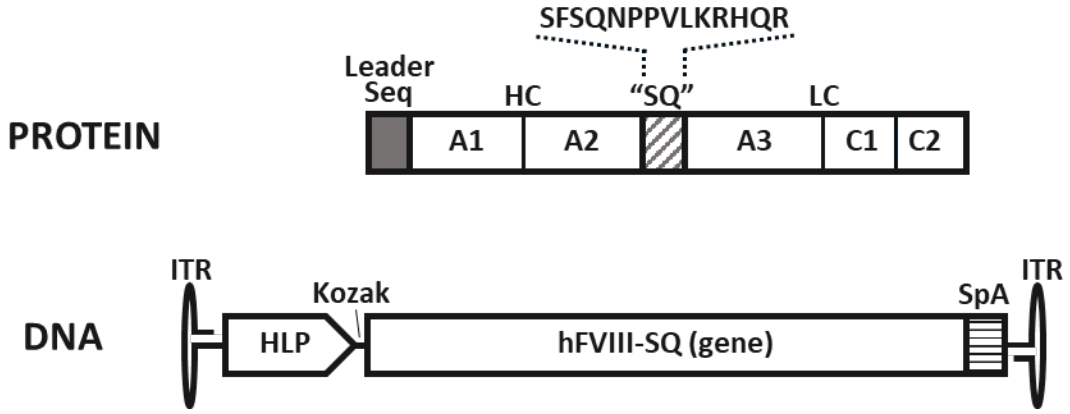
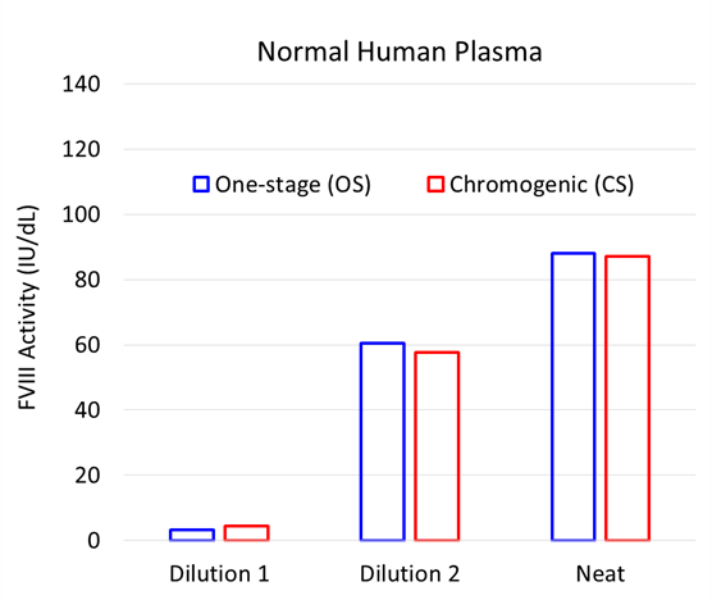
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Schematic of the BMN 270 Gene Therapy Vector Genome and Encoded Protein.



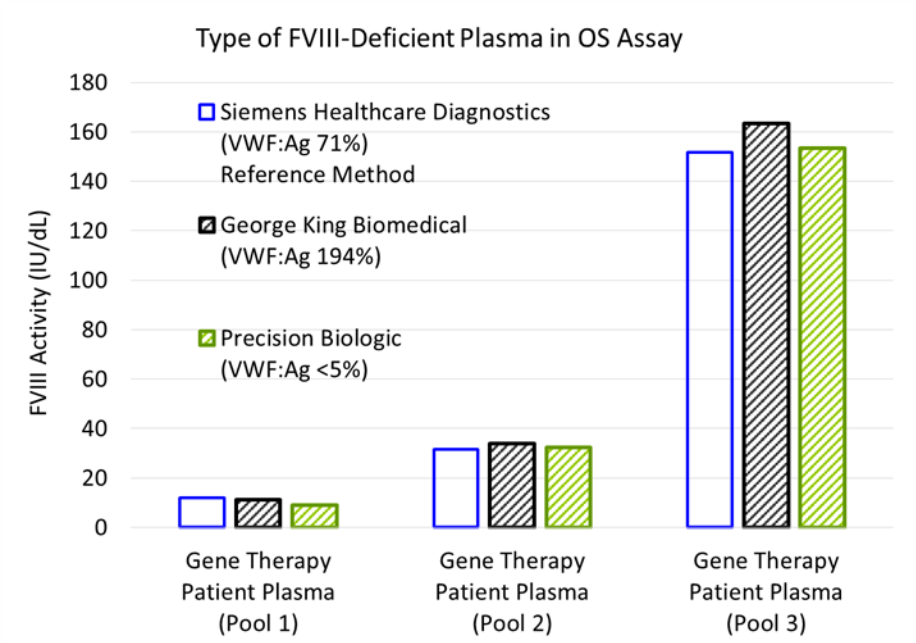
The BMN 270 (AAV5-hFVIII-SQ) gene therapy vector genome includes single-stranded DNA containing a hybrid human liver-specific promoter (HLP); a B-domain deleted human factor VIII (hFVIII-SQ) coding region (1); and a synthetic polyadenylation (SpA) signal (2). The expression cassette is flanked at its 5' and 3' ends by double-stranded AAV2-derived inverted terminal repeats (ITRs). The hFVIII-SQ coding region consists of a codon-optimized nucleic acid sequence encoding a secretory leader sequence (Leader Seq) as well as the A1, A2, A3, C1 and C2 FVIII protein domains. The B domain that is normally located between the A2 and A3 domains was replaced by a 14 amino acid "SQ" linker sequence, which was derived from the ends of the wild-type B-domain sequence. The resulting amino acid sequence is identical to that of the human FVIII reference sequence within the heavy (HC) and light (LC) chains of the protein. nt, nucleotide; aa, amino acid

