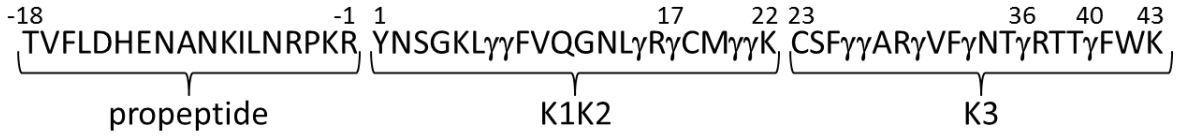
Supplementary tables

Table S1a γ-Carboxylation levels of K1K2 peptide (based on all K1K2 Gla peptides listed below) of various FIX samples determined by Lys-C peptide map. K1K2 contains 6 glutamic acid residues that are potential γ-carboxylation sites, represented by γ in the sequence, below; K1K2(6 Gla) is fully γ-carboxylated, K1K2(5 Gla) and K1K2'(5 Gla) are missing a single γ-carboxylation (at an unknown position or Glu17, respectively); no other under-γ-carboxylated species were detected. R-K1K2 peptides are derived from proFIX in which the pro-peptide has not been cleaved, and can be used to calculate the percentage of proFIX in the original sample.



K1K2 peptide (% Peak Area)	K1K2 (6Gla)	R-K1K2 (6Gla)	K1K2 (5Gla)	K1K2' (5Gla)	R-K1K2' (5Gla)	Total Gla
FIX WT	74.3	8.0	5.3	10.9	1.5	5.82
FIX Val	68.4	18.5	5.1	8.1	2.2	5.98

Propeptide % = 100×[R-K1K2(6Gla)+R-K1K2(5Gla)]/[R-K1K2(6Gla)+K1K2(6Gla)+R-K1K2'(5Gla)+K1K2(5Gla)+K1K2'(5Gla)]

Table S1b γ-Carboxylation level of K3 peptide of various FIX samples determined by Lys-C peptide map. K3 contains 6 potential γ-carboxylation sites; K3(6 Gla) is fully γ-carboxylated, K3(5 Gla), K3'(5 Gla), K3 (4 Gla) are missing γ-carboxylation at positions 36, 40, or both, respectively; no other under-γ-carboxylated species were detected.

K3 peptide (% Peak Area)	K3(6Gla)	K3(5Gla)	K3'(5Gla)	K3(4Gla)	Total Gla
FIX WT	63.2	25.8	3.4	7.5	5.56
FIX Val	73.2	14.3	3.7	8.8	5.64

Table S2 Detected Gla peptides and the peptide containing V107 (K9) in Lys-C peptide maps.

Peptide Number	From-To	Monoisotopic $[M+H]^+$ (theoretical)	Monoisotopic $[M+H]^+$ (observed)	Sequence
K1K2 (6Gla)	1- 22	2953.20	2953.42	YNSGKL**E**E**E**FVQGNL**E**R**E**C**C**M**E**E**E**K
K1K2 (5Gla)	1- 22	2909.20	2909.20	YNSGKL**E**E**E**FVQGNL**E**R**E**C**C**M**E**E**E**K
K1K2' (5Gla)	1- 22	2909.20	2909.44	YNSGKL**E**E**E**FVQGNL**E**R**E**C**C**M**E**E**E**K
R-K1K2 (6Gla)	1- 22	3109.30	3109.36	**R**YNSGKL**E**E**E**FVQGNL**E**R**E**C**C**M**E**E**E**K
R-K1K2' (5Gla)	1- 22	3065.30	3065.44	**R**YNSGKL**E**E**E**FVQGNL**E**R**E**C**C**M**E**E**E**K
K3 (6Gla)	23- 43	2959.19	2959.32	**C**SF**E**E**E**AR**E**VF**E**NT**E**RTT**E**FWK
K3 (5Gla)	23- 43	2915.19	2915.32	**C**SF**E**E**E**AR**E**VF**E**NT**E**RTT**E**FWK
K3'(5Gla)	23- 43	2915.19	2915.32	**C**SF**E**E**E**AR**E**VF**E**NT**E**RTT**E**FWK
K3 (4Gla)	23- 43	2915.19	2915.20	**C**SF**E**E**E**AR**E**VF**E**NT**E**RTT**E**FWK
K9	107-122	1913.84	1914.00	**V**V**C**S**C**TEGYRLAENQK

