Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Supplementary Appendix

Gene Therapy for Hemophilia B with a High Specific Activity Factor IX Variant

George LA, Sullivan SK, Giermasz A, Rasko JEJ, Samelson-Jones BJ, Ducore J, Cuker A, Sullivan LM, Majumdar S, Teitel J, McGuinn CE, Ragni MV, Luk AY, Hui D, Wright JF, Chen Y, Liu Y, Wachtel K, Winters A, Tiefenbacher S, Arruda VR, van der Loo J, Zelenaia O, Takefman D, Carr ME, Couto LB, Anguela XM, High KA

Clinical Investigators

George LA, Sullivan SK, Giermasz A, Rasko JEJ, Samelson-Jones BJ, Ducore J, Cuker A, Sullivan LM, Majumdar S, Teitel J, McGuinn CE and Ragni MV.

Supplementary Methods

AAV neutralizing antibody assay

A screen of hemophilia B (HB) participant sera for titers of neutralizing antibodies (NAbs) against the AAV-Spark100 capsid was performed using a cell-based assay.[1] This assay utilizes an AAV vector with the same capsid expressing a reporter gene encoding luciferase. Human Embryo Kidney 293 (HEK-293) cells were transduced with an AAV-luciferase vector alone (MOI ranging from 100-500) or with vector that had been mixed with participant serum samples in a range of dilutions. Following approximately twenty-four hours of incubation, luciferase expression was measured using a luminometer. The lowest dilution at which the luciferase expression was inhibited by ≥ 50% was reported as the sample NAb titer.

Vector Production and Titration

SPK-9001 vector was prepared under current good manufacturing practices. Vector was manufactured using helper virus-free transient transfection of HEK293 cells with plasmids (Aldevron, Minneapolis, MN) encoding the genes required for vector amplification and packaging. Vector particles are released from harvested cells by microfluidization and subjected to 0.2 μm filtration in order to separate the vector from cellular debris. The vector is further purified by cation exchange chromatography followed by density gradient centrifugation. The

vector product is then diafiltered by tangential flow filtration to achieve highly purified vector particles in a neutral buffered, isotonic formulation. This formulation contains a small amount (0.001%) of surfactant to prevent sticking of vector particles to plastic surfaces during storage and preparation prior to administration. The bulk product undergoes final sterilization by 0.2 μm filtration before final vial filling and labeling.[2,3] Lots are tested for identity, osmolality, potency, purity, pH, sterility, and appearance. SPK-9001 is formulated in sterile water containing 180 mM sodium chloride, 10 mM sodium phosphate, 0.001% Kolliphor P188 (also known as Lutrol F68, Pluronic F68, and Poloxamer 188) at pH 7.3. The purification process results in efficient separation of vector and empty capsids due to the inclusion of an isopycnic gradient ultracentrifugation step. From this gradient step, empty capsid particles were separately collected and similarly formulated in sterile water containing 180 mM sodium chloride, 10 mM sodium phosphate, 0.001% Kolliphor P188 at pH 7.3. The empty capsids were tested using a series of quality control methods to assure safety and purity, and their concentration determined by spectrophotometry as previously described.[4] Vector titer was determined as described in Wright *et al.* modified using SPK-9001 specific qPCR primers and probes.[5]

SPK-9001 vector and capsid sequence

SPK-9001 expression cassette:

AGGCTCAGAGGCACACAGGAGTTTCTGGGCTCACCCTGCCCCCTTCCAACCCCTCAG
TTCCCATCCTCCAGCAGCTGTTTGTGTGCTGCCTCTGAAGTCCACACTGAACAAACTT
CAGCCTACTCATGTCCCTAAAATGGGCAAACATTGCAAGCAGCAAACAGCAAACAC
ACAGCCCTCCCTGCCTGCTGACCTTGGAGCTGGGGCAGAGGTCAGAGACCTCTCTGG
GCCCATGCCACCTCCAACATCCACTCGACCCCTTGGAATTTCGGTGGAGAGGAGCAG
AGGTTGTCCTGGCGTGGTTTAGGTAGTGTGAGAGGGGTACCCGGGGATCTTGCTACC
AGTGGAACAGCCACTAAGGATTCTGCAGTGAGAGCAGAGGGCCAGCTAAGTGGTAC
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GGTTTCTGAGCCAGGTACAATGACTCCTTTCGGTAAGTGCAGTGGAAGCTGTACACT

