

# An Automated pre-Dilution Setup for Von Willebrand Factor Activity Assays

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## Abstract

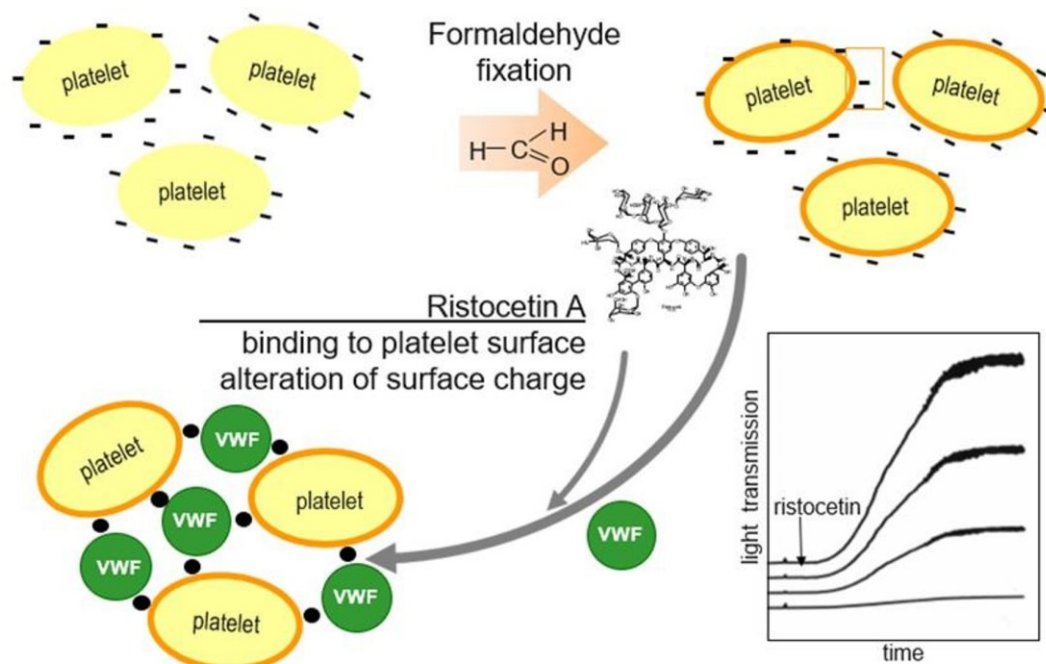
Accurate quantification of von Willebrand factor ristocetin cofactor activity (VWF:RCo) is critical for the diagnosis and classification of von Willebrand disease, the most common hereditary and acquired bleeding disorder in humans. Moreover, it is important to accurately assess the function of von Willebrand factor (VWF) concentrates within the pharmaceutical industry to provide consistent and high-quality biopharmaceuticals. Although the performance of VWF:RCo assay has been improved by using coagulation analyzers, which are specialized devices for blood and blood plasma samples, scientists still report a high degree of intra- and inter-assay variation in clinical laboratories. Moreover, high, manual sample dilutions are required for VWF:RCo determination of VWF concentrates within the pharmaceutical industry, which are a major source for assay imprecision. For the first time, we present a precise and accurate method to determine VWF:RCo, where all critical pipetting and mixing steps are automated. A pre-dilution setup was established on CyBio FeliX (Analytik-Jena) liquid handling system, and an adapted VWF:RCo method on BCS-XP analyzer (Siemens) is used. The automated pre-dilution method was executed on three different, most frequently used coagulation analyzers and compared to manual pre-dilutions performed by an experienced operator. Comparative sample testing revealed a similar assay precision (coefficient of variation = 5.9% automated, 3.1% manual pre-dilution) and no significant differences between the automated approach and manual dilutions of an expert in this method. While no outliers were generated with the automated procedure, the manual pre-dilution resulted in an error rate of 8.3%. Overall, this operator-independent protocol enables standardization and offers an efficient way of fully automating VWF activity assays, while maintaining the precision and accuracy of an expert analyst.

## Key features

- Automated pre-dilution setup for von Willebrand factor concentrates of various natures.
- Combination of a liquid handling system (CyBio FeliX) with a coagulation analyzer (BCS-XP).
- Simplifies method transfer to other laboratories.
- Basic training for CyBio FeliX and BCS-XP is required.