E: potential γ-carboxylation site; **C**: iodoacetamide alkylated Cys; **V:** Val 107; R: C-terminus of propeptide

Supplementary figures

Figure S1

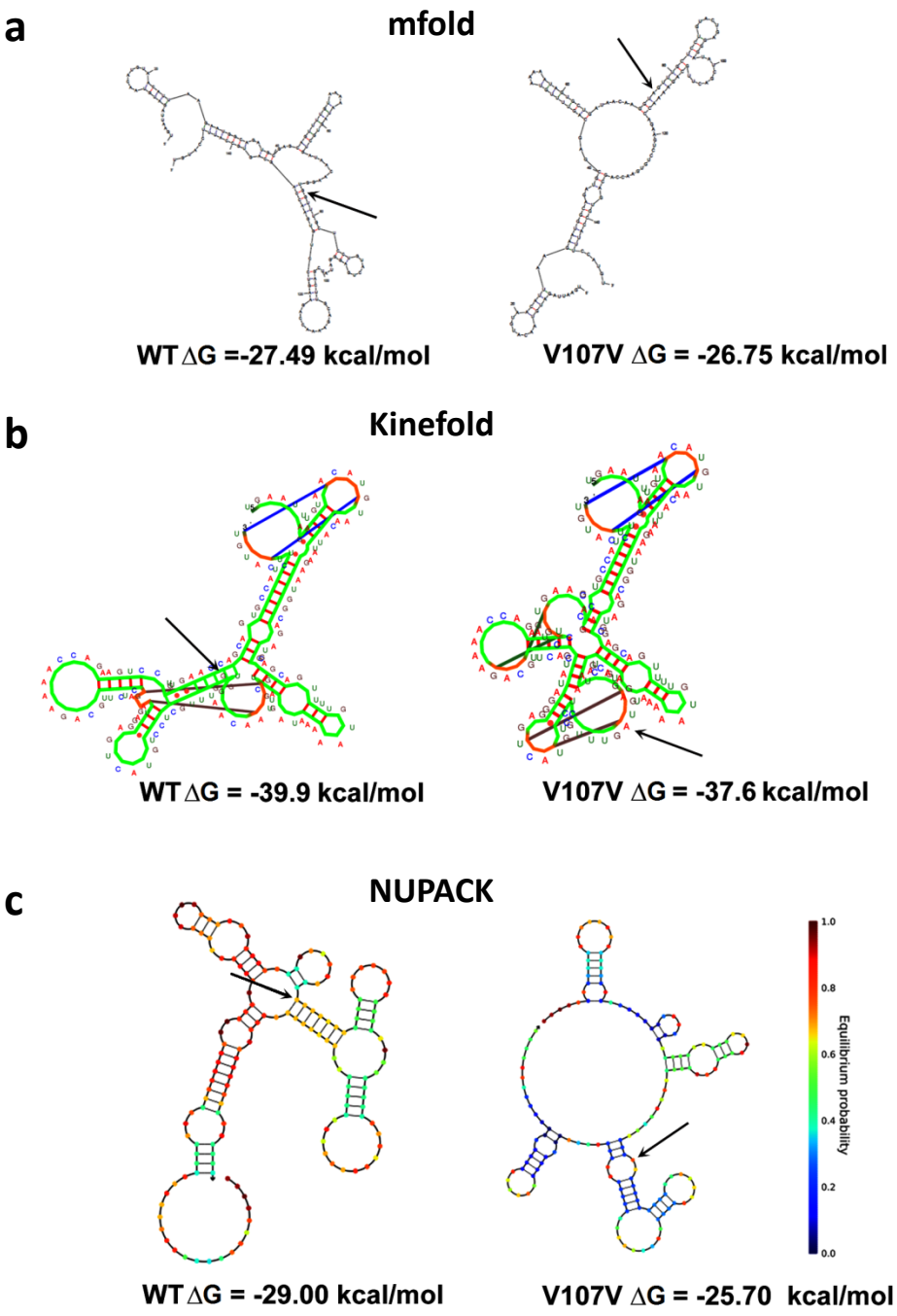