GCCCAGGCAAAGCGTCCGGGCAGCGTAGGCGGGCGACTCAGATCCCAGCCAGTGGA
CTTAGCCCCTGTTTGCTCCTCCGATAACTGGGGTGACCTTGGTTAATATTCACCAGCA
GCCTCCCCCGTTGCCCCTCTGGATCCACTGCTTAAATACGGACGAGGACAGGGCCCT
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AGCACCCAGGTTGGTAAGTACTGGTTCTTTGTTAGCTAGGTTTTCTTCTTCTTCACTT
TTAAAACTAAATAGATGGACAATGCTTATGATGCAATAAGGTTTAATAAACACTGTT
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ACAGTGGCCTTATTTACACAAAAAGTCTGATTTTAAGATATGTGACAATTCAAGGTT
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AAGCTTTTCATGGATTAGGAAAAAATCATTTTGTCTCTATCTCAAACATCTTGGAGTT
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TCCCATTTGGACAAACAGCATGTTCTCACAGTAAGCACTTATCACACTTACTTGTCA
ACTTTCTAGAATCAAATCTAGTAGCTGACAGTACCAGGATCAGGGGTGCCAACCCTA
AGCACCCCCAGAAAGCTGACTGGCCCTGTGGTTCCCACTCCAGACATGATGTCAGCT
GTGAAATCCACCTCCCTGGACCATAATTAGGCTTCTGTTCTTCAGGAGACATTTGTTC
AAAGTCATTTGGGCAACCATATTCTGAAAACAGCCCAGCCAGGGTGATGGATCACTT
TGCAAAGATCCTCAATGAGCTATTTTCAAGTGATGACAAAGTGTGAAGTTAAGGGCT
CATTTGAGAACTTTCTTTTTCATCCAAAGTAAATTCAAATATGATTAGAAATCTGACC
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GCAGTATTTGAGGGTGCCTCTGGTGGATAGAGCAACCTGCTTGCTGAGCACCAAGTT
TACAATCTACAACAATATGTTCTGTGCAGGGTTCCATGAAGGTGGTAGAGACAGCTG
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CTGGGATTATCAGCTGGGGTGAGGAGTGTGCTATGAAGGGAAAGTATGGGATCTAC
ACAAAAGTATCCAGATATGTGAACTGGATTAAGGAGAAAACCAAGCTGACTTGATG
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CTGAATTCCTGCAGCCAGGGGGATCAGCCTCTACTGTGCCTTCTAGTTGCCAGCCAT
CTGTTGTTTGCCCCTCCCCCTTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGT
CCTTTCCTAATAAAATGAGGAAATTGCATCACATTGTCTGAGTAGGTGTCATTCTATT
CTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCA
GGCATGCTGGGGATGCAGTGGGCTCTATGG

Spark100 capsid sequence:

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLGPF
NGLDKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGN
LGRAVFQAKKRVLEPLGLVESPVKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRL
NFGQTGDSESVPDPQPIGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGVGSSSGNW
HCDSTWLGDRVITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDF
NRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFT
DSEYQLPYVLGSAHQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQML
RTGNNFEFSYNFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFS
QAGPNNMSAQAKNWLPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNP
GVAMATHKDDEERFFPSSGVLMFGKQGAGKDNVDYSSVMLTSEEEIKTTNPVATEQYG
VVADNLQQQNAAPIVGAVNSQGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLM
GGFGLKHPPPQILIKNTPVPADPPTTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRW
NPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPIGTRYLTRNL

Vector preparation prior to infusion

The SPK-9001 investigational vector was combined in defined ratios with empty capsids. The empty capsid serves to adsorb circulating capsid antibodies, which can clear AAV vector particularly when vector is administered at low doses.[6] Using aseptic technique, a volume of human serum albumin (25%) is added to 250 mL normal saline, to achieve a final albumin

concentration of 0.25%, and gently mixed. Using a sterile syringe, an appropriate volume of vector was added to the intravenous infusion bag, and gently mixed. The intravenous infusion bag is connected to an intravenous catheter for administration to the patient over the course of one hour.

