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Near Real-time Immuno-optical Sensor for Diagnosing Single Point Mutation (A model System: Sensor for Factor V Leiden Diagnosis)

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Abstract

Factor V Leiden (FVL) is an abnormality of factor V (FV), a blood coagulation factor. It is a hereditary blood coagulation disorder with a high frequency (3–7% of general population). The most common type of FVL is caused by a single amino acid mutation and, therefore, its diagnosis is currently done only by DNA analysis, which takes a long time and is expensive. We have developed a rapid, accurate, and cost-effective, sandwich immuno-optical sensing method. To produce monoclonal antibodies against FV or FVL, having minimal cross-reactivity with the other molecule, a 20 amino acid sequence (20-mer) of FV or FVL at around the mutation site was utilized. The antibodies were screened first with the 20-mers and then with native FV or FVL molecules and they showed some cross reactivity. Using two antibodies having strongest affinity to either FV or FVL molecule, a FV and a FVL preferred sensors, were produced. After verifying that the levels of the antibody affinity to the two different molecules remained constant with changes in analyte concentration, a two-sensor system is developed to quantify FV and FVL in plasma samples. The system quantified the levels of FV and FVL at the maximum error of 0.5 µg/ml-plasma, in their physiological concentration range of 0–12 µg/ml-plasma. The levels of both molecules may provide us whether the patient has FVL or not but also the seriousness level of the disease (homozygous and different level of heterozygous).

Keywords

Factor V Leiden (FVL); Resistance to activated Protein C; Single point mutation; Thrombophilia; Hemostasis; Diagnosis

1. INTRODUCTION

In medical practices, accurate disease diagnoses and assessment of prognosis often relies on the information provided by sensors, i.e., detecting/quantifying disease representing molecules (biomarkers) in biosamples. Depending upon the nature of diseases, each sensor requires

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properties of the sensitivity, specificity, assay time, portability, user-friendliness, or assay cost. Biological samples usually contain many biomolecules that are structurally, chemically, physically, and/or functionally similar to the target biomarker. Therefore, sensing requiring high specificity utilizes mechanisms capturing potentially the targeted biomarker(s) only and one of most specific mechanisms is immuno-reaction. If a biomarker of interest has a high molecular-weight and is different from its homologue(s) in the sample only by one amino acid, then even an immuno-reaction may not be sufficiently specific. The disease diagnosis of single point mutation is, therefore, frequently done by DNA analysis, although it is expensive, time-consuming, and/or requires specially trained persons to perform the assay.

For a human to maintain normal physiological functions, it is crucial for blood to travel only inside blood vessels in an unobstructed manner (i.e., hemostasis). When an injury occurs, coagulation processes assist patching blood vessel walls, while the anticoagulant system ensures the blood fluidity within the vessels (Amiral and Fareed, 1996). Disturbances in hemostasis, either hemophilia or thrombophilia, can cause traumatic insults to the body. Nevertheless, thrombophilia may be more problematic since their symptoms are not often as obvious as hemophilia until life threatening incidents occur. Therefore, diagnosing thrombophilic disorders at an earliest stage is important to avoid serious health risks and to educate the affected individuals to avoid certain life styles potentially leading to risks.

Factor V Leiden [FVL; or resistance to activated Protein C (APC)] is the most common hereditary blood-coagulation disorder in the United States (5% of the Caucasian population and 1.2% of the African American population (Appleby and Olds, 1997; Makris, et al., 1997; Rao, et al., 1998; Haas, et al., 1998; Hull et al., 1986; Mattson and Crisan, 1998). The heterozygous form of FVL has been estimated to be 3–7% of Caucasians, while the homozygous state occurs in 1 in 5000 individuals (Rao, et al., 1998). This defect increases the risk of venous thrombosis 3–8 and 30–140 folds for the heterozygous and homozygous, respectively (Mattson and Crisan, 1998), and has been found in approximately 30% of patients who has shown thrombotic symptoms (Simantov, et al., 1996).