Supplementary Figure 2. FVIII Activity of Normal Human Plasma in OS and CS Assay.



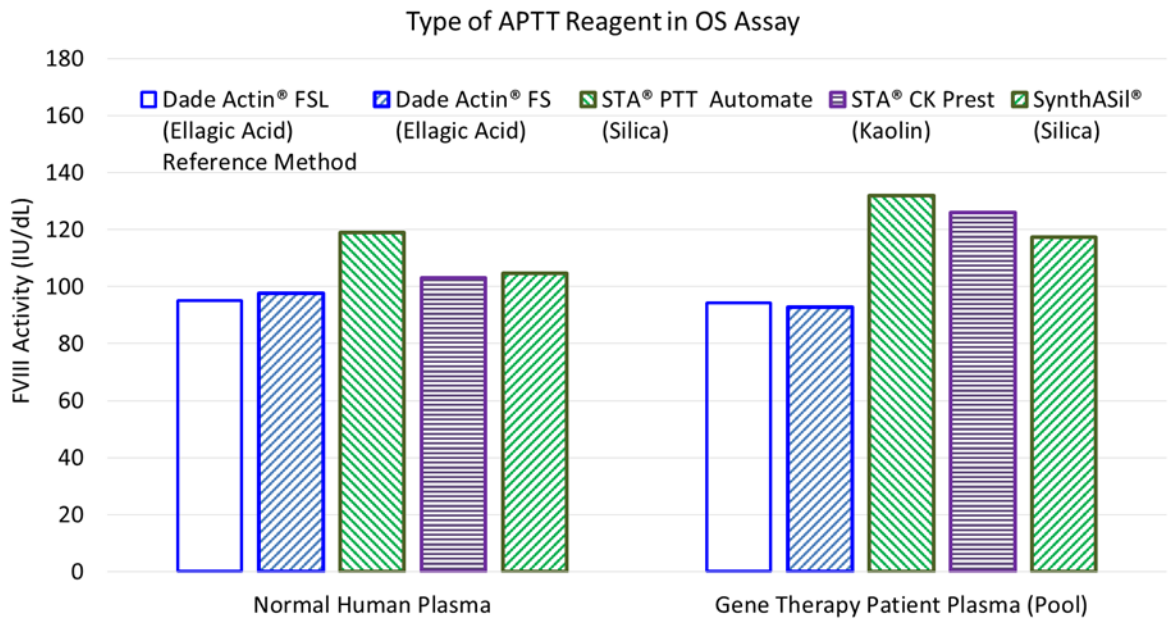
Pooled normal human plasma was either left undiluted (Neat) or diluted to two different FVIII activity levels (Dilution 1, Dilution 2) and tested in OS and CS assay. Similar OS and CS activity (≤ 25% relative difference, ≤ 3 IU/dL absolute difference) were observed at any given FVIII activity level. The OS/CS ratios ranged from 0.750 to 1.053 across all three samples. These results confirmed that both OS and CS assay can adequately detect different levels of endogenous, native FVIII, with little to no difference between the quantification in each assay.

Supplementary Figure 3. FVIII-Deficient Plasma Reagents with Different VWF Levels Yield Comparable OS Activity for Transgene-Produced FVIII-SQ.



