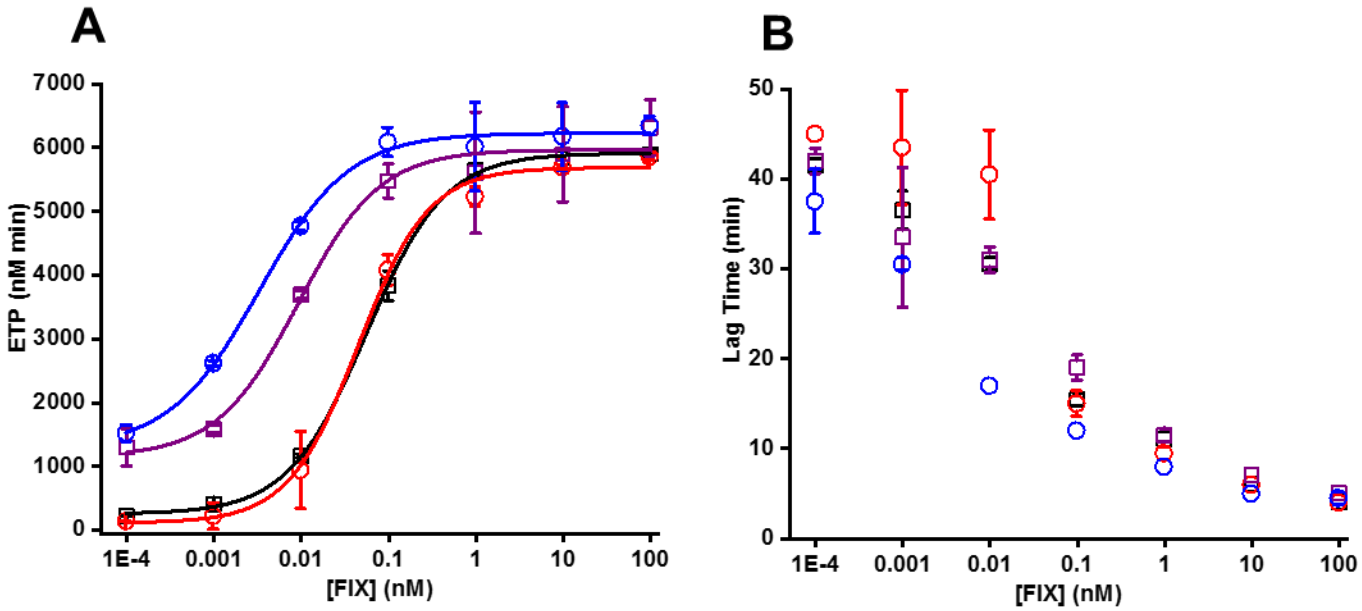


Supplementary Figure 3: Experimental scheme for the replacement of FVIIIa cofactor activity by emicizumab in plasma-based assays. The intrinsic Xase enzymatic complex is composed of FIXa-FVIIIa on a phospholipid membrane. FVIIIa binding to FIXa both increases FX affinity and as has an allosteric effect on the active site of the FIXa protease, which increases the rate of FX activation. In contrast, emicizumab principally increases the affinity of FX, with a much more modest effect on the rate of FX activation. In order to replace the endogenous FVIII/FVIIIa activity in HB plasma for plasma-based epxeriments, we added a cocktail of anti-FVIII/FVIIIa monoclonal antibodies (i.e. FVIII inhibitors, Supplementary Figure 4) that inhibit FVIII activity in normal human plasma to <1% normal effectively blocking the activity of the FIXa-FVIIIa-FX complex (top). In the presence of these anti-FVIII/FVIIIa antibodies, only the FIXa-emicizumab-FX complex is active; as such, all FXa generated occurs through the bottom pathway. (***Inset***) Domain representation of FIXa. The active site of FIXa is located in the protease domain, which sits atop a stalk composed of the Gla-, EGF1-, and EGF2-domains. The emicizumab binding site has been located on EGF1-domain of FIX.