Factor V (FV) is an essential factor in blood coagulation cascade (Colman, et al, 1993) at a molecular weight of 330 kDa with a heavy chain (MW = 105 kDa) and a light chain (MW = 71–74 kDa). Activated FV (FVa) is required for the clot formation on the wall of blood vessels (Tans, et al., 1997; Hockin, et al., 1999). Within the vessel, however, FVa needs to be inactivated by APC, to maintain the fluidity of the blood. FVL is caused by the substitution of a single amino acid arginine at 506 in the heavy chain in by glutamine (Bertina, et al., 1994). Both FV and FVL molecules have the same coagulation activity but FVa loses nearly all of its coagulation activity following 5 minutes of incubation with APC, FVL a loses only 50%. Moreover, following 60 minutes, FVL a still retained 10–20% of its activity. This impaired down-regulation of FVL a promotes the increased risk of thrombosis (Kalafatis, et al., 1995).

Currently, FVL diagnosis is made by DNA analysis or, indirectly, by the clotting test that measures the degree of prolongation of plasma clotting time after the addition of APC (Tripodi, et al., 1997). DNA analysis is, however, expensive and time-consuming, and the clotting test may not be specific only for FVL. In addition, there has not been any study results showing the levels of FV and FVL in heterozygous person's plasma and the effect of the levels of FV/ FVL on the person's hemostasis, because the method for quantifying these molecules is currently not available.

Immuno, fiber-optic biosensing systems (Ligler, et al., 1993) have been used in detecting and quantifying various biomarkers in biosamples, including multi-anticoagulants and cardiac markers in plasma (Spiker and Kang, 1999; Kwon, et al., 2002 and 2003; Tang, et al., 2004; Tang and Kang, 2006; Hong, et al., 2008). It performs a sandwich assay on the surface of an

optical fiber. The assay is rapid (< 15 minutes), specific, accurate, sensitive, and relatively cost-effective. By combining methods of developing antibodies against single point sites and the dual immuno-fiber-optic biosensing system, a novel method for a rapid, user friendly biosensing system for FVL diagnosis has been developed.

2. MATERIALS & METHODS

2.1. Materials and Instruments

Biosensing—A fluorometer (Analyte 2000) and the quartz fibers used for sensors were from Research International (Monroe, WA). Purified FV and a monoclonal antibody against the light chain of FV molecule were purchased from Haematologic Tech. (Essex Junction, VT) and another monoclonal antibody against FV light chain was from Fitzgerald (Concord, MA). FV free plasma was from American Diagnostica Inc. (Stamford, CT). The homozygous patient plasma was obtained from a FVL homozygous patient, following the rules and regulations by the University of Louisville - Institutional Review Board. Fluorophore Alexa Fluor® 647 (AF647) was purchased from Invitrogen (Carlsbad, CA). ImmunoProbe™ Biotinylation Kits, avidin, hydrofluoric acid, phosphate buffered saline (PBS), triethylamin, γ -maleimideobutyric acid N-hydroxysuccinimide ester, (3-mercaptopropyl) – trimethoxysilane were from Sigma-Aldrich (St. Louis, MO).

Enzyme linked immunoassay—Bovine Serum Albumin (BSA) and *o*-phenylenediamine dihydrochloride (OPD) were from Sigma-Aldrich. Fc specific, horse raddish peroxidase conjugated rabbit anti-mouse IgG (HRP-IgG) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The ELISA plate was from Dow corning (NY) and the reader was from Bio-Rad (Hercules, CA).

2.2. Methods

Biosensing—All sensors were prepared following the protocol established by previous researchers (Spiker and Kang, 1999; Kwon, et al., 2002; Tang and Kang, 2006). Briefly, the immobilization of monoclonal antibody was done *via* the reaction of avidin and biotin (Savage, et al, 1992). The antibody against the 20 mers for FV or FVL (1° MAb) was immobilized on the fiber surface by the avidin-biotin linkage and then the fiber is enclosed in a sensing chamber, forming a sensor. Sensors can be re-used 3–6 times, with a short regeneration step after each assay (Kwon, et al., 2002).

For an assay, a liquid sample is injected into the chamber and the FV and/or FVL is captured by the 1° MAb. All liquids are applied with convection at a linear velocity of 1.2 cm/s, to facilitate faster molecular transport (Tang and Kang, 2004). After the antigen-antibody reaction is complete PBS buffer is applied to remove unbound bio-molecules. Then the fluorophore AF647 conjugated antibody against FV/FVL light chain (2° MAb) is applied and reacted, forming sandwich complex. Excitation light (635 nm) is applied to the sensor and the emitted fluorescence (667 nm) is measured by the fluorometer and the fluorescence intensity is correlated with the amount of FV/FVL in the sample.