Binding to VWF stabilizes FVIII in plasma and extends its half-life (3); however, VWF binding can also suppress FVIII activation in OS assays and delay APTT-based clotting times because the rate of FVIII release from VWF determines its availability for activation (4, 5). The OS assay described in this manuscript used an immunodepleted FVIII-deficient plasma reagent (Siemens Healthcare Diagnostics), which contains less than normal levels of VWF, likely due to its partial removal via binding to FVIII during immunodepletion. To test whether the use of FVIII-deficient plasma with less than normal VWF levels led to increased FVIII activity in the OS assay, three different types of FVIII-deficient substrate plasma were compared: Immunodepleted FVIII-deficient plasma from Siemens Healthcare Diagnostics (VWF:Ag 71%), congenital FVIII-deficient plasma from George King Biomedical (VWF:Ag 194%), and immunodepleted FVIII-deficient plasma from Precision Biologic (VWF:Ag < 5%). The OS activity for transgene-produced FVIII-SQ measured in pooled plasma samples from gene-therapy participants (Pool 1, 2, and 3) was similar across all three types of FVIII-deficient substrate plasma, with a CV < 15% between measurements taken at different VWF levels. Therefore, the lower than normal VWF level in immunodepleted FVIII-deficient plasma does not account for the higher OS activity of transgene-produced FVIII-SQ.

Supplementary Figure 4. The Type of APTT Reagent Used in the OS Assay Does Not Explain the Higher OS Than CS Activity of Transgene-Produced FVIII-SQ.



Different OS assay kits utilize different APTT reagents that contain different surface activators, some of which can lead to aberrant measurements for certain recombinant factor replacement products (6, 7). To compare different OS assay kits for transgene-produced FVIII-SQ, pooled gene-therapy participant plasma was tested using 5 different APTT reagents that differed in the type of surface activator (right): Dade Actin® FSL and Dade Actin® FS (Siemens Healthcare Diagnostics) contained ellagic acid, STA®-PTT Automate (Diagnostica Stago) and HemosIL® SynthASil® (Instrumentation Laboratory) contained silica, and STA® CK Prest (Diagnostica Stago) contained kaolin. Together, these commonly used APTT reagents cover over 90% of CAP accredited laboratories in the US. The APTT reagent routinely used in gene therapy trials described herein was Dade Actin® FSL (Reference Method). Pooled normal human plasma (CRYOcheck™ Normal Reference Plasma, Lot # 7143) was also tested with each of the five APTT reagents for comparison (left). To eliminate any potential bias resulting from different primary standard calibrators in each OS assay kit, a secondary calibrator (SSC lot 4) was co-run in each assay and the reported test results were corrected accordingly if SSC lot 4 recovery

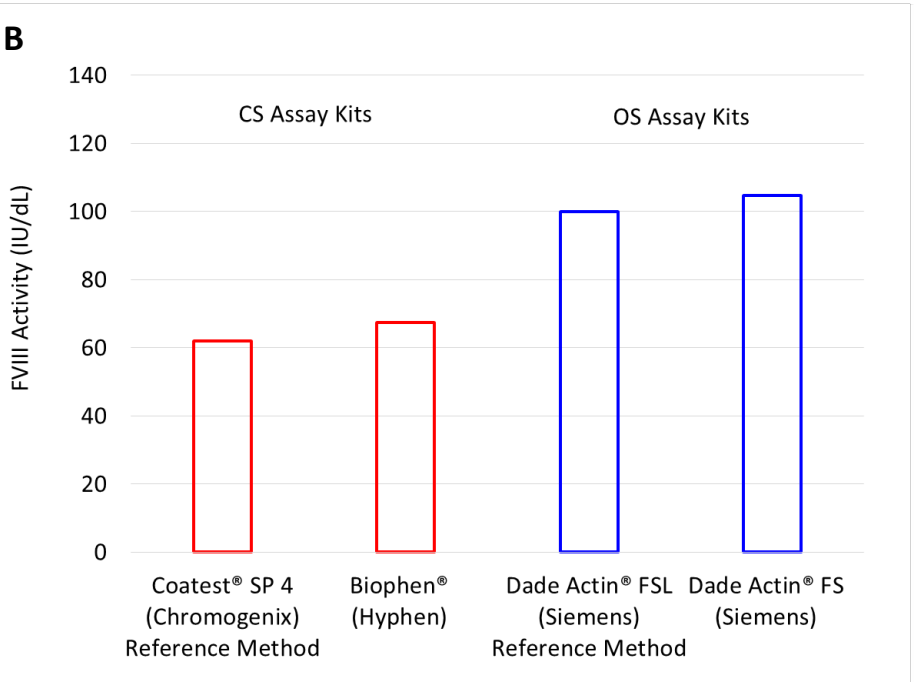
showed an |RE| > 10%. In summary, while there was some variability in OS measurements obtained with different APTT reagents, the use of silica or kaolin as alternative surface activators did not result in lower OS activity for transgene-produced FVIII-SQ compared to the OS activity measured when using ellagic acid. Therefore, the APTT reagent used in BMN 270 gene-therapy trials described herein was not responsible for the higher OS than CS activity of transgene-produced FVIII-SQ.

Supplementary Figure 5: Bovine and Human FIXa and FX Reagents Yield Comparable CS Activity for Transgene-Produced FVIII-SQ.