**Keywords:** Automation, Blood coagulation, Ristocetin cofactor, VWF:RCo, Von Willebrand disease, Von Willebrand factor

## Graphical overview



Sample dilution on CyBio FeliX



VWF:RCo measurement on BCS-XP

**VWF:RCo assay principle and measurement setup.** Platelets (yellow ellipsoids) with negative surface charge (- -) are treated with formaldehyde, which partly denatures the cell surface and thus stabilizes platelets for use as assay reagents. Stabilized platelets (dark-yellow-framed yellow ellipsoids) are then brought in contact with ristocetin A (chemical structure shown; black dots), which binds to the platelet surface and facilitates binding of VWF (green circles). The graphs show an example of quantitative determination of platelet agglutination by measurement of light transmission, where increasing amounts of VWF increase light transmission over time. The photo in the left-bottom corner shows the CyBio FeliX setup for VWF sample dilution and the photo in the right-bottom corner displays the BCS-XP system, which is used for VWF:RCo measurements.

## Background

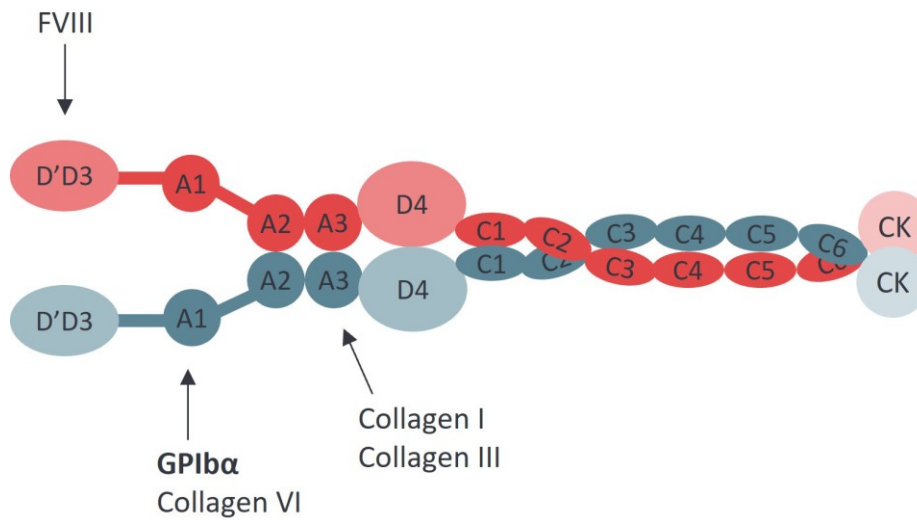
Von Willebrand factor (VWF) is the largest soluble plasma protein in all species with a blood clotting system; it has essential functions in blood coagulation, control of bleeding, and angiogenesis [1,2]. Shear stress in the bloodstream plays a crucial role in the functional characteristics of VWF. While, for example, high shear rates are needed for the binding of VWF to GPIIb $\alpha$ , the shear rate needs to be below a critical level for effective binding to coagulation factor

VIII (Figure 1) [3]. VWF plays an important role in the pathology of various diseases, including bacterial infections and cardiovascular conditions [4,5]. Another example where VWF is highly involved is in diseases resulting from inherited or immune-mediated deficiencies of ADAMTS13, a metalloprotease that serves as the natural regulator of VWF size and function through specific proteolysis [6,7]. Furthermore, most recently, an association between a VWF/ADAMTS13 imbalance and the prolonged presence of highly thrombogenic ultra-high-molecular-weight (UHMW) VWF multimers was identified in patients with severe COVID-19 [8,9]. As a consequence, the VWF:RCo assay is now widely performed for research and the differential diagnosis of diseases in which VWF and ADAMTS13 are involved in pathological mechanisms and/or serve as valid biomarkers guiding therapies. Deficiencies or defects of VWF result in von Willebrand disease (VWD), the most frequent inherited bleeding disorder with a prevalence of 1 in 1000 individuals. VWD is a heterogeneous disease with different genetic subtypes called type 1, type 2 (with subtypes 2A, 2B, 2M, and 2N), and type 3 [10,11].