Monoclonal Antibodies Against 20mers—Twenty amino acid sequences (20mers) of FVL and FV molecules at around the region of the mutation sites were generated by Peptide International (Louisville, KY). Generation of hybridoma cells against 20mers and production/purification of the monoclonal antibodies against 20mers were done by Iowa State University Hybridoma Facility, Iowa.

ELISA—To test the affinity of the antibodies generated, ELISA was performed as follows: 96 wells of an ELISA plate were incubated with 100 μ l of FV in plasma (2 μ g-FV/ml-FV free

plasma) or 100 μ l of homozygous FVL plasma (2 μ g/ml), overnight. The well was blocked with 250 μ l of 1% BSA for 90 minutes at room temperature, then 100 μ l of anti-FV antibodies (1 μ g/ml) was applied on the first column wells and a 1/2 serial-dilution was performed. After incubation at 37 °C for 90 minutes, 100 μ l of 1:1000 HRP-IgG was applied for 20 minutes at 37 °C. After washing the plate and adding 100 μ l of OPD solution to each well, the plate was incubated at room temperature for 30 minutes, and then optical density was measured at 450 nm.

3. RESULTS AND DISCUSSIONS

3.1. Production Monoclonal Antibodies against FV and FVL

Developing monoclonal antibodies against a particular amino acid site in a large bio-molecule is extremely difficult, if not impossible, because, in hybridoma generating process, there is very little control over selecting this small and particular site. This may be the main reason that neither pure FVL molecule, nor the antibody against FVL without cross-reacting with FV is currently available. To increase the probability of generating antibodies against the mutation site of FVL and the corresponding site of FV, a 20 amino acid sequence (20mer) of FV [H-I-C-K-S-R-S-L-D-R-**R**-G-I-Q-R-A-A-D-I-E-Q-NH₂] or FVL [H-I-C-K-S-R-S-L-D-R-**Q**-G-I-Q-R-A-A-D-I-E-Q-NH₂] with the mutation site (Jenny, et al., 1987; Ren, et al., 2008) at the center of the sequence was used for antibody generation. The 20mers were conjugated with a carrier protein to increase the immunogenicity. The conjugated molecules were then injected to mice and hybridoma cell lines were generated (Kwon, et al., 2003). The resulting hybridoma cell lines were then screened, by ELISA, using 20mers, to select the ones showing high affinity for the 20-mer of FVL with no affinity to the 20-mer of FV molecule, and *vice versa*. The cells selected were then cultivated and the antibodies produced were again screened with the 20-mers again. Then the screened antibodies were tested with homozygous FVL plasma (purified FVL is not available) and commercially available, purified FV molecules, by ELISA. The antibodies were, however, found to have some cross-reactivity with the other molecule (mutually exclusive). Therefore, the entire process of monoclonal antibody generation was repeated to generate the mutually exclusive antibodies. For the second attempts, again, the antibodies that we initially screened with 20-mers without cross reacting with the other 20-mers showed some cross reactivity with the other native molecule.

After realizing the level of difficulties in producing the desired antibodies, the approach for developing our sensor system was revised from having mutually exclusive, a FV and a FVL sensor system (Fig. 1a) to the one with two sensors with constant affinities for FV and FVL molecules. The logics was that, as long as the antibody immobilized on one sensor keeps the level of affinities to these two different molecules constant, with the change in the levels of FV and FVL in the sample, then the two readings from the two sensors for a sample can be a basis for quantifying their levels (Fig. 1b). The quantification may be done simply by solving two equations with two unknowns (levels of FV and FVL). Thus, we selected two antibodies for our sensing system: one with a higher affinity for FV (*FV preferred*) and the other with a higher affinity for FVL molecule and a lower affinity to FV (*FVL preferred*).

3.2. Development of FV Preferred and FVL Preferred Sensors

Three antibody candidates showing higher affinity to FVL and another three showing higher affinity to FV were selected. Each antibody was immobilized on an optical fiber and the affinity of the sensor to FVL or FV was studied using samples at an arbitrary chosen concentration, 5 μ g-FV or -FVL/ml-plasma. This study was necessary because antibodies tend to change their affinity to the antigen after they are immobilized on solid surface, possibly due to the change in their three dimensional configuration during the immobilization process. Among the six antibodies tested, the sensor with antibody 5G3 showed the highest relative

affinity for FVL, while the one with antibody 1D4 showed the highest for FV. These two were, therefore, selected to be the 1°MABs for the FVL and the FV preferred sensors, respectively.