Cellular Immunology Studies

Peripheral blood mononuclear cell (PBMCs) responses to the AAV capsid and FIX-R338L expressed protein were measured using IFN-γ ELISpot as previously described.[1,7,8] Each condition is tested in triplicate unless otherwise specified. Participant cryopreserved PBMCs are adjusted to a concentration of $2x10^6$ cells/mL in AIM-V 3% HI-FBS culture medium. A volume of 100 µL of the cell suspension is added to the wells containing antigens and mitogens.

PBMC reactivity was tested against the following antigens and controls: AAV capsid, the FIX-R338L mutation, wild type FIX, the FIX-A148T polymorphism and lambda phage sequences present in the transgene plasmid. Each protein tested is screened using a peptide library of 15-mers overlapping by 10 amino acids that span the entire protein for a total of 146, 3, 91, 4, 151 peptides, respectively (Eunoia Biotech). Individual peptides are suspended in 50% acetonitrile 0.1% acetic acid to a final stock concentration of 5mg/mL. AAV peptides are organized in 6 pools (AAV-P1-6), wild-type FIX is organized into 3 pools (FIX-P1-P3), FIX-R338L is a single pool, each form of the polymorphic FIX residue is a single pool and Lambda is organized into 6 pools (Lambda P1-P6). Each pool is tested at a concentration of ~5µg/ml per peptide. Lymphocyte culture medium was used as negative control, and a mixture of PMA (0.05 µg/mL)

and ionomycin (1 μg/mL) was used as a positive control. For each plate, a reference quality control (QC) PBMC sample was tested against both HCMVGr2 antigen and CEF, a pool of 32 epitopes from CMV, EBV and flu viruses binding to several common HLA alleles (Cellular Technology Limited). The reference QC sample was required to meet previously determined acceptance criteria for both of these antigens in order for results to be considered valid.

Enrollment Criteria:
Inclusion:

1. Be able to understand the purpose and risks of the study and provide signed and dated informed consent and authorization to use protected health information (PHI) in accordance with national and local privacy regulations;

2. Be male and ≥18 years of age;

3. Have hemophilia B with ≤2 IU/dL (≤2%) endogenous FIX activity levels as documented from the central laboratory at the time of screening. If the screening result is >2% due to insufficient washout from the FIX product, then the severity of hemophilia B may be confirmed by documented historical evidence from a certified clinical laboratory demonstrating ≤2% FIX coagulant activity (FIX:C) or from a documented genotype known to produce clinically severe phenotype of hemophilia B;

4. Have had ≥50 prior exposure days (EDs) to any recombinant and/or plasma-derived FIX protein products based on historical data from participant's records/history;

5. a. Prophylaxis participants: Have had bleeding events and/or infusion with FIX products during the last 12 weeks, as documented in the participants' medical records; or b. On-demand participants: Have had ≥4 bleeding events in the last 52 weeks and/ or chronic hemophilic arthropathy (pain, joint destruction and loss of range of motion) in one or more joints;

6. Have no prior history of hypersensitivity or anaphylaxis associated with any FIX

or IV immunoglobulin administration;

7. Have no measurable FIX inhibitor as assessed by the central laboratory; or documented no prior history of FIX inhibitor after 50 EDs (family history of inhibitors will not exclude the participant) and no clinical signs or symptoms of decreased response to FIX administration;

8. Have acceptable laboratory values sampled at screening and reviewed prior to Day 0:

   - Hemoglobin ≥11 g/dL;

   - Platelets ≥100,000 cells/μL;

   - AST, ALT, alkaline phosphatase ≤2x ULN;

   - Bilirubin ≤3x ULN*; [*Bilirubin levels above the laboratory's normal range are acceptable in individuals with a documented history or laboratory evidence of Gilbert's Disease.]