Besides genetic analysis, differential diagnosis of VWD requires the determination of VWF activity by different functional assays, of which the von Willebrand factor Ristocetin cofactor activity (VWF:RCo) assay is the most important. Ristocetin A is an antibiotic effective against Gram-positive bacteria and mycobacteria. Its use was discontinued due to its association with thrombocytopenia. However, this effect is utilized to, at least partially, mimic primary hemostasis in vitro by facilitating the binding of VWF to GPIIb/IIIa, leading to platelet agglutination [12]. A recently published survey among expert laboratories revealed that the VWF:RCo assay is the most commonly established VWF assay worldwide [13]. It is needed for the diagnosis of all VWD subtypes except for type 2N. While the other subtypes are, in part, characterized by lower VWF:RCo levels, type 2N typically exhibits normal VWF:RCo. Instead, this subtype involves reduced binding to coagulation factor VIII [14]. The VWF:RCo assay is not only the standard functional assay for the diagnosis of VWD but is required for potency assignment to VWF concentrates within the pharmaceutical industry [15–17]. The European Pharmacopoeia defined this assay and international VWF standard samples to determine the VWF activity of VWF-containing drug products. The assigned activity is then used to specify the dosage of these biopharmaceuticals [18]. Besides its broad utilization, the assay requires technical expertise, is labor intensive, and has a high degree of inter- and intra-assay variation [15]. Recently issued international clinical guidelines call for further research improving the VWF:RCo assay, which “has greater variability, resulting in the potential for misdiagnosis and/or misclassification” [14].

The performance of the VWF activity assays has already been improved and simplified for operators by automation on dedicated coagulation analyzers [19,20]. These devices are commonly used worldwide to determine VWF activity [17,21–23]. In contrast to diagnostic plasma samples, plasma-derived VWF (e.g., Immunate® and Biostate®) and recombinant VWF (rVWF) products (Vonvendi® or Veyvondi®) are highly concentrated. To obtain the concentration range prescribed by the European Pharmacopoeia for the measurement of VWF:RCo, these biopharmaceuticals need to be diluted, which is mainly performed manually [18].

Here, we demonstrate an approach to determine the VWF:RCo of VWF concentrates, where all critical pipetting and mixing steps are automated. We have established a pre-dilution setup for VWF concentrates on a CyBio FeliX system (Analytik-Jena). This liquid handling system (LHS) is versatile, compact, and affordable, making it interesting not only for the pharmaceutical industry but also for academia. Despite its benchtop size of only 650 mm × 450 mm × 700 mm, it can be employed for a wide range of applications. For example, it is used for DNA purification, transformation of bacterial cells, and purification of Aβ peptides for the diagnosis of Alzheimer's disease [24–26]. The combination of the LHS and the adapted method on BCS-XP provides a precise and accurate determination of VWF activity in samples of various natures of VWF concentrates, and VWF-containing samples, without requiring specific training for the VWF:RCo assay. This ensures accurate diagnosis and potency assignment of VWF as a drug substance and drug product. Moreover, this approach offers an operator-independent method, which may also reduce errors compared to manual dilutions. With fewer errors, fewer measurements need to be repeated, resulting in reduced hands-on time for operators. Furthermore, fewer errors result in reduced reagent consumption, thereby increasing cost efficiency. In addition, implementation of the automated pre-dilution steps simplifies method transfer to other laboratories and lays the foundation for the full automation of various VWF assays.



**Figure 1. Schematic domain structure of von Willebrand factor according to Lenting et al. [1].** Binding domains of coagulation factor (FVIII), platelet glycoprotein Ib alpha (GPIIb), and different collagens are shown.

## Materials and reagents

### Reagents

1. Distilled water
2. Imidazole + 1% albumin buffer (Baxter AG Vienna, catalog number: 1501390)
3. BC von Willebrand reagent (Siemens, catalog number: OUBD37)

### Equipment

1. CyBio FeliX (Analytik-Jena, catalog number: OL5015-100-24)
2. BCS-XP (Siemens)
3. Adapter 24 tubes, passive cooling function (Analytik-Jena, catalog number: 844-00136-0)
4. Waste box (Analytik-Jena, catalog number: 844-00430-0)
5. TipRack 96/1,000  $\mu$ L (Analytik-Jena, catalog number: OL3317-11-140)
6. CyBio Robo tip tray 96/1,000  $\mu$ L (Analytik-Jena, catalog number: OL3810-25-871)
7. 12-channel adapter (Analytik-Jena, catalog number: OL3316-14-340)
8. Support; 37 mm height (Analytik-Jena, catalog number: OL3317-11-120)
9. Axygen 8-row v-bottom high-profile reservoir (VWF, catalog number: 47743-966)
10. Protective cap (Analytik-Jena, catalog number: OL3316-11-200)
11. 96-deep-well plate (VWR, catalog number: 736-0607)
12. 5 mL glass flasks (Siemens, catalog number: 10873438)
13. Magnetic stirrer (Siemens, catalog number: 10642244)
14. Pipettes and tips
15. 1.5 mL tubes

## Software and datasets

1. CyBio FeliX script (shown in Supplementary information 1)
2. BCS-XP tests (shown in Supplementary information 2)
3. Microsoft Excel