As stated in the Method section, for the second antibody (2°MAB) conjugated with a fluorophore, we decided to use a commercially available antibody that was generated to specifically target the light chain of FV/FVL molecule. By this way, the epitope for 1°MAB can be far from that for 2°MAB (fluorophore-linked, anti-light chain in Fig. 2a), so that binding of the target molecule to 1°MAB minimally interferes that to 2°MAB and the 2°MAB reaction kinetics for both FVL and FV are the same or very similar, merely acting as the signal mediator.

Next, we have studied whether the sensor with the selected 1°MAB antibody has a constant affinity to FVL and FV molecules with the change in the levels of the other molecule in the sample. To adjust the concentration of FV in the plasma sample, predetermined amount of purified FV was added in FV-free plasma. For FVL plasma, first, the amount of FVL in the homozygous plasma was quantified by ELISA using two different antibodies with epitopes in the light chain of FV, with purified FV as the standard. To adjust the levels of FV and FVL in a plasma sample, appropriate amounts of FV plasma and FVL plasma were mixed.

Figure 2 (a) and (b) show the signal intensities of FVL and FV preferred sensors at various concentrations of only FV or FVL in the sample. The concentration range tested is 0–12 µg/ml-plasma, the usual range for FV/FVL in plasma. The reaction times for the samples and the 2° Mab were both 10 minutes, to provide sufficient reaction time. The length of the sensors was 3 cm. The reaction time and sensor length were based on our previous experiences with Protein C (the average normal concentration in plasma, 4 µg/ml) sensor development (Tang and Kang, 2005). For both FV and FVL preferred sensors, the relationship between the analyte concentration and the signal intensity was linear. For the FVL preferred sensor, the slopes for the factor levels vs. signal intensity were 8.6 for FVL and 5.4 for FV (Fig. 2a). For the FV preferred sensor, the slopes for FV and FVL were 9.3 and 6.8, respectively (Fig. 2b).

Next, the response of the sensors to the mixture of both molecules (i.e., samples with both FV and FVL in plasma), was studied, to see how the presence of FVL molecule affects the affinity of the FV preferred sensor for FV, and *vice versa* for the FVL preferred sensor. For this test, since there needs to be various ratio of the mixture of FV and FVL, we fixed the concentration of one type of molecule constant and varied that of the other. For instance, for testing FVL preferred sensor, the concentration of FV in the sample was set to be constant at 8 µg/ml-plasma (arbitrary chosen) and the concentration of FVL was varied between 1–12 µg/ml-plasma, and *vice versa* for FV preferred sensor. For the FVL preferred sensor (Fig. 2c), with FV at 8 µg/ml-plasma in the sample, the signal intensities of the mixture still showed a linear relationship with the changes in the FVL concentration. The slope slightly decreased from 8.6 to 8.4 (2%) from the one with only FVL in the sample. For all concentrations tested, the signals were very much in parallel to the one with only FVL, with an increase by 43 pA, the signal intensity generated by the sample with only FV at 8 µg/ml. This results shows that the signal intensity of the mixture is the addition of the signal generated by FV and that by FVL, and that FV in samples at various ratios does not significantly affect the affinity of the FVL sensor to FVL. Similarly, Fig. 2d confirms that FVL present in the sample at various ratios affect the affinity of FV preferred sensor to FV very little. There was a minor decrease in the slope (from 9.3 to 8.9, ~ 4%) and also, for all samples, the signal intensity increased by 54, the signal intensity by only FVL only at 8 µg/ml. In sum, within the physiologically meaningful range, it was found that the FV and the FVL molecules in a sample contribute the sensor signal intensity for both FVL and FV preferred sensors independently.