   - Creatinine ≤2.0 mg/dL.

9. Agree to use reliable barrier contraception until three consecutive semen samples after the administration of SPK-9001 are negative for vector sequences.

Exclusion:

1. Have active hepatitis B or C, and HBsAg, HBc, HBV-DNA positivity or HCV-RNA viral load positivity, respectively. Negative viral assays in two samples, collected at least six months apart, will be required to be considered negative. Both natural clearers and those who have cleared HCV on antiviral therapy are eligible;

2. Currently on antiviral therapy for hepatitis B or C;

3. Have significant underlying liver disease, as defined by pre-existing diagnosis of portal hypertension, splenomegaly, encephalopathy, reduction below normal limits of serum albumin, or evidence of significant liver fibrosis (fibrosis stage ≥ 3) within the past 6 months prior to or at screening as determined by any of the following diagnostic modalities: Metavir ≥ 3 by liver biopsy, FibroScan score >8.3 kPa, FibroTest/FibroSURE >0.48 or AST-to-Platelet Ratio Index (APRI) >1;

4. Have serological evidence of HIV-1 or HIV-2 with CD4 counts ≤200/mm^3. Participant who are HIV-positive and stable, with an adequate CD4 count (>200/mm^3) and undetectable viral load (<50 gc/mL) measured twice in the six months prior to enrollment, on an antiretroviral drug regimen are eligible to enroll;

5. Have anti-AAV-Spark100 neutralizing antibody titers ≥1:5;

6. Have history of chronic infection or other chronic disease that the Investigators consider to constitute an unacceptable risk;

7. Have participated in a previous gene therapy research trial within the last 52 weeks or in a clinical study with an investigational drug within the last 12 weeks;

8. Any concurrent clinically significant major disease, or any other unspecified reasons that, in the opinion of the Investigator, makes the participant unsuitable for participation in the study;

9. Unable or unwilling to comply with the schedule of visits and study assessments described in the clinical protocol.

Target joint definition:

The definition of target joint is outlined within the study protocol section 9.5.1 of protocol version 5.1 (available online). A target joint is defined as a major joint (e.g. hip, elbow, wrist, shoulder, knee, and ankle) into which repeated bleeding occurs (frequency of 3 or more bleeding events into the same joint in consecutive 3-month period) and/or with symptoms of pre-existing joint involvement (e.g. synovitis, persistent swelling, effusion, limitation of range of motion).

Contributions:

LAG, AYL, MEC and KAH designed the study. XA and KAH designed the investigational vector. FJW, JvdL and OZ produced the investigational vector. LAG, SKS, AG, JEJR, BJSJ, JD, AC, LMS, SM, DH, YC, YL and AW gathered the data. LAG, SKS, AG, JEJR, BJSJ, JD, AC, LMS, SM, JT, CM, MVR, AYL, DH, JFW, YC, YL, KW, AW, ST, VRA, DT, MEC, LBC, XA and KAH analyzed the data. LAG, SKS, AG, JEJR, BJSJ, JD, AC, LMS, SM, JT, CM, MVR, AYL, DH, JFW, YC, YL, KW, AW, ST, VRA, DT, MEC, LBC, XA and KAH vouch for the data and the analysis. LAG, XA and KAH wrote the paper and all authors provided critical content revisions. LAG wrote the first draft of the manuscript. All co-authors decided to publish the paper. Clinical investigators (LAG, SKS, AG, JEJR, BJSJ, JD, AC, LMS, SM) signed a confidentiality agreement indicating trial results could be published irrespective of study outcome, but they could not discuss results before data were made public by presentations at a scientific meetings.