## Procedure

The protocol consists of two major parts: the automated dilution using the liquid handling system CyBio FeliX and the VWF:RCo assay on the BCS-XP. The CyBio FeliX system dilutes the VWF samples to 1 IU/mL VWF:RCo. The diluted samples are then transferred manually to the BCS-XP, where the automated VWF:RCo assay is executed, and the agglutination of the platelets in the reagent is measured. Data analysis is performed in Microsoft Excel. For a better understanding of the general functioning of the CyBio FeliX and BCS-XP and how to work with these devices, example videos (not specific to this protocol) are provided in Supplementary Information 3.

### A. Pre-dilution using CyBio FeliX

1. Bring the samples and the imidazole + 1% albumin buffer to room temperature (RT).
2. Turn on the CyBio FeliX system.
3. Put 1,000  $\mu$ L tips in the 96/1,000 tip rack:
  - a. Complete rows A and B.
  - b. Row C: C5–C12.
  - c. The remaining wells stay empty.
4. Put the samples and empty 1.5 mL tubes (left open) in the 24-tube adapter (Table 1). The diluted samples are pipetted into the empty tubes in the same order as the undiluted samples (Table 1).

**Table 1. Scheme of the 24-tube adapter for CyBio FeliX.** The respective VWF sample, VWF standard sample, VWF control sample, and empty tubes are placed as shown in the table.

	1	2	3	4	5	6
A	Standard sample	Sample 3		Empty tube	Empty tube	
B	Control sample	Sample 4		Empty tube	Empty tube	
C	Sample 1	Sample 5		Empty tube	Empty tube	
D	Sample 2	Sample 6		Empty tube	Empty tube	

5. Fill 12 mL of imidazole + 1% albumin buffer in well 1 of the 8-row v-bottom reservoir.
6. Equip the CyBio FeliX system:
  - a. Position 1: 96/1,000 tip rack
  - b. Position 3: Waste box
  - c. Position 4: 12-channel adapter
  - d. Position 6: 37 mm adapter for protective cap
  - e. Position 7: 24-tube adapter
  - f. Position 8: 96-deep-well plate
  - g. Position 9: 8-row v-bottom reservoir
7. Execute the VWF dilution script. (The script setup is shown in Supplementary Information 1.)

### B. VWF:RCo measurement using BCS-XP

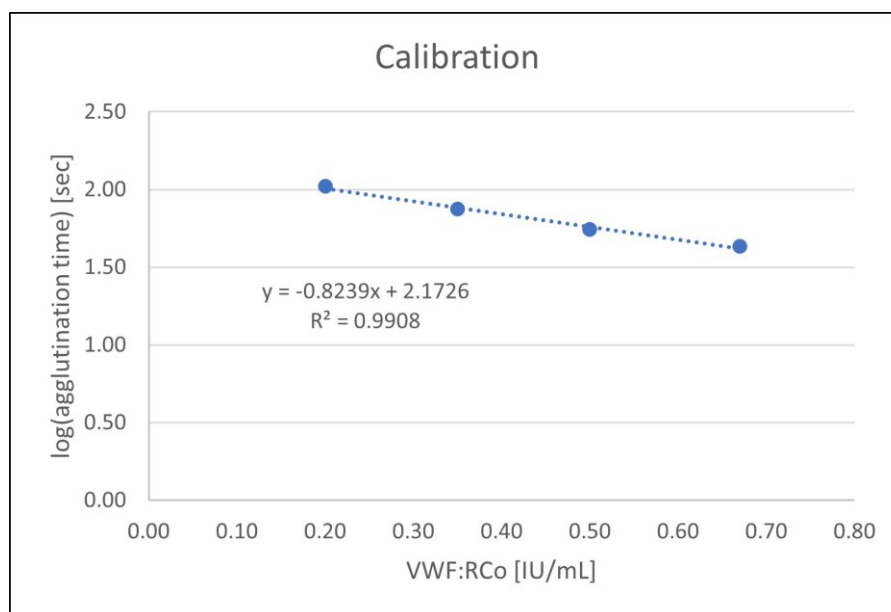
1. Bring the BC VWF:RCo reagent to RT, reconstitute in 4 mL of distilled water, put a stirrer in the flask, and place it, without cap, in a cool rack of the BCS-XP system.