A

CS Assay Kit Feature	Coatest® SP 4 (Chromogenix) Reference Method	Biophen® (Hyphen)
Exogenous FIXa and FX	Bovine	Human
Phospholipids	Purified	Synthetic
Chromogenic substrate	S-2765	SXa-11
Exogenous thrombin added?	No	Yes, human

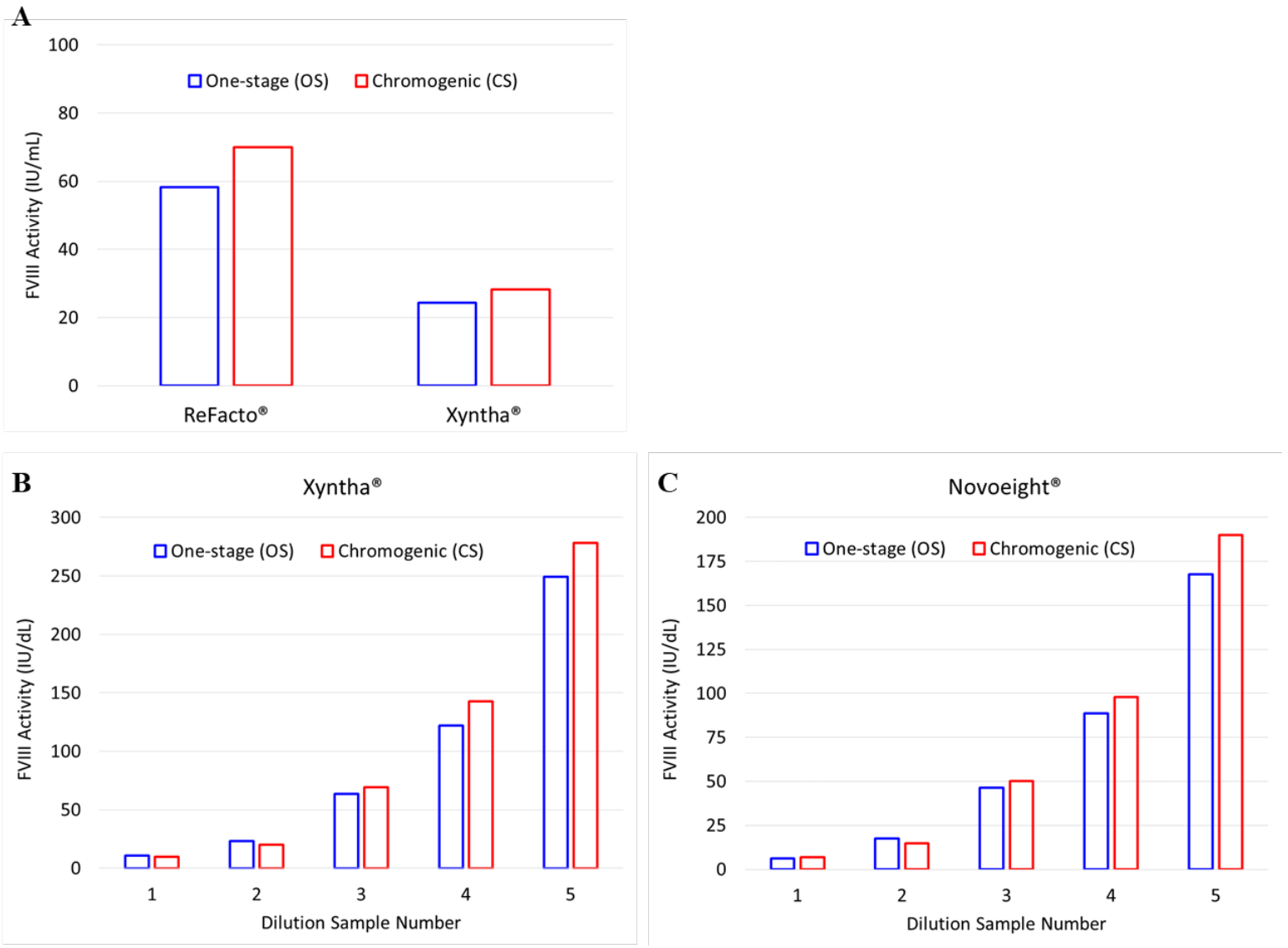
B



CS assay kits use either human or bovine FIXa and FX reagents, which are exogenously added in excess. The CS assay in BMN 270 gene-therapy trials described herein was based on the Coatest® SP 4 kit (Chromogenix) that contains bovine FIXa and FX. To test whether the use of these bovine reagents led to lower CS than OS activity for transgene-produced FVIII-SQ, the Coatest® SP kit was compared to the Biophen Chromogenic VIII kit (Hyphen) that contains human FIXa and FX. (**A**) The two CS assay kits also differed in their source of phospholipids,