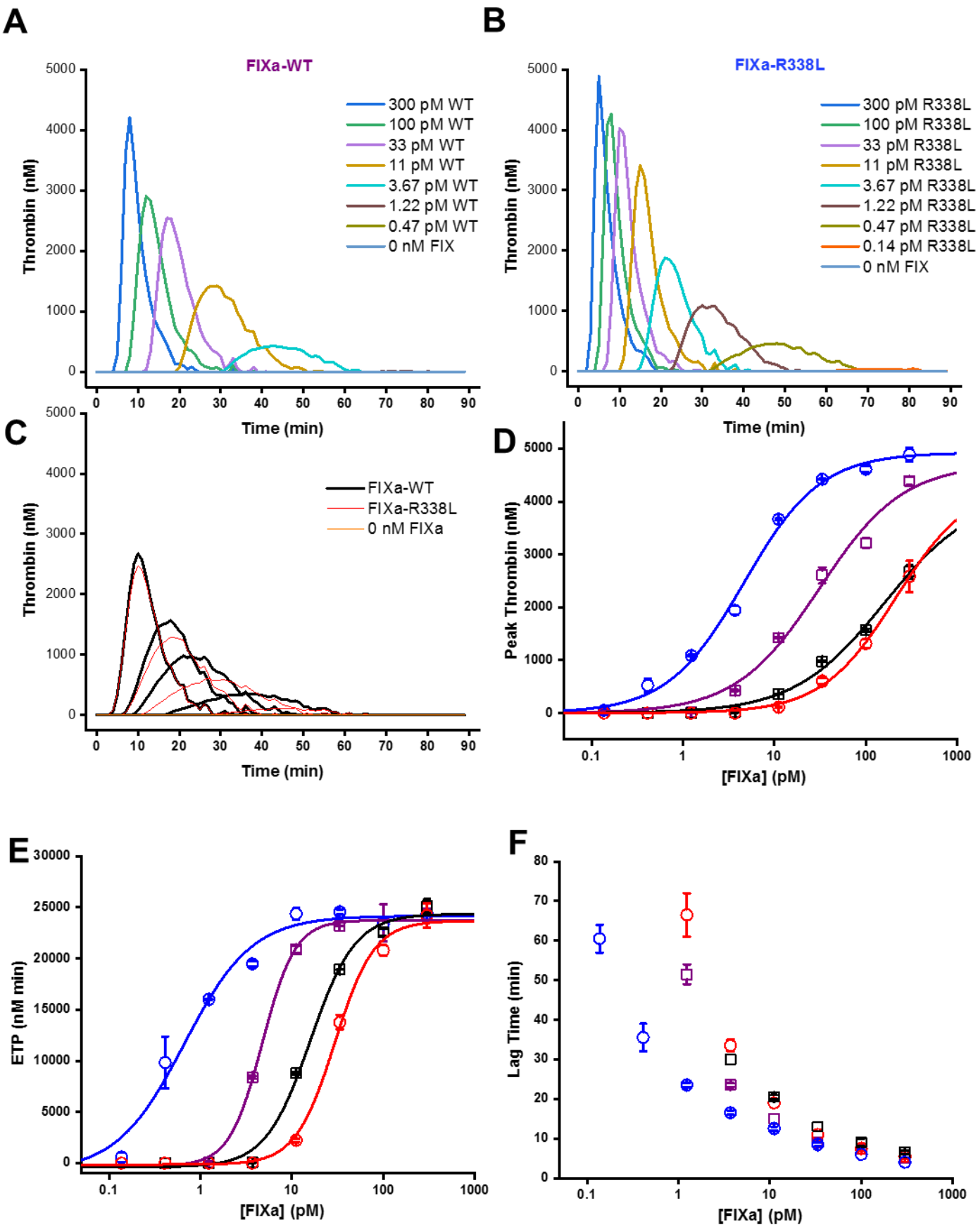


Supplementary Figure 4: Development of plasma-based assay with emizicumab substituted for FVIII/FVIIIa cofactor activity. (**A**) We assessed the inhibitory effect of a series commercial anti-FVIII/FVIIIa monoclonal antibodies (from GMA) with a modified aPTT-based clotting. Here, we mixed pooled normal human plasma (NHP) from George King with a variety of concentrations and