2. Place a 5 mL flask filled with imidazole + 1% albumin buffer, without cap, in a RT rack of the BCS-XP system.
3. After the automated dilution, the samples are in the empty tubes in the same order as before. Place the samples (in the open tubes) in a sample rack of the BCS-XP system.
4. Execute the VWF:RCo tests for all samples, including standard and control samples (test setups are shown in Supplementary Information 2):
  - a. rVWF:RCo\_0.67
  - b. rVWF:RCo\_0.50
  - c. rVWF:RCo\_0.35
  - d. rVWF:RCo\_0.20
5. Transfer the raw data to a Microsoft Excel sheet (calculation is shown in Data analysis section).

## Data analysis

The VWF:RCo is calculated by linear regression in Microsoft Excel. The measured agglutination time is logarithmically transformed. The calibration comprises four VWF dilutions of the standard sample (Figure 2). Additionally, four dilutions are measured from the samples. Only the agglutination times from sample dilutions that fall within the calibration line are utilized to calculate the VWF:RCo. Subsequently, the mean of these activities is calculated and used as the final result for each sample.

The raw data from the BCS-XP system is presented in seconds (agglutination time). Transfer the respective raw data into the yellow fields of the Microsoft Excel evaluation sheet and enter the dilution factor of the samples in the respective green field (an example of the evaluation sheet is shown in Figure 3, and an example Microsoft Excel evaluation sheet including formulas can be found in Supplementary information 4).



**Figure 2. Example of the calibration line.** Generated by plotting the known VWF:RCo against the corresponding measured agglutination time (logarithmically transformed). A linear regression analysis was performed.



calibration						calibration	
target VWF:RCo [IU/mL]	agglutination time [sec]	log (agglutination time) [sec]				slope	
0.20						intercept	
0.35						dilution factor	
0.50						R <sup>2</sup>	
0.67							
control sample						control sample	
target VWF:RCo [IU/mL]	agglutination time [sec]	log (agglutination time) [sec]	diluted VWF:RCo [IU/mL]	VWF:RCo (IU/mL)		dilution factor	
0.20						mean VWF:RCo [IU/mL]	
0.35							
0.50							
0.67							
sample aliquot 1						sample aliquot 1	
target VWF:RCo [IU/mL]	agglutination time [sec]	log (agglutination time) [sec]	diluted VWF:RCo [IU/mL]	VWF:RCo (IU/mL)		dilution factor	
0.20						mean VWF:RCo [IU/mL]	
0.35							
0.50							
0.67							

**Figure 3. Example of the Microsoft Excel evaluation sheet.** Raw data are transferred to yellow fields and the dilution factor of the samples is input into the green fields. The grey fields are used for calculations. An example evaluation sheet, including the formulas, is provided in Supplementary Information 4.

## Validation of protocol

### Von Willebrand factor samples

The recombinant von Willebrand factor (rVWF) used in this study is co-expressed with recombinant coagulation factor VIII in Chinese hamster ovary cells. It is highly purified by several downstream processes and formulated in a protein-free buffer [27]. This rVWF shows remarkable similarity in many aspects to the structure and function of the native protein [28]. It was licensed by the US Food and Drug Administration (FDA) in 2015 and by the European Medical Agency (EMA) in 2018.

### Statistical analysis

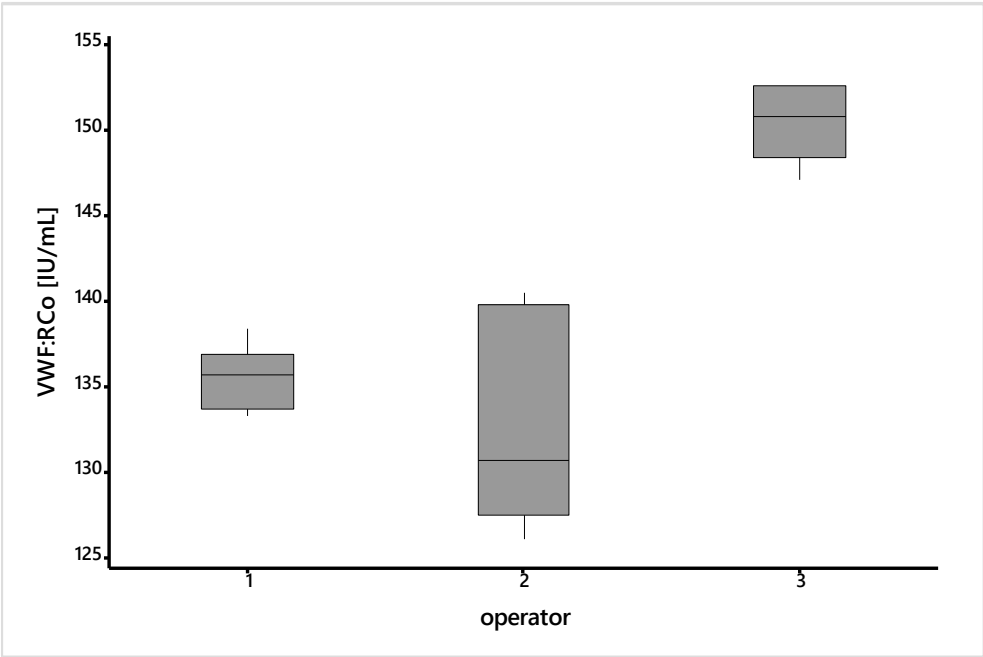
Minitab® 21.2 was used for statistical analysis. Significant differences between operators, pre-dilution procedures, and BCS-XP systems were calculated using one-way ANOVA with a confidence level of 95%. Anderson-Darling normality test was carried out. Data were tested for outliers using Tukey's Fence test. All data showed normal distribution ( $p > 0.05$ ) within groups. As stated in the corresponding table, outliers were not included in the calculations.