Figure S2

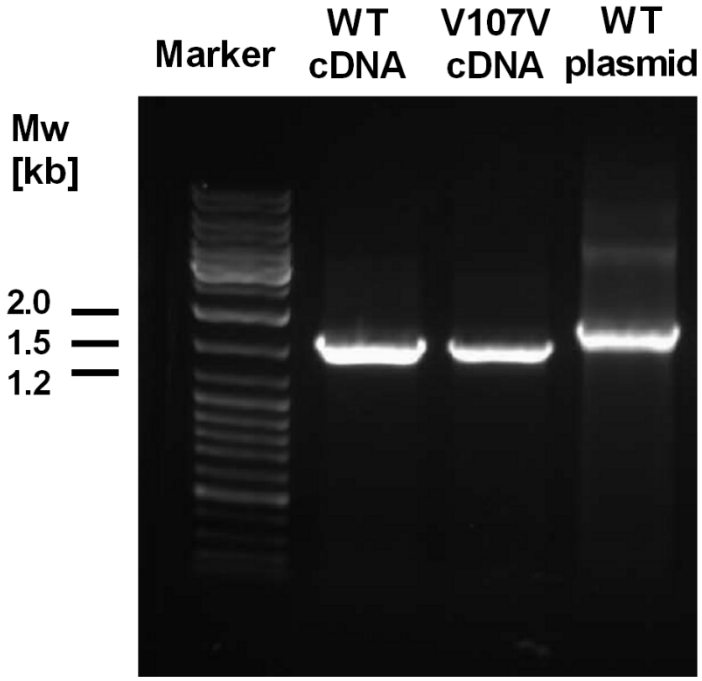


Figure S3

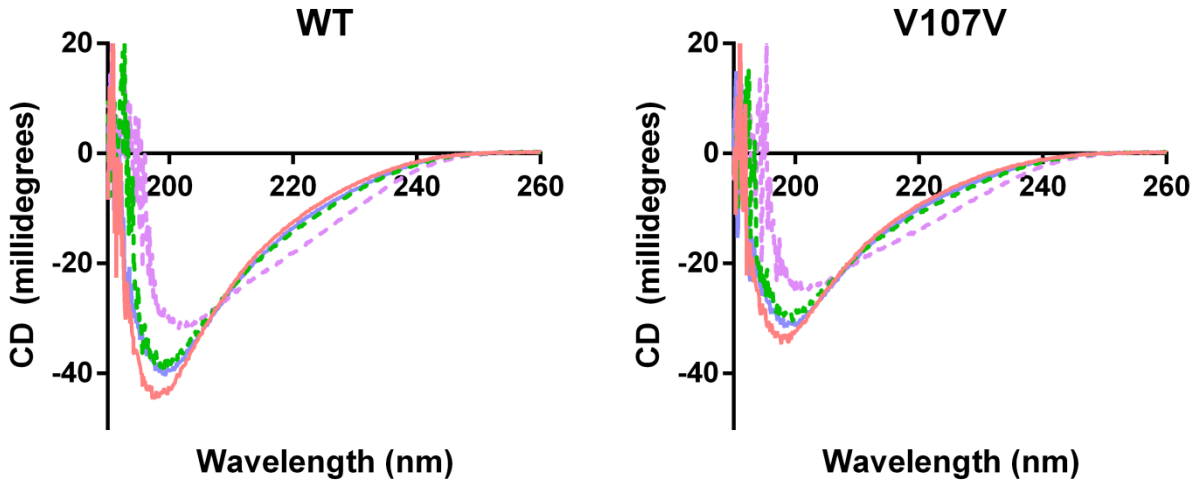

WT
V107V
CD (millidegrees)
20
0
-20
-40
200
220
240
260
Wavelength (nm)