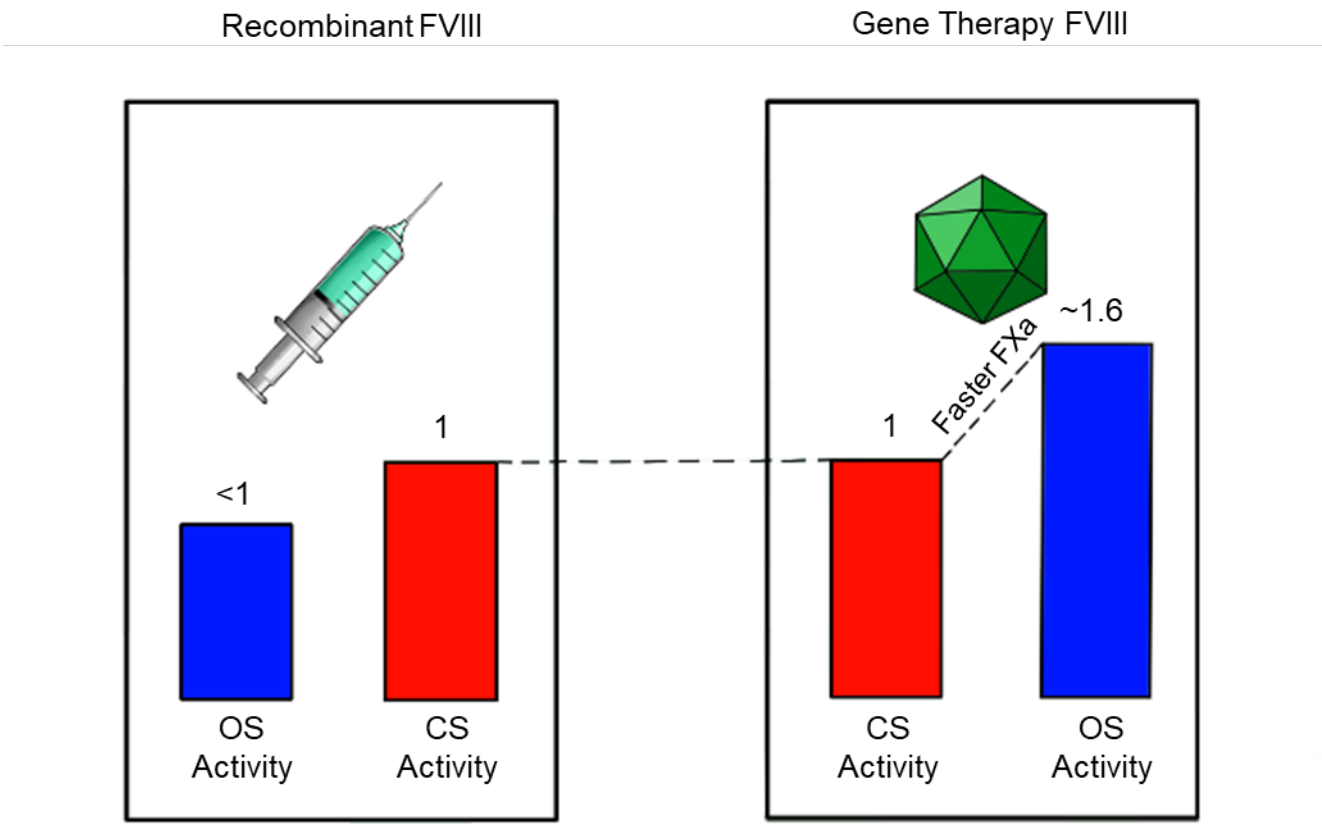
type of chromogenic FXa substrate, and the absence (Coatest® SP 4 kit) or presence (Biophen kit) of exogenously added human thrombin. (**B**) Plasma samples derived from a BMN 270 gene-therapy participant on the same visit day were tested with each assay in different laboratories and yielded similar CS activity (|RE| < 10%, using the Coatest® SP 4 kit as the reference). Thus, there was no noticeable impact of using bovine vs human FIXa and FX reagents in the CS assay. As a control, the same sample was also tested by OS assay in each laboratory, using similar APTT reagents (Dade Actin® FSL or Dade Actin® FS) containing the same surface activator (ellagic acid). The degree of difference between the OS and CS FVIII activity was highly similar in each laboratory, reflected in similar OS/CS ratios (1.609 and 1.553). In conclusion, the use of bovine vs human FIXa and FX reagents in the CS assay did not sufficiently explain the OS/CS difference for transgene-produced FVIII-SQ.

Supplementary Figure 6. FVIII Activity of Recombinant BDD-FVIII Products in OS and CS Assay.