The trial was conducted under an FDA IND with the guidance of an independent data safety monitoring board consisting of David Lillicrap MD (chair), Mark Skinner JD, Daniel Solomon PhD, Jeanne Lusher MD

Supplementary Results

Study Participants

One participant withdrew consent for family planning reasons. A second participant who met all inclusion criteria, but shared an HLA haplotype implicated in an immune response experienced by participant 7, was deferred pending demonstration that the immune response could be controlled by a tapering course of steroids.

Participant 3 bleeding events

At baseline, participant 3 had significant underlying hemophilic arthropathy. He self-reported 4 bleeding events into 2 target joints (left ankle- 1 bleed, right knee- 3 bleeds). Prior to study enrollment, recurrent orthopedic evaluation had recommended a right knee replacement that was the site of 3 of his bleeding events. At the time of self-reported hemarthrosis, participant 3's FIX:C was either approximately 30% or, in 2 of 4 cases, estimated to be in the normal range at FIX:C >50% due to the use of exogenous factor IX (EHL-FIX) (outlined in Supplementary Table 2).

Prednisone course of participants 7 and 9

Participant 7 received oral steroids for a total of 130 days. Prednisone 60mg daily was initiated on day 64 post vector and was maintained at this dose through day 77 post vector. Thereafter, participant 7 was gradually tapered off prednisone as follows: 40mg daily day 78-99, 30mg daily

day 100- 112, 20 mg daily day 113-126, 15mg daily day 127-135, 10mg daily day 136-150, 7.5mg daily day 151-161, 5mg daily day 162-171, 2.5mg daily day 172-183, 2.5mg every other day 184-194 post vector.

Participant 9 received oral steroids for a total of 119 days. Prednisone 60mg was initiated on day 34 and maintained at this dose through day 49 post vector. Thereafter, participant 9 was gradually tapered off steroids as follows: 40mg daily day 50-61, 30mg daily 62-78, 20mg daily day 79-90, 15mg daily day 91-106, 10mg daily day 107-124, 5mg daily day 125-140, 2.5 mg daily day 141-153 post vector.

Figure S1:

Decreasing vector copy number per microgram of DNA in participant peripheral blood mononuclear cells (PBMCs) over time following vector administration. Decrease in vector copies over time is consistent with loss of episomal vector genomes.