Comparison of manual dilutions by three operators

In total, 18 rVWF sample aliquots were analyzed. Each of the three operators measured six sample aliquots. The mean VWF:RCo of all samples across all three operators was 139.5 IU/mL and the coefficient of variation (CV) was 6.3%. The CVs of the measurements of the single operators ranged from 1.4% to 4.6% (Table 2). There was a significant ( $p < 0.001$ ) difference between operator 3 compared to the other operators. The variations of the operators are shown in Figure 4.

**Table 2. Comparison of three operators.** rVWF sample aliquots were diluted to 1 IU/mL and VWF:RCo was measured on BCS-XP. Operator 3 showed a significant ( $p < 0.001$ ) difference compared to the other operators. Statistical calculations were carried out using Minitab® 21.2.  $n = 1$  with 6 technical replicates.

Sample aliquot	Operator 1 [IU/mL]	Operator 2 [IU/mL]	Operator 3 [IU/mL]
1	133.9	128.0	148.8
2	136.0	126.1	152.6
3	133.3	129.9	149.8
4	135.4	140.5	151.8
5	138.4	139.5	147.1
6	136.4	131.6	152.6
Mean VWF:RCo [IU/mL]	135.6	132.6	150.5
CV [%]	1.4	4.6	1.5
Total mean VWF:RCo [IU/mL]	139.5		
Total CV [%]	6.3		



**Figure 4. Boxplots of the VWF:RCo measured by three operators.** Each operator manually diluted six rVWF sample aliquots to 1 IU/mL and measured the VWF:RCo on a BCS-XP system. The graph was created using Minitab® 21.2.  $n = 1$  with 6 technical replicates for each operator.

Comparison of three BCS-XP systems

In total, six rVWF sample aliquots were analyzed. The CVs of the sample aliquots, which were split up and measured on each of the three devices, ranged from 0.4% to 3.1% (Table 3). There was no significant ( $p = 0.296$ ) difference between the three instruments.



**Table 3. Comparison of three BCS-XP systems.** rVWF sample aliquots were manually diluted to 1 IU/mL VWF:RCo. Each aliquot was separated into three parts for measurement on each of the devices. No significant ( $p = 0.296$ ) difference between the three devices was shown. Statistical calculations were carried out using Minitab® 21.2.  $n = 1$  with 6 technical replicates on each of the three devices.

Sample aliquots	BCS-XP 1 [IU/mL]	BCS-XP 2 [IU/mL]	BCS-XP 3 [IU/mL]	Mean VWF:RCo [IU/mL]	CV [%]
1	121.5	123.3	125.3	123.4	1.6
2	128.4	128.0	127.4	128.0	0.4
3	127.8	126.4	131.0	128.4	1.8
4	131.0	138.8	137.1	135.6	3.0
5	127.4	132.9	132.0	130.8	2.3
6	127.5	135.1	134.1	132.2	3.1