(**A**) To confirm assay performance for recombinant B-domain deleted (BDD) FVIII-SQ, the OS and CS activities for ReFacto® and Xyntha® were determined, which showed an OS/CS ratio of 0.834 and 0.862, respectively, reflecting the lower OS than CS activity described in the literature (8, 9). This was further evaluated using multiple dilution levels (n = 5) of Xyntha® (**B**) and Novoeight® (**C**), covering a range of concentrations within the high and low calibration curves. Both Xyntha® and Novoeight® represent recombinant B-domain-deleted FVIII replacement products: Xyntha® is identical in sequence to ReFacto®, while Novoeight® contains a different linker region replacing the B domain. The determined OS/CS ratios ranged from 0.855 to 1.182 across all dilutions for both recombinant products. Note that lower OS than CS activity was measured for each product in more concentrated samples (Sample Dilutions 3, 4, and 5) as per usual, while slightly higher OS than CS activity was measured in less concentrated samples (Sample Dilutions 1 and 2), as described in the literature for ReFacto® (10). In summary, both OS and CS assay delivered expected results for plasma samples containing recombinant BDD-FVIII products. This suggested that the absence of the B domain *per se* is unlikely to explain the higher OS than CS activity of transgene-produced FVIII-SQ.

Supplementary Figure 7. Graphical Abstract Comparing the Activity of Recombinant and Gene Therapy FVIII Products.



SUPPLEMENTARY TABLES

Supplementary Table 1. Calibrator Verification Against the 6th WHO International Standard Plasma[a]

	OS FVIII Activity (IU/dL), Inter-run Mean	%RE	CS FVIII Activity (IU/dL), Inter-run Mean	%RE	OS/CS ratio
6th WHO International Standard Plasma (NIBSC 07/316), nominal FVIII Activity: 68.0 IU/dL	66.8	-1.8	67.7	-0.5	0.987

[a]Both OS and CS activity assay utilized the same normal plasma calibrator. To ascertain correct calibrator value assignment, the plasma calibrator was verified independently in each assay against the 6th WHO International Standard Plasma (NIBSC lot 07/316) that represents a nominal FVIII activity of 68.0 IU/dL (11). Calibration verification was performed during assay method validation prior to clinical sample testing, and the average FVIII activities measured for the 6th WHO International Standard Plasma in OS and CS assay are shown here. Both assays generated near-identical results with an OS/CS ratio of 0.987, thus demonstrating there were no apparent deviations in calibrator value assignments. Calibrator verification is part of regular assay maintenance and is performed at least each time a new calibrator lot is qualified in each assay.

Supplementary Table 2. Summary of FVIII Assay Correlations from a Clinical Laboratory Field Study in BMN 270 Clinical Trial 270-201

Clinical Lab	OS APTT Reagent	OS Phospholipids; Surface Activator	CS Assay Kit	CS FIXa; FX Reagents	CS FXa Substrate	Number of Subjects	OS/OS Slope, Local/Central *(data points, R^2)*	CS/CS Slope, Local/Central *(data points, R^2)*	OS/CS Slope *(data points, R^2)*
Central Lab	Siemens Dade Actin FSL	soy, rabbit; ellagic acid	Chromogenix Coatest® SP4 FVIII	bovine; bovine	S-2765	13	NA	NA	1.656 *(629, 0.963)*
1266	HemosIL SynthASil	synthetic; colloidal silica	NA	NA	NA	1	1.221 *(54, 0.952)*	NA	NA
1545	Siemens Dade Actin FS	soy; ellagic acid	Hyphen Biophen FVIII:C	human; human	SXa-11	5	1.077[a] *(219, 0.946)*	1.098[a] *(169, 0.975)*	1.850[a] *(350, 0.974)*
1546	Tcoag/Stago Triniclot APTT HS	porcine, chicken; micronized silica	Technoclone Technochrom FVIII:C	human; bovine	FXa-1	3	1.022[a] *(95, 0.976)*	1.074[a] *(101, 0.961)*	1.665 *(149, 0.941)*
1580	HemosIL SynthASiL	synthetic; colloidal silica	Hyphen Biophen FVIII:C	human; human	SXa-11	4	1.193 *(156, 0.947)*	0.924 *(149, 0.900)*	1.739 *(204, 0.909)*
1583	Siemens Dade Actin FS	soy; ellagic acid	Hyphen Biophen FVIII:C	human; human	SXa-11	1	0.822[a] *(22, 0.837)*	0.941 *(20, 0.738)*	1.634[a] *(26, 0.976)*
1745	HemosIL SynthASil	synthetic; colloidal silica	Technoclone Technochrom FVIII:C	human; bovine	FXa-1	2	1.121 *(20, 0.941)*	1.166[a] *(15, 0.983)*	1.531 *(20, 0.867)*

[a] Based on outlier-removed data. Outliers were identified as observations with studentized residual > 2 and DFBETAS statistics for slope parameter > 2.