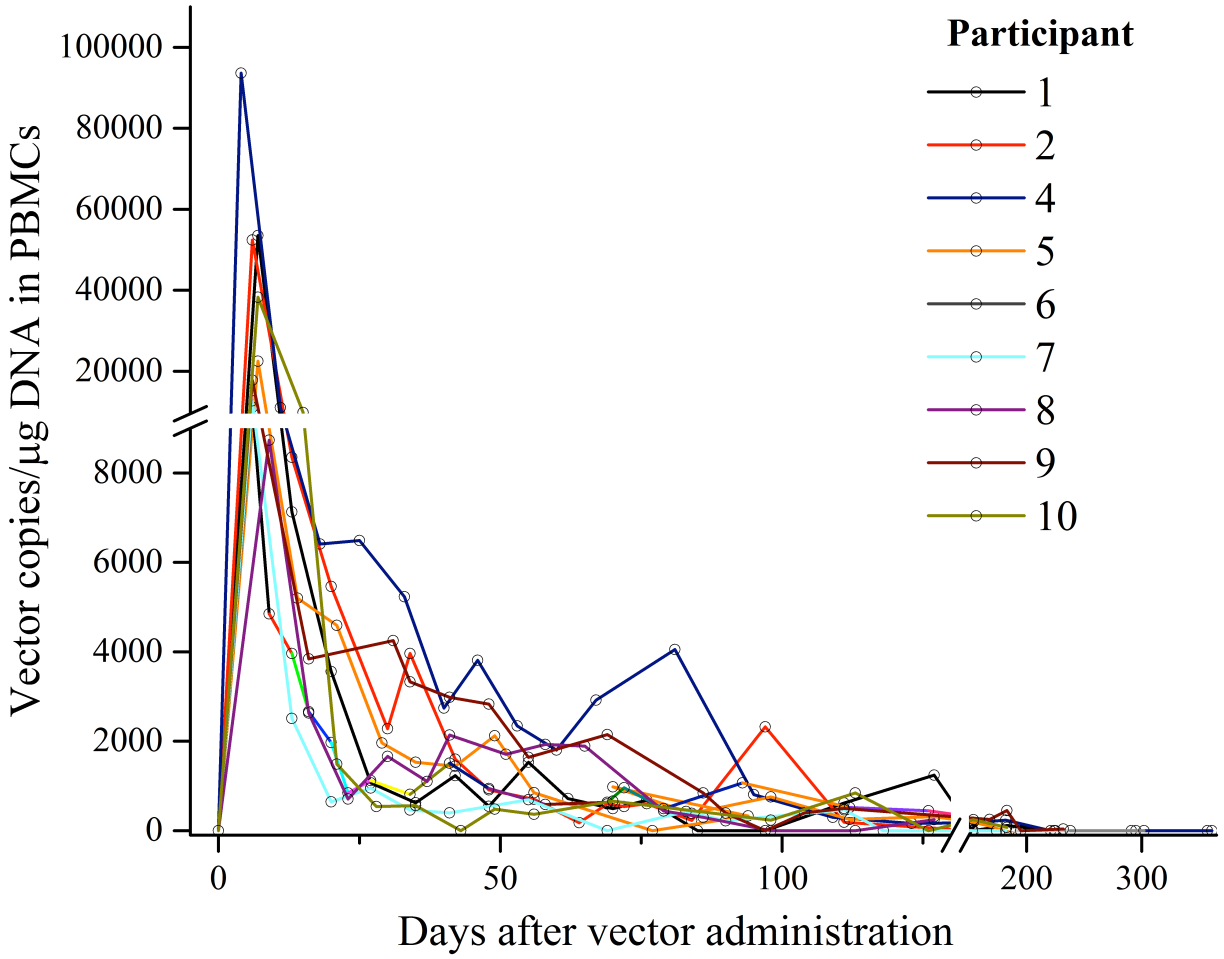

Figure S2

The laboratory course of the participants who did not have an immune response to the adeno-associated virus (AAV) vector. Longitudinal values for factor IX activity (FIX:C), alanine aminotransferase (ALT), aspartate aminotransferase (ALT), and interferon-γ enzyme-linked immunosorbent spot (ELISPOT) assay evaluation of participant peripheral blood mononuclear cells (PBMCs) to AAV capsid peptides and the transgene (FIX-R338L). ELISPOT results are shown as number of spot forming units (SFU) per 1 million PBMCs wherein values >50 SFU or 3-fold above the media control (dotted line) are considered positive. Panels A-G outline data for participants 1-6, 8 and 10, respectively.

