Supplement

Table S-1. Specific Activity of FVIII variants

FVIII variants	Activity (%)
WT	100.0
R121C/L2302C	86.4
A108I	73.7
A108I/D519A	77.7
A108I/D519V	69.5
A108I/E665A	98.8
A108I/E665V	83.8
A108I/E1984A	43.4
A108I/E1984V	94.1
R121C/L2302C/D519A	60.7
R121C/L2302C/D519V	79.5
R121C/L2302C/E665A	0.0
R121C/L2302C/E665V	22.2
R121C/L2302C/E1984A	63.4
R121C/L2302C/E1984V	90.3
D519V/E665V	90.0
A108I/D519V/E665V	76.5

Activity was measured by FXa generation assay [8] and expressed as relative values compared to WT activity. The single letter code is used to designate amino acid residues, R (Arg), C (Cys), E (Glu), D (Asp), A (Ala) and V (Val).

Table S-2. Thrombin generation assay parameter values

	Latent Time (min)	Peak Time (min)	Peak Value (nM)	ETP (nM·min)
WT	10.6 ± 0.4 (1.00)	17.7 ± 0.2 (1.00)	56.8 ± 5.7 (1.00)	1009 ± 61 (1.00)
A108I	9.9 ± 0.7 (0.93)	17.0 ± 0.5 (0.96)	66.3 ± 5.5 (1.16)	1164 ± 109 (1.15)
A108I/D519V	11.7 ± 0.2 (1.10)	19.5 ± 1.0 (1.10)	73.8 ± 4.2 (1.30)	1318 ± 101 (1.31)
A108I/E665V	10.4 ± 0.4 (0.98)	18.4 ± 0.9 (1.04)	121.0 ± 14.1 (2.13)†	1675 ± 166 (1.66)†
D519V/E665V	8.3 ± 0.2 (0.78)†	13.9 ± 0.1 (0.78)†	171.6 ± 11.4 (3.02)†	1808 ± 131 (1.79)†
A108I/D519V/E665V	9.1 ± 0.3 (0.86)*	14.4 ± 0.2 (0.81)†	183.9 ± 6.6 (3.23)†	1832 ± 135 (1.82)†

Thrombin generation assays in the presence of 0.25 nM FVIII, 0.5 pM rTF, and 4 μM PSPCPE vesicles were performed and parameter values were calculated according to the method [7]. Data represents the average values ± standard deviation of triplicate samples. Values in parentheses are relative to the WT value. Statistical analysis was performed by one-way ANOVA with Dunnett analysis. The single letter code is used to designate amino acid residues, E (Glu), D (Asp), A (Ala) and V (Val).
*$p < 0.05$ or $^{\dagger}p < 0.01$ compared to the value of WT.

Fig. S-1. Stability studies comparing WT FVIII and Ala108Ile/Asp519Val/Glu665Val variant. (A) FVIII activity decay at elevated temperatures. FVIII (4 nM) was incubated at 57ºC and at the indicated times aliquots were removed and activity was measured by FXa generation assays [8]. Data were fitted to a single exponential decay curve by non-linear least squares regression. WT FVIII (circles) decayed to ~40% the initial activity level in 6-7 min at 57ºC. On the other hand the Ala108Ile/Asp519Val/Glu665Val variant (diamonds) retained >70% activity for >30 min. Overall, the first order rate constant for the mutant obtained by curve-fit were reduced by ~12-fold compared to the WT FVIII value, showing enhanced stability when the mutations were combined. (B) Inhibition of FVIII by guanidinium. FVIII (50 nM) in 0-1.2 M guanidinium chloride was incubated for 2 hrs at 23ºC, diluted 1/50 and FVIII activity was measured by FXa generation. Using the range of linear responses (~0.6-1.2 M), data points were fitted by a linear equation and the IC_{50} values were obtained. The variant showed an ~36% higher IC_{50} value compared with WT). (C) FVIIIa decay. Thrombin-activated FVIIIa (1.5 nM) was incubated at 23ºC, aliquots were taken at indicated time points and activity was measured by FXa generation assay. Data were fitted to a single exponential decay curve by non-linear least squares regression. These reaction conditions resulted in the loss of ~50% of WT FVIIIa activity at ~4 min after thrombin activation, while ~10% activity remained after 16 min. FVIIIa activity of Glu665Val/Ala108Ile was more stable, retaining ~70% activity after 16 min. The estimated first order rate constant of the mutant was reduced by ~11-fold compared with WT FVIII. Symbols denote WT (circles) and Ala108Ile/Asp519Val/Glu665Val (diamonds). Each point represents the value averaged from three separate determinations. Standard deviation values for the data points were within 15% of the average values.