Supplementary Table 3. Summary of FVIII Assay Correlations from a Clinical Laboratory Field Study in BMN 270 Clinical Trial 270-301

Clinical Lab	OS APTT Reagent	OS Phospholipids; Surface Activator	CS Assay Kit	CS FIXa; FX Reagents	CS FXa Substrate	Number of Subjects	OS/OS Slope, Local/Central *(data points, R^2)*	CS/CS Slope, Local/Central *(data points, R^2)*	OS/CS Slope *(data points, R^2)*
Central Lab	Siemens Dade Actin FSL	soy, rabbit; ellagic acid	Chromogenix Coatest® SP4 FVIII	bovine; bovine	S-2765	22	NA	NA	1.534 *(649, 0.958)*
1727	Siemens Dade Actin FS	soy; ellagic acid	Hyphen Biophen FVIII:C	human; human	SXa-11	1	1.031 *(45, 0.915)*	1.129 *(45, 0.915)*	1.292 *(47, 0.883)*
1469	HemosIL SynthASiL	synthetic; colloidal silica	Hyphen Biophen FVIII:C	human; human	SXa-11	2	1.172 *(88, 0.968)*	0.964 *(52, 0.869)*	1.848 *(92, 0.923)*
1583	Siemens Dade Actin FS	soy; ellagic acid	Hyphen Biophen FVIII:C	human; human	SXa-11	2	0.986 *(27, 0.695)*	1.303 *(28, 0.583)*	1.503[a] *(27, 0.956)*
1745	HemosIL SynthASil	synthetic; colloidal silica	Technoclone Technochrom FVIII:C	human; bovine	FXa-1	1	1.395 *(17, 0.951)*	1.065 *(11, 0.963)*	2.013 *(14, 0.968)*
0157	Stago C. K. PREST 5	rabbit (Cephalin); kaolin	NA	NA	NA	1	1.237 *(22, 0.983)*	NA	NA
0181	Siemens Dade Actin FSL	soy, rabbit; ellagic acid	Siemens Chromogenic FVIII:C	bovine; bovine	Pefachrome 5279	1	1.153 *(26, 0.936)*	0.961 *(2, 1.000)*	1.524 *(2, 1.000)*
0188	Stago C. K. PREST 5	rabbit (Cephalin); kaolin	Tcoag/Stago TriniCHROM FVIII:C	bovine; bovine	Pefachrome 5279	2	0.962 *(20, 0.821)*	0.975 *(19, 0.808)*	1.556 *(19, 0.646)*

1633	Siemens Dade Actin FS	soy; ellagic acid	Chromogenix Coamatic FVIII	bovine; bovine	S-2765	3	1.185[a] *(89, 0.918)*	0.783 *(75, 0.882)*	1.883 *(96, 0.856)*
1664	Siemens 'Mixed Reagent'	NA	NA	NA	NA	2	1.062 *(15, 0.951)*	NA	NA
1746	Stago Sta-PTT	rabbit (Cephalin); silica	Chromogenix Coamatic FVIII	bovine; bovine	S-2765	1	1.192 *(19, 0.850)*	NA[b]	NA[b]

[a] Based on outlier-removed data. Outliers were identified as observations with studentized residual > 2 and DFBETAS statistics for slope parameter > 2.

[b] Unable to perform regression due to insufficient FVIII data points ($n \leq 1$).

SUPPLEMENTARY METHODS

Expression of FVIII-SQ in CHO, HEK 293, and HepG2 Cells

Recombinant human FVIII-SQ was produced in stably transfected CHO-S (Thermo-Fisher), HEK 293F (Thermo-Fisher) and HepG2 (ATCC) cells, cultured with 10 μg/ml Puromycin in CDCHO medium (Thermo-Fisher), 0.5 μg/ml Puromycin in 293 Freestyle medium with L-glutamine, and 10 μg/ml Puromycin in EMEM/10% FBS medium, respectively. Supernatants were collected from cells grown to confluency in T75 or T150 flasks (Corning) for 3 to 4 days. For FVIII-SQ purification, a 5L fixed-bed reactor (Eppendorf) was inoculated with stable HepG2 cells in EMEM medium/10% FBS. After establishment on the fixed bed, the medium was changed to Freestyle 293/10% FBS and culture parameters were controlled by BioFlo320 (Eppendorf). Supernatant was collected every 3 to 4 days, 0.2 μm filtered, and snap-frozen until purification.