**Pre-dilutions by CyBio FeliX compared with manual pre-dilutions on BCS-XP**

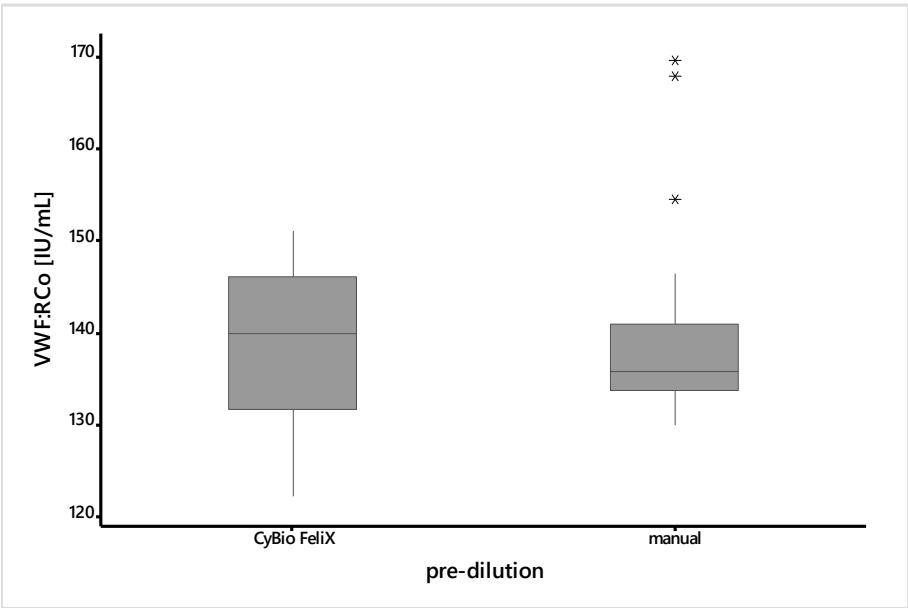
The precision of the CyBio FeliX pre-dilution script compared with manually pre-diluted samples was evaluated by analyzing 36 rVWF sample aliquots each. The automated pre-dilution procedure resulted in a mean activity of 139.0 IU/mL (Table 4), and the manual pre-dilution showed a mean of 136.3 IU/mL (Table 5). The mean CV of the samples pre-diluted by the CyBio FeliX system was 5.9%, and the mean CV of manually pre-diluted samples was 3.1%. The CV within one run of the manually pre-diluted samples ranged from 1.2% to 3.4% (Table 5) and of the automated samples from 1.2% to 5.5% (Table 4). The variations of both pre-dilution procedures are shown in Figure 5. The manually pre-diluted samples exhibited three outliers, yielding an error rate of 8.3% (Table 5). There was no significant difference ( $p = 0.098$ ) between an experienced operator, who executed this assay many times, and the automated dilution procedure. Thereby, the CyBio FeliX approach provides a method that is as precise and accurate as an expert without requiring specific training for this assay. While significant differences can occur between operators who are not specifically trained for this method (as shown in the respective section above), the automated dilution showed reproducible results. Furthermore, it is less error-prone, as indicated by the error rate of 8.3%.

**Table 4. VWF:RCo [IU/mL] of rVWF sample aliquots on six days.** The samples were pre-diluted on a CyBio FeliX liquid handling system to 1 IU/mL, and VWF:RCo was measured on BCS-XP. Statistical calculations were carried out using Minitab® 21.2. Data were tested for outliers using Tukey's Fence test. No outliers were observed.  $n = 6$  with 6 technical replicates on each of six days.

Sample aliquots	Day 1 [IU/mL]	Day 2 [IU/mL]	Day 3 [IU/mL]	Day 4 [IU/mL]	Day 5 [IU/mL]	Day 6 [IU/mL]
1	144.7	145.5	147.3	149.7	139.4	130.7
2	137.9	147.7	149.2	148.6	122.1	127.5
3	138.9	143.3	149.2	151.1	132.7	131.9
4	138.0	134.0	146.3	143.1	140.4	131.0
5	125.6	141.1	144.4	145.2	133.3	130.5
6	142.7	133.6	131.7	148.6	125.8	130.8
Mean VWF:RCo [IU/mL]	137.9	140.9	144.7	147.7	132.3	130.4
CV [%]	4.8	4.2	4.6	2.0	5.5	1.2
Total mean VWF:RCo [IU/mL]	139.0					
Total CV [%]	5.9					

**Table 5. VWF:RCo [IU/mL] of rVWF sample aliquots on six days.** The samples were pre-diluted manually by one operator to 1 IU/mL and VWF:RCo was measured on BCS-XP. Statistical calculations were carried out using Minitab® 21.2. Data were tested for outliers using Tukey’s Fence test. The outliers are marked in bold and were excluded from the calculations of mean and CV. The error rate was calculated by dividing the number of outliers by the total number of observations. n = 6 with 6 technical replicates on each of six days.