Fig. S-2. Thrombin generation assay profile. Thrombin generation assays were performed in the presence of 0.25 nM FVIII, 0.5 pM rTF, and 4 μM PSPCPE vesicles according to the method [7]. Each thrombin generation curve traces the average values of triplicate measurements and data were summarized in Table S-2. Thrombin generation initiated at ~10-12 min for WT, Ala108Ile, Ala108Ile/Asp519Val, and Ala108Ile/Glu665Val variants, while Asp519Val/Glu665Val and Ala108Ile/Asp519Val/Glu665Val variants initiated significantly earlier (~8-9 min). The former variants reached the peak thrombin generation at ~17-20 min while the latter variants peaked somewhat earlier (~14 min). All of the mutants examined showed increased peak thrombin values and endogenous thrombin potential (ETP) values compared with the WT values. Small increases of ~15-30% in peak thrombin and ETP values were observed for the Ala108Ile and Ala108Ile/Asp519Val variants. However, more significant increases (113% and 66% increases in peak thrombin value and ETP value, respectively) were observed for the Ala108Ile/Glu665Val variant, whereas ~200% and ~80% increases in peak thrombin and ETP values, respectively, were observed for both the Asp519Val/Glu665Val and Ala108Ile/Asp519Val/Glu665Val variants. Taken together, the Asp519Val/Glu665Val double mutation enhanced thrombin generation potential while Ala108Ile contributed an incremental increase in the enhancement to thrombin generation potential. The reason(s) for the reduced time to peak observed with the high stability variants relative to WT is not clear.