Purification of FVIII-SQ From HepG2 Cells

All purification steps were performed on ÄKTA avant chromatography system and UNICORN 6.4 software (GE Healthcare). Medium was thawed in a 37°C water bath and placed on ice for ultrafiltration/diafiltration (UF/DF) using a Vivaflow 200 30kDa MWCO PES crossflow cassette (Sartorius). Medium was 3X concentrated and buffer-exchanged with Buffer A (30 mM Tris, 20 mM L-Histidine, 20 mM $CaCl_2$, 30 mM NaCl, and 0.02% Tween 80 at pH 7.0 and conductivity 9.1 to 9.3 mS/cm). UF/DF medium was 0.2 μm filtered and cation exchange chromatography was performed using SP Sepharose Fast Flow resin (GE Healthcare) equilibrated in Buffer A. After loading medium, the SP resin was washed with Buffer A, followed by elution of FVIII-SQ with Buffer B (30 mM Tris, 20 mM L-Histidine, 20 mM $CaCl_2$, 1.5 M NaCl, and 0.02 % Tween 80 at pH 7.0 and conductivity 115 to 130 mS/cm). UF/DF was performed on the eluted fractions containing active FVIII-SQ using a Vivaflow 50 30kDa MWCO PES crossflow cassette (Sartorius). The elution was 2X concentrated and buffer-exchanged with Buffer C (10 mM L-Histidine, 20 mM $CaCl_2$, 300 mM NaCl, and 0.02% Tween 80 at pH 7.0). Affinity chromatography was performed using VIII Select agarose resin (GE Healthcare) equilibrated in Buffer C. After loading, the VIII Select resin was washed with Buffer D (20 mM L-Histidine, 20 mM $CaCl_2$, 300 mM NaCl, and 0.02% Tween 80 at pH 6.5), then Buffer E (20 mM L-Histidine, 20 mM $CaCl_2$, 1.0

M NaCl, and 0.02% Tween 80 at pH 6.5), followed by elution of FVIII-SQ with Buffer F (20 mM L-Histidine, 20 mM $CaCl_2$, 1.5 M NaCl, 0.02% Tween 80, and 50% ethylene glycol at pH 6.5). UF/DF was performed with the eluted fractions containing active FVIII-SQ using Vivaspin 15 30kDa MWCO PES centrifugation membrane. The purified FVIII-SQ material was concentrated, buffer-exchanged with Buffer G (9.67 mM L-Histidine, 1.7 mM $CaCl_2$, 308 mM NaCl, 8.74 mM Sucrose, and 0.01% Tween 80 at pH 7.0), 0.2 μm filtered, and snap-frozen.

SUPPLEMENTARY REFERENCES

1. McIntosh J, Lenting PJ, Rosales C, Lee D, Rabbanian S, Raj D, Patel N, Tuddenham EG, Christophe OD, McVey JH, Waddington S, Nienhuis AW, Gray JT, Fagone P, Mingozzi F, Zhou SZ, High KA, Cancio M, Ng CY, Zhou J, Morton CL, Davidoff AM, Nathwani AC. 2013. Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. Blood 121:3335-44.
2. Choi JH, Yu NK, Baek GC, Bakes J, Seo D, Nam HJ, Baek SH, Lim CS, Lee YS, Kaang BK. 2014. Optimization of AAV expression cassettes to improve packaging capacity and transgene expression in neurons. Mol Brain 7:17.
3. Peyvandi F, Garagiola I, Baronciani L. 2011. Role of von Willebrand factor in the haemostasis. Blood Transfus 9 Suppl 2:s3-8.
4. Buyue Y, Liu T, Kulman JD, Toby GG, Kamphaus GD, Patarroyo-White S, Lu Q, Reidy TJ, Mei B, Jiang H, Pierce GF, Sommer JM, Peters RT. 2014. A single chain variant of factor VIII Fc fusion protein retains normal in vivo efficacy but exhibits altered in vitro activity. PLoS One 9:e113600.
5. Butenas S, Parhami-Seren B, Mann KG. 2009. The influence of von Willebrand factor on factor VIII activity measurements. J Thromb Haemost 7:132-7.
6. Rosen P, Rosen S, Ezban M, Persson E. 2016. Overestimation of N-glycoPEGylated factor IX activity in a one-stage factor IX clotting assay owing to silica-mediated premature conversion to activated factor IX. J Thromb Haemost 14:1420-7.
7. Gu JM, Ramsey P, Evans V, Tang L, Apeler H, Leong L, Murphy JE, Laux V, Myles T. 2014. Evaluation of the activated partial thromboplastin time assay for clinical monitoring of PEGylated recombinant factor VIII (BAY 94-9027) for haemophilia A. Haemophilia 20:593-600.
8. Pfizer/Wyeth-ReFacto. Annex I - Summary of Product Characteristics for ReFacto AF 250 IU powder and solvent for solution for injection.
9. Peyvandi F, Oldenburg J, Friedman KD. 2016. A critical appraisal of one-stage and chromogenic assays of factor VIII activity. J Thromb Haemost 14:248-61.
10. Morfini M, Cinotti S, Bellatreccia A, Paladino E, Gringeri A, Mannucci PM, ReFacto ASG. 2003. A multicenter pharmacokinetic study of the B-domain deleted recombinant factor VIII concentrate using different assays and standards. J Thromb Haemost 1:2283-9.
11. Hubbard AR, Hamill M, Beeharry M, Bevan SA, Heath AB, IX SSCS-CoFVF, von Willebrand factor of I. 2011. Value assignment of the WHO 6th International Standard for blood coagulation factor VIII and von Willebrand factor in plasma (07/316). J Thromb Haemost 9:2100-2.