Figure S4

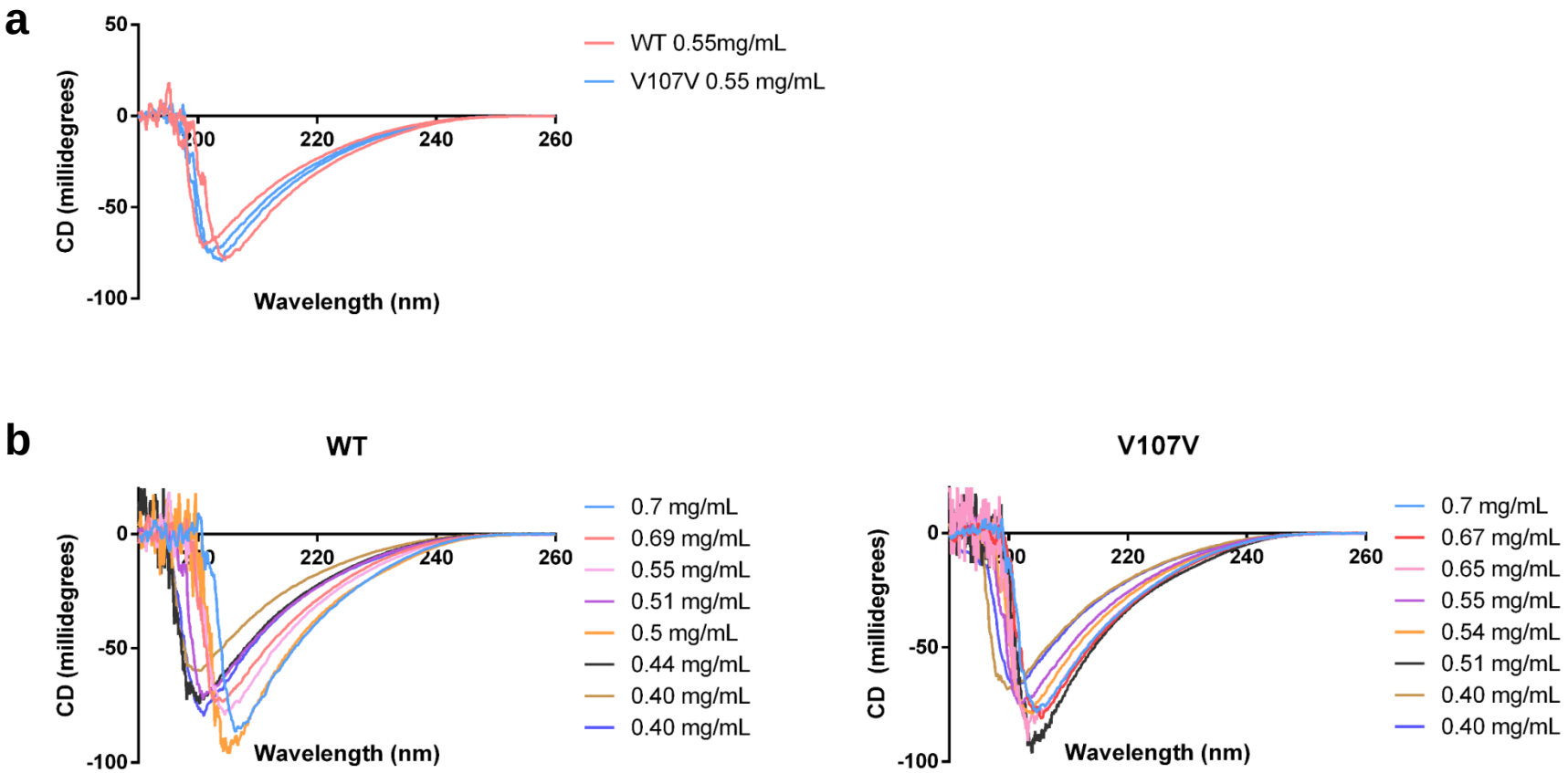

a
50
0
-50
-100
CD (millidegrees)
220
240
260
Wavelength (nm)
WT 0.55mg/mL
V107V 0.55 mg/mL
b
WT
0
-50
-100
CD (millidegrees)
220
240
260
Wavelength (nm)
0.7 mg/mL
0.69 mg/mL
0.55 mg/mL
0.51 mg/mL
0.5 mg/mL
0.44 mg/mL
0.40 mg/mL
0.40 mg/mL
V107V
0
-50
-100
CD (millidegrees)
220
240
260
Wavelength (nm)
0.7 mg/mL
0.67 mg/mL
0.65 mg/mL
0.55 mg/mL
0.54 mg/mL
0.51 mg/mL
0.40 mg/mL
0.40 mg/mL