Fig. S-1A

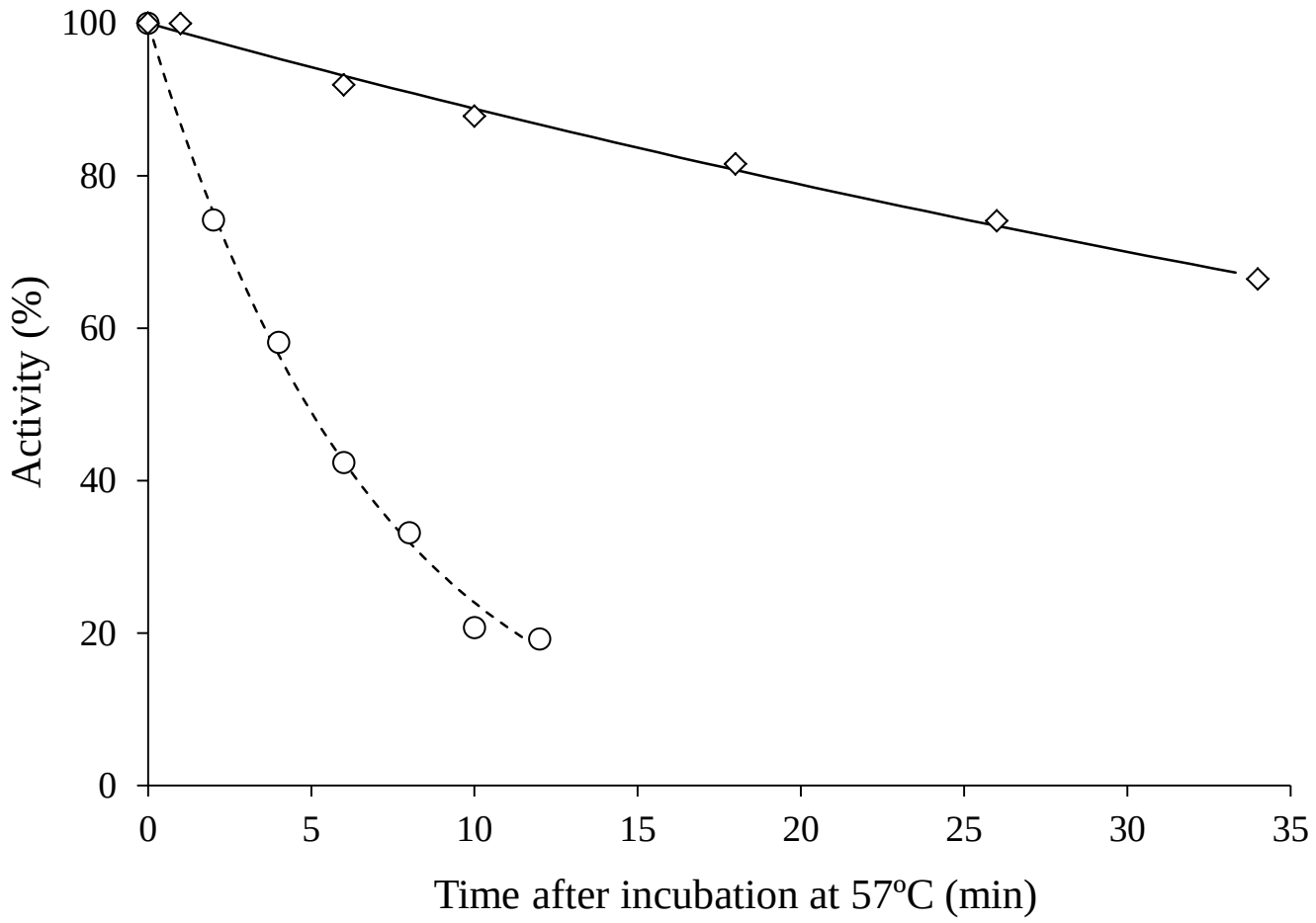

100
80
60
40
20
0
Activity (%)
0
5
10
15
20
25
30
35
Time after incubation at 57ºC (min)