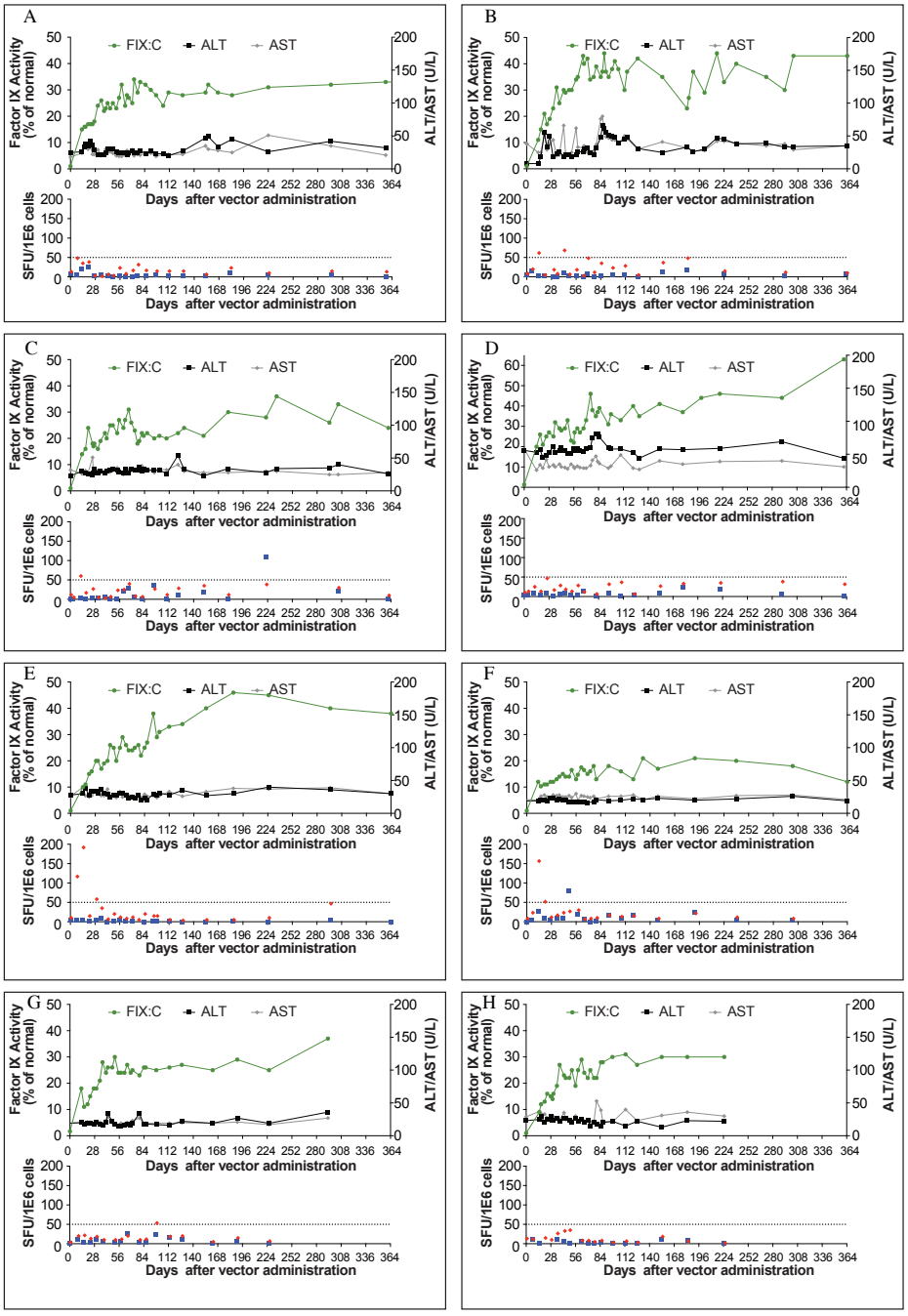

Figure S3:

Comparison of participant factor IX activity (FIX:C) determined by chromogenic assay versus one-stage assay. Factor IX activity was concurrently determined by both assays using participant plasma. One-stage FIX:C and chromogenic FIX:C were determined using a silica based aPTT reagent (TriniCLOT^{TM} aPTT S; TCoag, Ireland) and a commercially available chromogenic kit (ROX Factor IX; Rossix AB, Sweden), respectively. Seven distinct participant plasma samples over a range of FIX:C demonstrated a linear relationship. The dotted line is linear fit (y=0.5789x-0.6729, $r^{2=}$ 0.99).