Figure S5

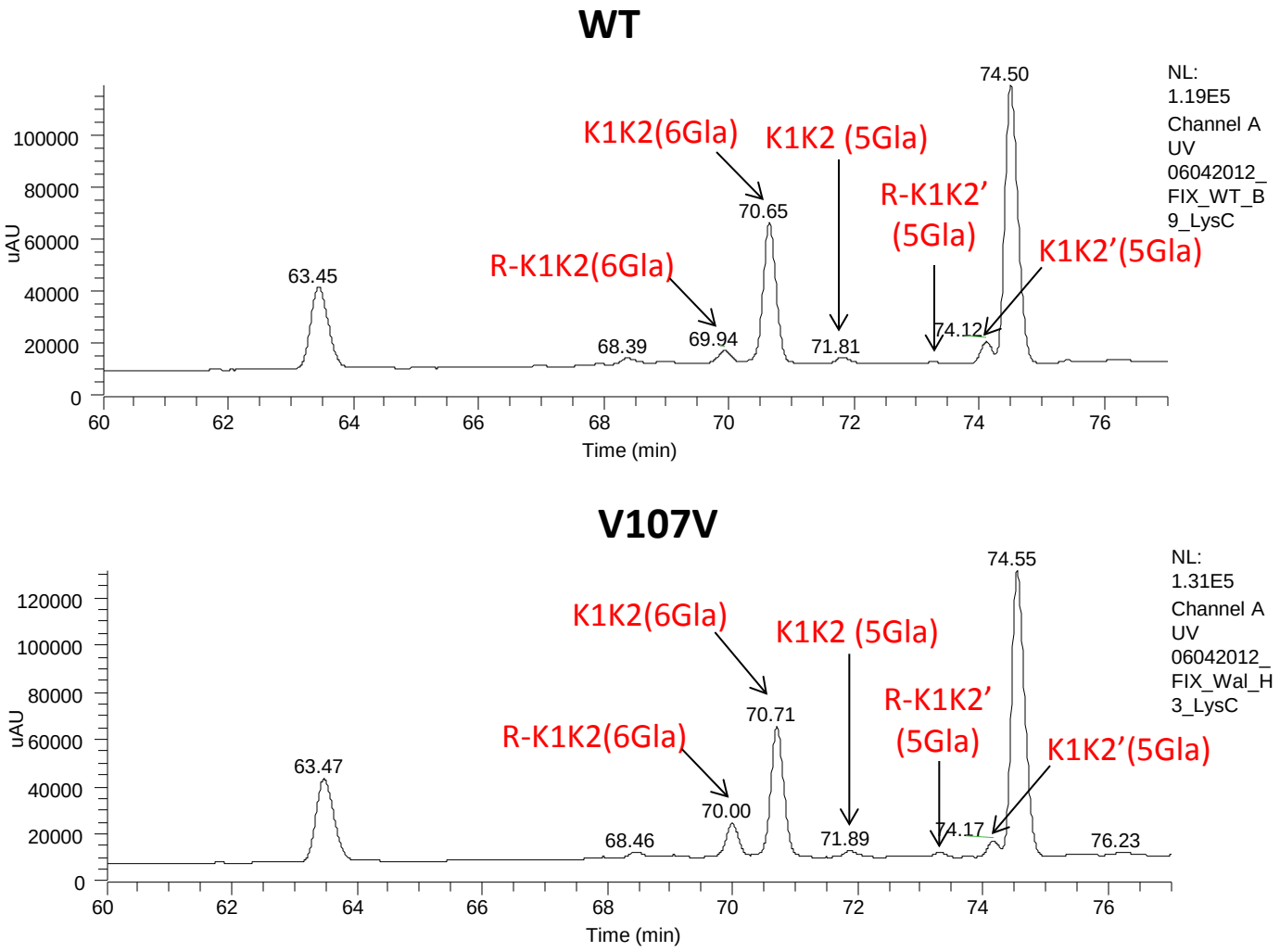