3.3. Quantification of FV and FVL in Blood Plasma Sample

Since our sensors may not sense FV and FVL molecules separately, to quantify them, a mathematical manipulation was needed. The results above showed that the relationships between the two sensor signals and the levels of FV and FVL in samples are linear, and the presence of one type of the molecule in a sample does not significantly change the sensor affinity to the other. A simple mathematical model representing the relationship between the signal intensity and the levels of FV and FVL in a sample can be then expressed as follows:

$$SI_1 = A_1 C_{FV} + B_1 C_{FVL} \quad (1)$$

$$SI_2 = A_2 C_{FV} + B_2 C_{FVL} \quad (2)$$

where, SI is the signal intensity (pA) generated by a sensor; A is the slope of standard curve for FV, in pA/(μg/ml); B is the slope of standard curve for FVL, in pA/(μg/ml); C is the concentrations of FV or FVL in the sample, in μg/ml; Subscripts 1 and 2 represent FV preferred and FVL preferred sensors, respectively. By rearranging the equations 1 and 2, the levels of FV (C_{FV}) and FVL (C_{FVL}) in a sample can be expressed as equations 3 and 4, respectively.

$$C_{FV} = \frac{B_2 SI_1 - B_1 SI_2}{B_2 A_1 - B_1 A_2} \quad (3)$$

$$C_{FVL} = \frac{A_2 SI_1 - A_1 SI_2}{A_2 B_1 - A_1 B_2} \quad (4)$$

The process of developing a sensing system for quantifying FV and FVL in a plasma sample may be summarized as follows: (1) The sensing system is composed of a FV preferred and a FVL preferred sensors, which were developed utilizing two types of monoclonal antibodies specifically developed against the mutation regions of FV and FVL; (2) For a constant sensor size with constant assay protocol, standard curves for purified FV or FVL in plasma samples were generated to obtain the slope values, A_1 , A_2 , B_1 , and B_2 ; (3) For an actual blood plasma sample assay, a plasma sample is applied to the dual sensor system and a set of sensing signal intensities (SI_1 , and SI_2) are obtained. With the signal intensities and the four slope values, the amount of FV and FVL in the plasma sample can be quantified using equations (3) and (4).

In our sensing system, we had developed a system consisting one sensor with higher affinity to FV and the other to FVL. It should, however, be noted that, as long as there are two sensors possessing relatively constant affinities to FV and FVL, with changes in the ratio of FV to FVL in the sample, the levels of FV or FVL in the sample can be quantified using the equations (3) and (4). In addition, even for the case of varying sensor affinity with the presence of the other molecule in the sample, as long as the change of affinity can be explicitly expressed one should be able to quantify FV and FVL, although a non-linear mathematical model needs to be established for this case.

3.4. Optimization of the Sensing Protocol

As previously stated, the study up to this point was performed using 3 cm sensors with the sample and 2° Mab reaction time 10 minutes each, allowing the sensing to be completed within

25 minutes. The plasma sample size for two 3 cm sensors requires less than 0.5 ml. Since the FV/FVL concentrations are mostly above the range of our anticoagulant sensing and the reaction time for the anticoagulants or fluorophore conjugated 2° Mab are much shorter than 10 minutes (Tang, et al., 2006), we felt that the reaction time (i.e., assay time) may be further reduced. For this type of diseases, real-time or near real-time diagnosis is not necessary but a faster assay time can reduce the expense involved in personnel. We have, therefore, performed a study to optimize (or minimize) the assay time. The study was performed first by varying the sample incubation time from 1–10 minutes with a constant 2°Mab incubation time of 10 minutes (Fig. 3a). Also, 2°Mab incubation was varied from 1 to 10 minutes after a constant sample incubation time of 10 minutes (Fig. 3b). For the sample incubation time (Fig. 3a) for both FV and FVL sensors, the reactions completed at around 3 minutes. For the 2°Mab reaction (Fig. 3b), for both sensors, the reaction took only 2 minutes to complete. Therefore, the total time of 5 minutes appeared to be sufficient for the two incubations, resulting in the total assay, including various washing steps, to be completed within 8 minutes.