Fig. S-1B

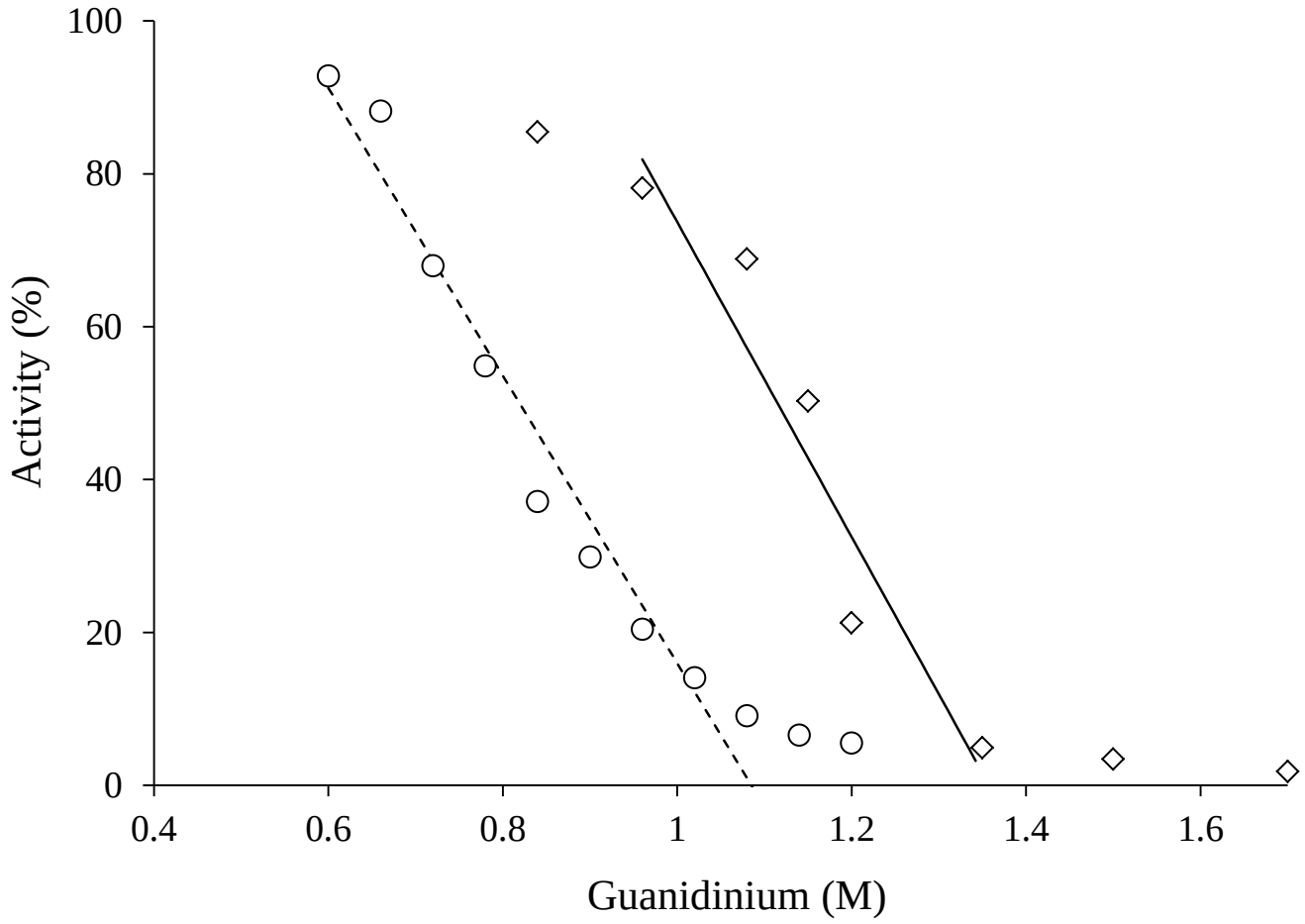


Fig. S-1C

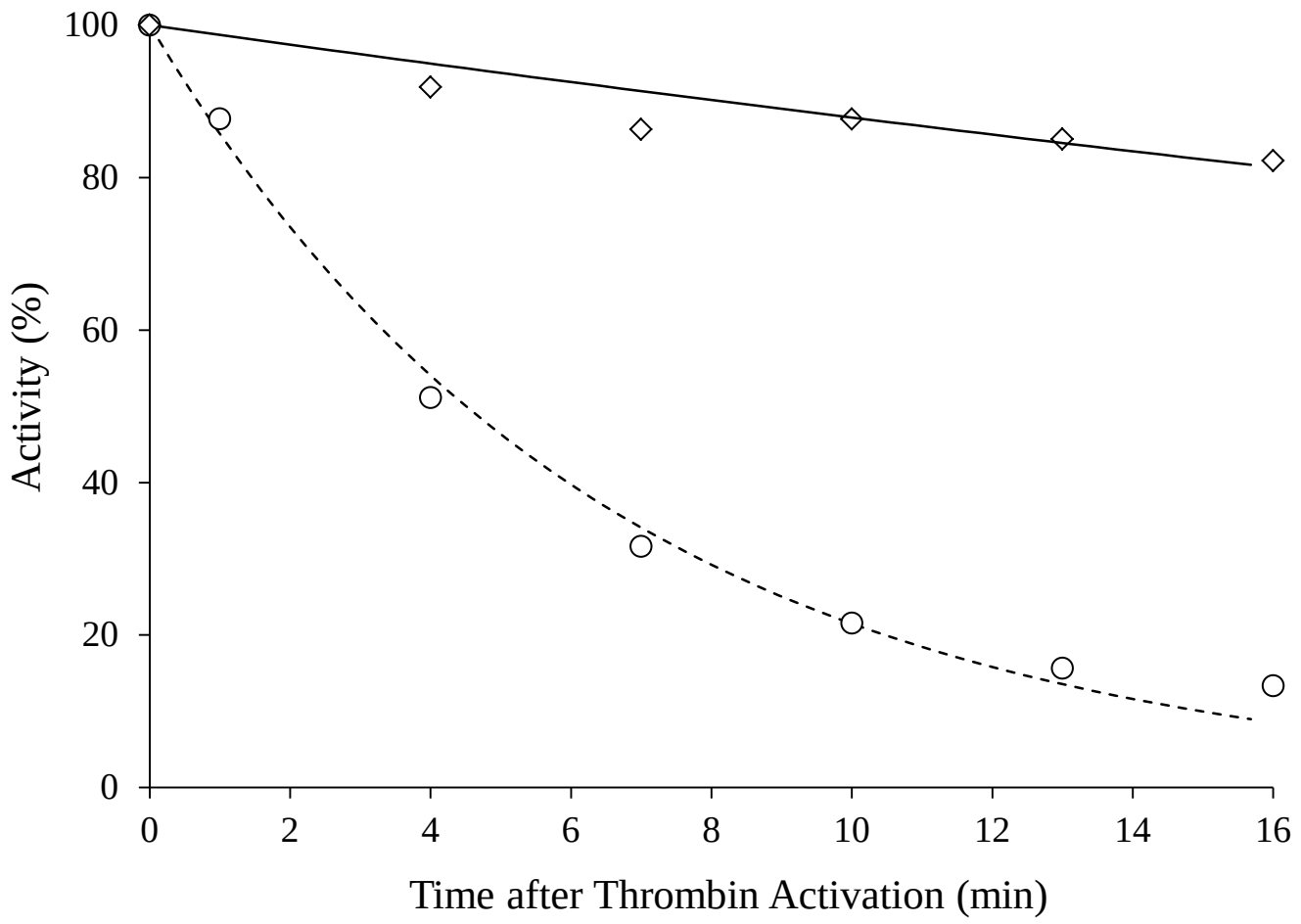
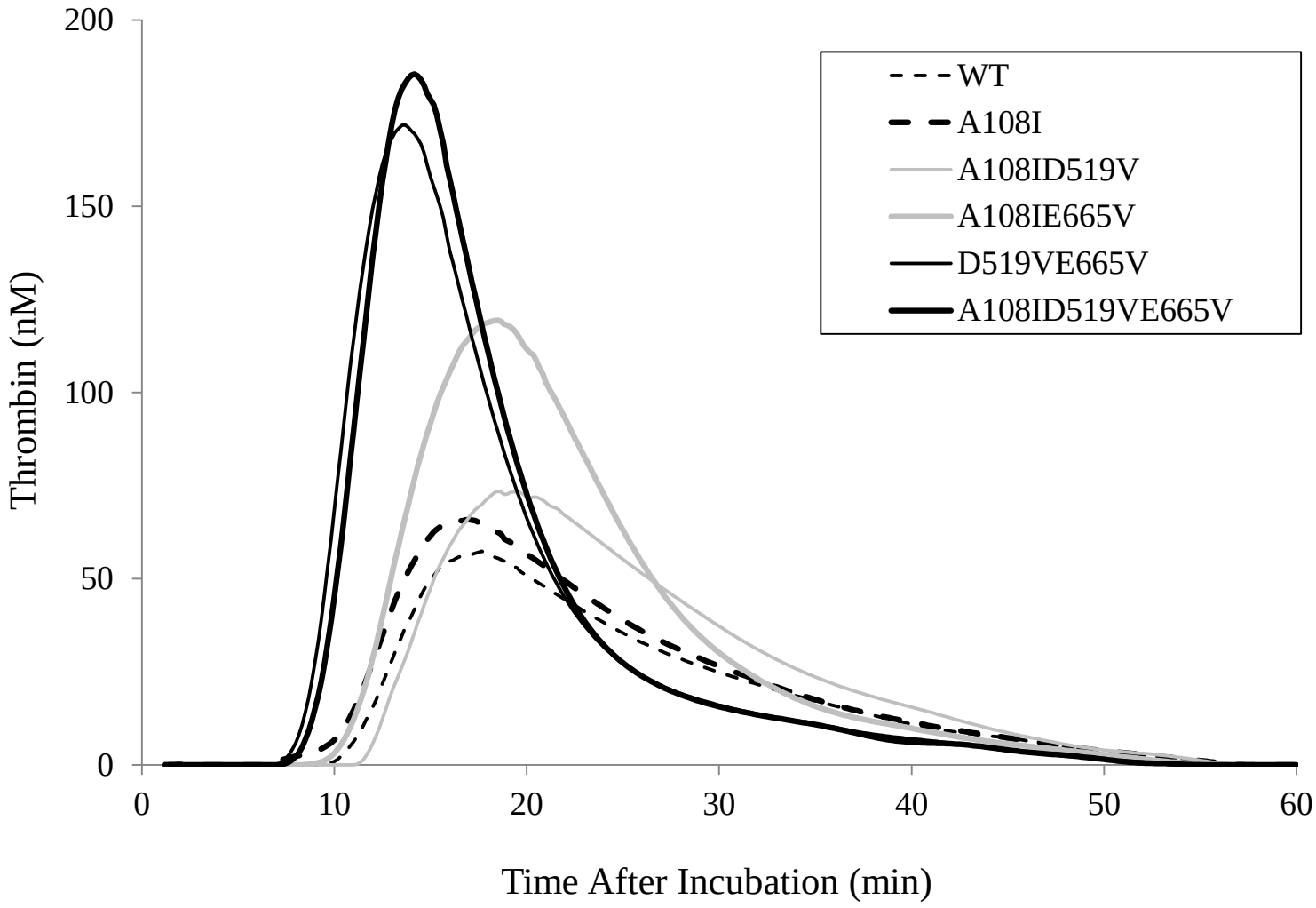

100
80
60
40
20
0
Activity (%)
0
2
4
6
8
10
12
14
16
Time after Thrombin Activation (min)

Fig. S-2


200
150
100
50
0
Thrombin (nM)
0
10
20
30
40
50
60
Time After Incubation (min)
WT
A108I
A108ID519V
A108IE665V
D519VE665V
A108ID519VE665V