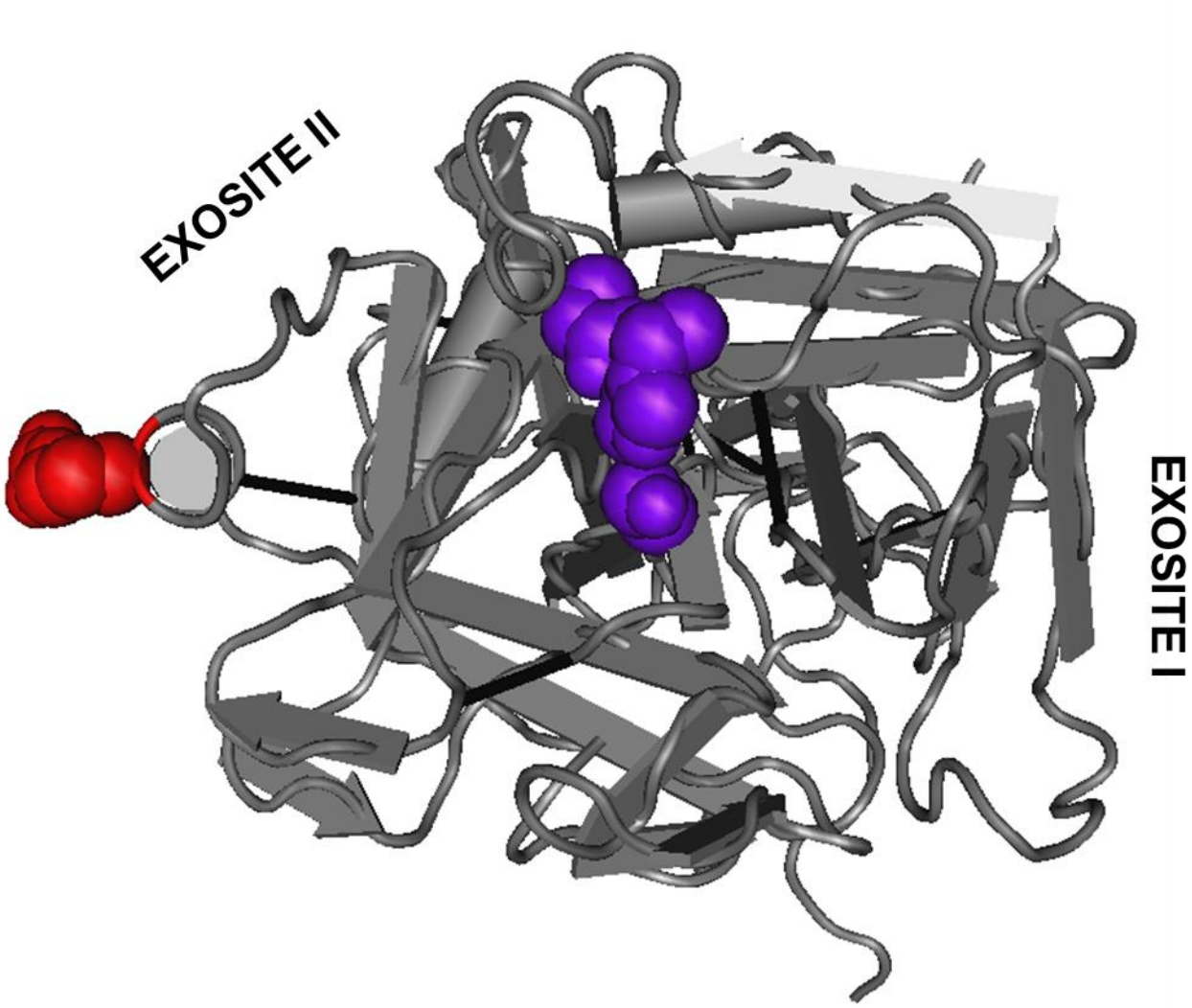
combinations of anti-FVIII antibodies and measured the clot time. In the absence of antibodies, NHP mixed only with HBS had a clot of 34 secs. We observe that the combination of 50 µg/mL GMA-8009 and 5 µg/mL GMA-8021 (arrow) maximally prolonged the clot time and was similar to 20-fold higher concentrations of GMA-8009. GMA-8009 (1D4) and GMA-8021 (2-76) are reported to have 7 BU/µg and 38 BU/µg, respectively, and target different epitopes of the FVIII A2-domain (Markovitz et al., Blood 2013 121.14: 2785). Each point is a single measurement and the mean is represented as a solid line. We did not observe an increase in the inhibitory effect if the sample was incubated at 37°C for 2 hrs. We also tested combinations with GMA-8013, -8017, -8022, and -8029 (*data not shown*). (**B**) The residual FVIII activity in NHP combined with 50 µg/mL GMA-8009 and 5 µg/mL GMA-8021 (FVIII inhibitor cocktail) with or without an additional 200% rFVIII was determined with an aPTT-based clotting assay using HA plasma and a standard curve relating dilutions of NHP to clot time. We observe that residual FVIII activity with this FVIII inhibitor cocktail, with or without an additional 200% rFVIII, was less than 1% normal activity, comparable to severe HA. rFVIII at 50% normal activity was the positive control. Each circle is a distinct clotting time measurement, bars represent mean of data, and error bars are ±SD. (**C**) Concentration dependence of the ability of emizicumab to restore the clot time in NHP inhibited with 50 µg/mL GMA-8009 and 5 µg/mL GMA-8021. Data points are the mean (n=2-4) and error bars represent ±SD. The therapeutic concentration of emicizumab is about 380 nM. (**D**) Example of standard curves with commercial rFIX relating FIX activity (U/mL) with aPTT – based clot time in unmodified HB plasma (black) and HB plasma with 100 nM emicizumab and 50 µg/mL GMA-8009 and 5 µg/mL GMA-8021 (red). Though the curve for HB plasma with emicizumab replacing endogenous FVIII/FVIIIa is shifted to the left compared to unmodified HB plasma, there is still a well-defined relationship between FIX activity and aPTT clot time. The left-shifted curve for emicizumab is expected as emicizumab does not require activation as FVIII does.