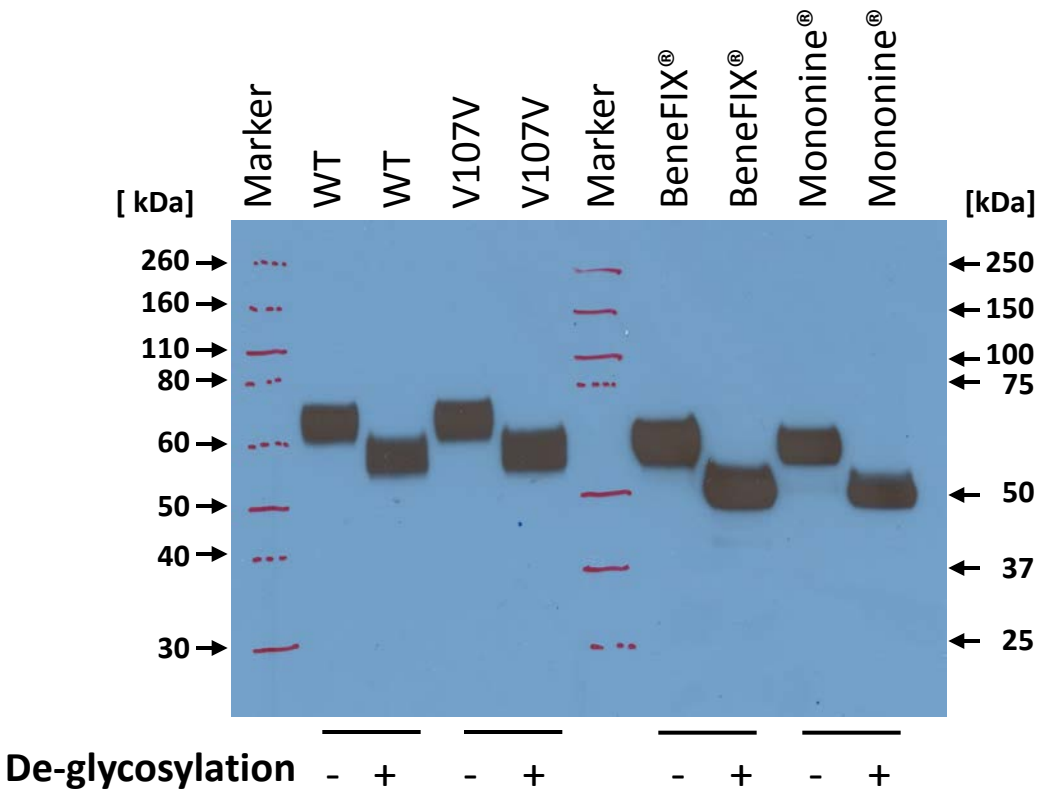