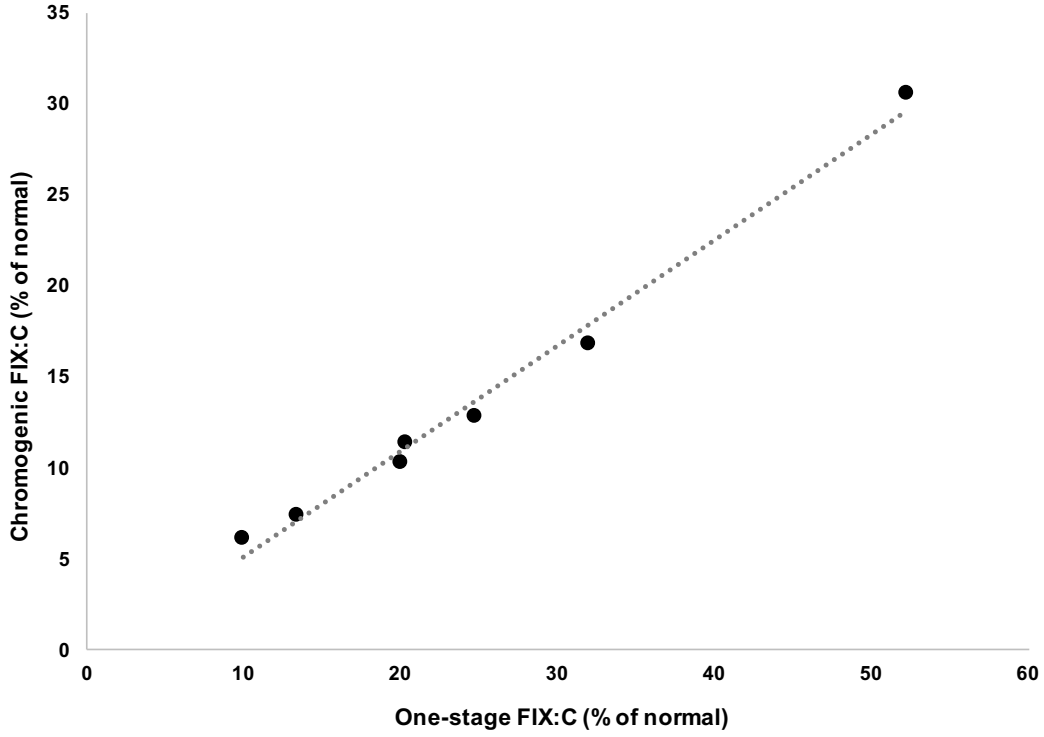

Table S1

Duration (weeks) until two consecutive negative results for vector shedding. Evaluation for vector DNA is performed in participant semen, urine, saliva, serum and peripheral blood mononuclear cells (PBMCs) by polymerase chain reaction. P indicates pending.

Supplementary Table 1. Participant time (weeks) to two connesecutive negative vector shedding samples										
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8	Subject 9	Subject 10
Semen	5	5	12	8	12	5	16	4	8	12
Urine	2	8	3	2	3	2	2	2	2	3
Saliva	6	4	6	5	5	5	4	4	5	4
Serum	26	6	18	14	10	8	5	4	14	5
PBMC	42	26	32	42	32	32	22	P	P	P

Table S2

Outline of participant 3's self-reported bleeding events relative to factor IX activity (FIX:C). Participant 3 reported 4 bleeding events following vector administration wherein FIX:C was either in the mild range (approximately 30% of normal) or estimated to be within normal limits (FIX:C >50% of normal) due to administration of exogenous extended half-life factor IX (EHL-FIX) product. Estimated (*) FIX:C is based on known participant 3 recovery and survival studies (1 IU/kg of EHL-FIX raised FIX:C 1.1%, terminal half-life 80 hours).

Supplementary table 2.Outline of participant 3's self-reported bleeding events relative to factor IX activity (FIX:C)

Site of bleed	Days post vector administration	Most recent FIX:C (days post vector FIX:C measured)	Dose of exogenous factor in IU/kg (days post vector administered)	Estimated FIX:C (source of FIX:C)
left ankle	2	68% (1)	100 (0)	>50% (EHL-FIX product)
right knee	245	36% (235)	N/A	36% (transgene)
right knee	265	36% (235)	N/A	36% (transgene)
right knee	271	36% (235)	50 (265) 50 (267)	>50% (transgene and EHL-FIX product)

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