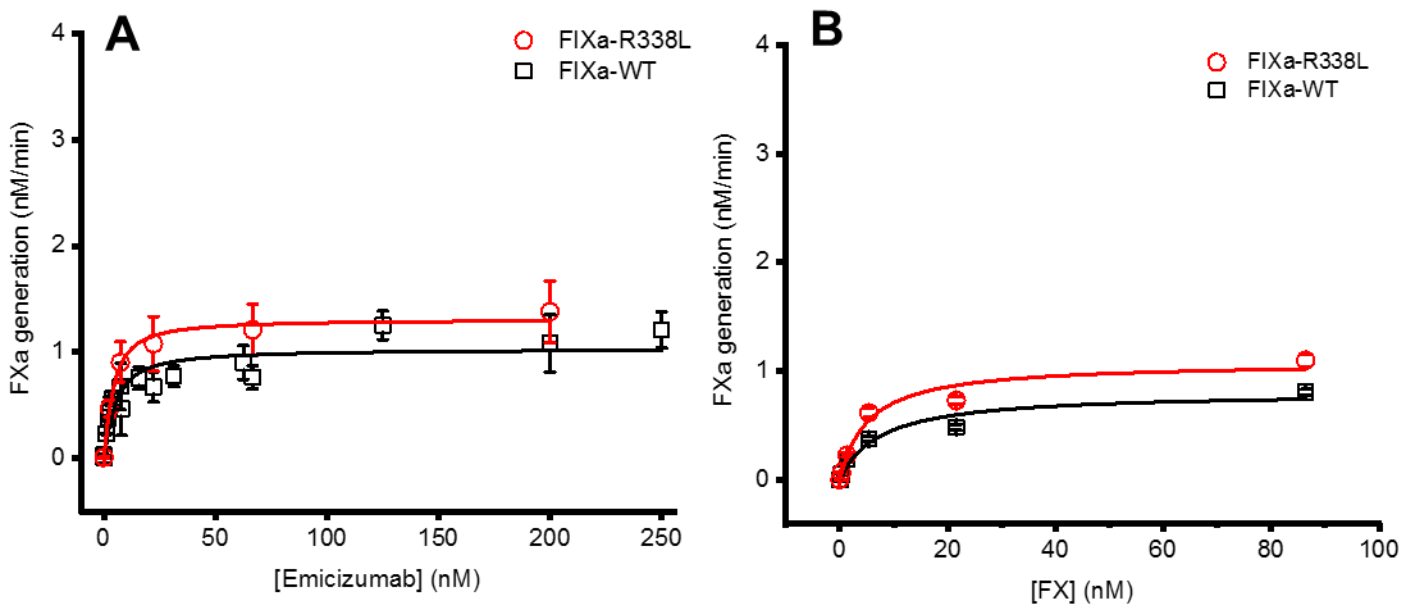
Supplementary Figure 5: ETP and lag time of rFIX-WT and rFIX-R338L in HB plasma and HB plasma with emicizumab. The (**A**) ETP, and (**B**) lag time from the thrombogram in Figure 5 as a function of added FIX-WT (□), FIX-R338L (○), FIX-WT with emicizumab (□), and FIX-R338L with emicizumab (○). Error bars represent ±SD of duplicate measurements. Data points were empirically fitted to a logistic plot (solid lines) to estimate the EC50s ($R^2 \geq 0.98$ for all plots) that are included in Table 2. Data is representative of 2-4 experiments.