3.5. Quantification of FV and FVL in Plasma Samples

With the finalized dual sensing system (3 cm FV and FVL preferred sensors and 8 minute assay time), its performance was tested for six samples at arbitrary selected ratios of FV and FVL. The tested ratios of FV/FVL were 10:2; 9:3; 6:6; 3:9; 2:10; and 0:12. The sample volume required for the assay was less than 0.5 ml and each sample was assayed at least three times. Table 1 displays the results of the measurements compared to the actual amount of FV/FVL in the samples, along with respective errors. For the six samples, the maximum absolute error was 0.5 µg/ml with the maximum standard deviation of 0.8 µg/ml. The error and standard deviations may be reduced more, as the system becomes automated. With this measurement accuracy, both homozygous and heterozygous FVL can be diagnosed in a much more cost effective manner in a near real-time, and without the need for DNA analysis.

The future studies include automation of sensor preparation including antibody immobilizations, converting the two sensors sensing system to a dual channel sensing chip, and automation of assay procedures so that the operator only need to inject the sample to a sample port to obtain two digital values for FV and FVL levels within 10 minutes.

4. CONCLUSIONS

The amino acid sequences of FV and FVL are different by only one amino acid. In order to increase probability for generating antibodies specific only to FV or only to FVL, 20-mers around the mutation site for each molecule were used for hybridoma generation. Two antibodies with higher affinity to FV and to FVL were selected as 1°Mab for the FV and the FVL preferred sensors, respectively. For a sample containing both FV and FVL, the total signal intensity was found to be the addition of the signals generated by FV and by FVL. The signals from these two sensors were used to quantify FV and FVL in a plasma sample accurately with the maximum error of 0.5 µg/ml. Also, the entire assay can be completed within 8 minutes.

Inherited thrombophilic diseases can lead individuals affected to various detrimental complications, which can cause tremendous burden to the individuals and the family and also to national health care system. Early diagnosis of these diseases can educate the affected individuals to prevent from having life threatening episodes. This instrument for FVL diagnosis is expected to be helpful for large populations who are not aware of their hidden disease status, at a cost effective manner.

It should be noted that this unit is also capable of diagnosing FV deficiency (hemophilia), with *no* further modification. The same principles may also be applied for developing diagnosis tools for other diseases with a single or a few point mutation(s).

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Fig. 1. Schematic diagrams displaying the principle of (a) mutually exclusive, a FVL and a FV sensors and (b) a FVL and a FV preferred sensors. For system (a), the FV molecule in the sample does not react with FVL sensor and *vice versa*. For system (b), FV molecule has some affinity to FVL preferred sensor but not as strong as to FV, and *vice versa* for FV preferred sensor. For both systems, the fluorophore linked 2°Mab binds to the light chain, away from the 1°Mab binding site (i.e., heavy chain).

Fig. 2. Standard curves for FVL and FV reacted with (a) FVL preferred and (b) FV preferred sensors. (c) Signal intensities of the FVL preferred sensor with the change in FVL concentration in the samples containing 8 µg/ml of FV, and (d) the FV preferred sensor with the change in FV concentration in the sample containing 8 µg/ml of FVL.

Fig. 3. Signal intensities of FVL and FV sensors, with changes in the incubation time of (a) sample and (b) 2°MAb.

Table 1
Quantification of FV and FVL in six samples by the dual sensing unit and comparison of experimental values with the actual amount of analyte

sample No.	analyte	actual conc. # (µg/ml)	measured conc. ## (µg/ml)	Mean error (µg/ml)	sample No.	analyte	actual conc. # (µg/ml)	measured conc. ## (µg/ml)	Mean error (µg/ml)
1	FV	10	9.6±0.2	-0.4	4	FV	3	3.5±0.3	0.5
	FVL	2	2.3±0.3	0.3		FVL	9	8.8±0.8	-0.2
	Total *	12	11.9±0.5	-0.1		Total *	12	12.3±0.5	0.3
2	FV	9	9.3±0.7	0.3	5	FV	2	1.8±0.5	-0.2
	FVL	3	3.0±0.1	0		FVL	10	10.2±0.8	0.2
	Total *	12	12.3±0.7	0.3		Total *	12	12.0±0.5	0
3	FV	6	6.1±0.1	0.1	6	FV	0	-0.5±0.3	-0.5
	FVL	6	5.7±0.3	-0.3		FVL	12	12.5±0.8	0.5
	Total *	12	11.7±0.1	-0.3		Total *	12	12.0±1.1	0

Adjusted levels of FV and FVL in samples, using purified FV, homozygous FVL plasma, and FV free plasma.

The FV and FV levels quantified by the dual sensing system.

* Total = FV + FVL