WT
74.50
NL:
1.19E5
Channel A
UV
06042012_
FIX_WT_B
9_LysC
K1K2(6Gla)
K1K2 (5Gla)
R-K1K2'
(5Gla)
K1K2'(5Gla)
R-K1K2(6Gla)
70.65
63.45
68.39
69.94
71.81
74.12
uAU
Time (min)
V107V
74.55
NL:
1.31E5
Channel A
UV
06042012_
FIX_Wal_H
3_LysC
K1K2(6Gla)
K1K2 (5Gla)
R-K1K2'
(5Gla)
K1K2'(5Gla)
R-K1K2(6Gla)
70.71
63.47
70.00
68.46
71.89
74.17
76.23
uAU
Time (min)

Figure S6


Marker
WT
WT
V107V
V107V
Marker
BeneFIX®
BeneFIX®
Mononine®
Mononine®
[kDa]
[kDa]
260
160
110
80
60
50
40
30
250
150
100
75
50
37
25
De-glycosylation
- + - + - + - +

Supplementary figure legends

Supplementary Figure S1. *In silico* analyses of mRNA folding and stability for the wild-type *F9* and the GTG>GTA (Val107Val) variant. RNA structure and free energy prediction were derived from (**a**) mfold,[1] (**b**) Kinefold[2] and (**c**) NUPACK[3]. Wild-type and Val107Val FIX structures correspond to the 151 nt mRNA fragment region centered on the mutation site. The folding patterns with the lowest free energy among multiple candidates are shown for each case.

Supplementary Figure S2. Agarose gel analysis of *F9* cDNA samples amplified by RT-PCR from the cells expressing WT or Val107Val FIX and original plasmid containing truncated intron 1 (299 nt). The wild-type and mutant *F9* ORF cDNA showed an identical sequence of 1386 nt without intron except for the mutation site.

Supplementary Figure S3. Thermal unfolding and refolding CD spectrums. Thermal unfolding and refolding CD spectrums of wild-type (left) vs. Val107Val variant (right) (~0.4 mg/mL concentration) with initial temperature at 5 °C (red), 25 °C (green, dashed), 55 °C (light purple, dashed) and final temperature at 5 °C after cooling (dark purple).

Supplementary Figure S4. CD spectrums of wild-type vs. Val107Val variant at constant temperature using various concentrations of the proteins: (**a**) CD measurements of two separate preparations of wild-type and Val107Val FIX with concentrations of 0.55 mg/mL indicate some variability in the CD (millidegrees) and wavelength (nm) of the spectra. (**b**) CD was performed on preparations of wild-type (left panel) and Val107Val FIX (right panel) with a concentration range of 0.7-0.4 mg/mL, indicating a strong concentration-dependent pattern.

Supplementary Figure S5. Part of UV chromatograms (214 nm) of Lys-C peptide map of FIX wild-type (WT) and FIX (Val107Val) variants. The relevant peaks (described in supplementary tables S1 and S2) are indicated.

Supplementary Figure S6. Glycosylation assessment of FIX. The untreated (-) and deglycosylated (+) purified wild-type FIX,Val107Val FIX, BeneFIX® and Mononine® were run under SDS-PAGE reducing conditions and visualized by immune blotting with a FIX specific monoclonal antibody (9D).

REFERENCES

1. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;31(13):3406-15.
2. Xayaphoummine A, Bucher T, Isambert H. Kinefold web server for RNA/DNA folding path and structure prediction including pseudoknots and knots. *Nucleic Acids Res* 2005;33(Web Server issue):W605-10.
3. Zadeh JN, Steenberg CD, Bois JS, Wolfe BR, Pierce MB, Khan AR, Dirks RM, Pierce NA. NUPACK: Analysis and design of nucleic acid systems. *J Comput Chem* 2011;32(1):170-3.