A
FIXa-WT
300 pM WT
100 pM WT
33 pM WT
11 pM WT
3.67 pM WT
1.22 pM WT
0.47 pM WT
0 nM FIX
Thrombin (nM)
Time (min)
B
FIXa-R338L
300 pM R338L
100 pM R338L
33 pM R338L
11 pM R338L
3.67 pM R338L
1.22 pM R338L
0.47 pM R338L
0.14 pM R338L
0 nM FIX
Thrombin (nM)
Time (min)
C
FIXa-WT
FIXa-R338L
0 nM FIXa
Thrombin (nM)
Time (min)
D
Peak Thrombin (nM)
[FIXa] (pM)
E
ETP (nM min)
[FIXa] (pM)
F
Lag Time (min)
[FIXa] (pM)

Supplementary Figure 6: Thrombin generation of activated rFIXa-WT and rFIXa-R338L in HB plasma and HB plasma with emicizumab. FIXa-WT (**A**) and FIXa-R338L (**B**) dependent thrombin generation in unmodified HB plasma. (**C**) FIX-WT (black) and FIX-R338L (red) dependent thrombin generation in HB plasma conditioned with 100 nM emicizumab and anti-FVIII antibodies. The same FIX protein dilutions were used for experiments in HB plasma (A and B) and HB plasma with emicizumab (C). Thrombograms are the mean of n=2 measurements. (**D**) Peak thrombin, (**E**) ETP, and (**F**) lag time as a function of added FIXa-WT (□), FIXa-R338L (○), FIXa-WT with emicizumab (□), and FIXa-R338L with emicizumab (○). Points represent the mean (n=2) and error bars are ± SD. Data points were empirically fitted to a logistic plot (solid lines) to estimate the EC50s ($R^2 \geq 0.98$ for all plots) that are included in Table 2. Data is representative of 2-4 experiments.



Supplementary Figure 7: Structure of FIXa-WT. Ribbon rendering of published partial structure of FIXa-WT (PDB code 1RFN) showing the protease domain in the standard orientation. The enzymatic activate-site is highlighted with the amino acids of catalytic triad (H221, D269, S365) shown in purple. R338 is shown in red. Though the FVIIIa-binding site is only partially defined, it likely overlaps with exosite II and likely includes R338.



Supplementary Figure 8: Enzyme kinetics FX activation of rFIXa-WT and rFIX-R338L with emicizumab. (**A**) Emicizumab binding to 3 nM FIXa assayed by FIXa-emicizumab complex FXa generation, initial [FX] is 90 nM. Points represent the mean (n=2-3) and error bars ±SD. Solid lines are fittings of a quadratic binding equation ($R^2 \geq 0.84$). Data is representative of 2 experiments. (**B**) Enzyme kinetics of the activation of FX by 3 nM FIXa with 200 nM emicizumab. Points represent the mean (n=2) and error bars are ±SD. Solid lines are Michaelis-Menten fittings ($R^2 \geq 0.94$). Parameters are included in Table 1.