Sample aliquots	Day 1 [IU/mL]	Day 2 [IU/mL]	Day 3 [IU/mL]	Day 4 [IU/mL]	Day 5 [IU/mL]	Day 6 [IU/mL]
1	144.7	144.7	133.9	130.1	130.9	136.2
2	135.5	135.5	136.0	135.2	133.9	133.4
3	138.9	138.9	133.3	134.0	131.3	133.7
4	<b>169.6</b>	<b>169.6</b>	135.4	141.9	131.7	<b>154.4</b>
5	142.4	142.4	138.4	134.3	133.9	140.9
6	136.2	136.2	136.4	130.0	134.7	139.2
Mean VWF:RCo [IU/mL]	139.5	140.4	135.6	134.3	132.7	136.7
CV [%]	2.8	3.4	1.4	3.3	1.2	2.4
Total mean VWF:RCo [IU/mL]	136.3					
Total CV [%]	3.1					
Error rate [%]	8.3					



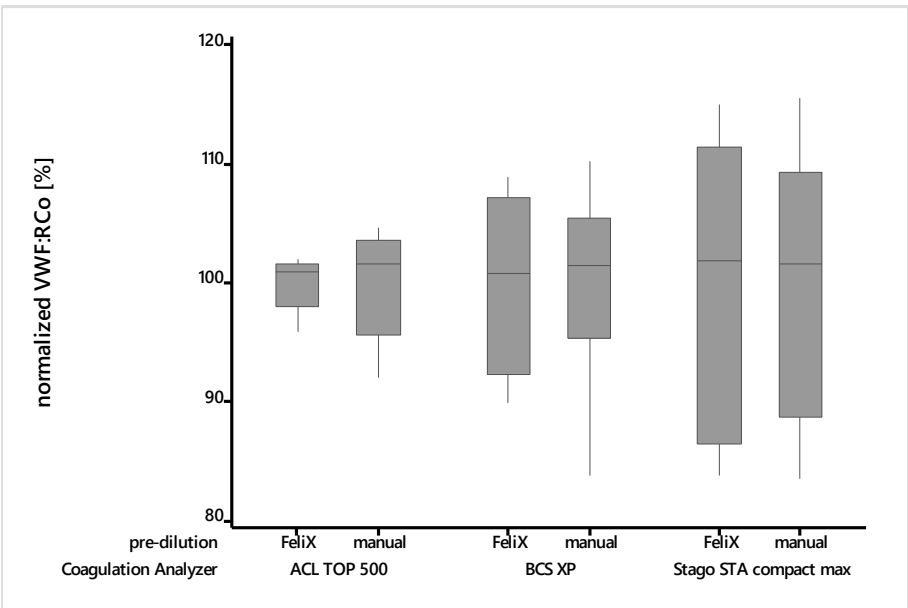
**Figure 5. Comparison of two rVWF pre-dilution procedures.** rVWF sample aliquots (n = 36) were diluted by a CyBio FeliX liquid handling system and manually by one operator. VWF:RCo was measured on BCS-XP. There is no significant difference (p = 0.098) between manual dilutions and those performed by the CyBio FeliX system. The graph was created using Minitab® 21.2. n = 1 with 6 technical replicates on each of six days.

**Pre-dilutions by CyBio FeliX compared with manual pre-dilutions on different coagulation analyzers**

The performance of the pre-dilution script on the CyBio FeliX system was assessed on three different coagulation analyzers [BCS-XP (Siemens, Germany), Stago STA compact max (Stago, France), ACL TOP 500 (Instrumentation Laboratory, USA)]. In total, 36 rVWF sample aliquots were analyzed. On each of the devices, six sample aliquots pre-diluted manually as well as six sample aliquots pre-diluted by CyBio FeliX were measured. The mean VWF:RCo was calculated and normalized to the mean separately for both pre-dilution procedures on each device. The summary of the results is shown in Table 6. The two pre-dilution methods show similar variations on each device, as shown in Figure 6.

**Table 6. Comparison of pre-dilutions by a CyBio FeliX liquid handling system with manual pre-dilutions of rVWF sample aliquots.** The samples were diluted to 1 IU/mL and VWF:RCo was measured on three different coagulation analyzers: ACL TOP (Instrumentation Laboratory, USA), BCS-XP (Siemens, Germany), and Stago STA Compact Max (Stago, France). The VWF:RCo was normalized to the mean separately for both pre-dilution procedures on each device. Statistical calculations were carried out using Minitab® 21.2. n = 1 with 6 technical replicates on each of the three devices.

Coagulation analyzer	Pre-dilution	Normalized VWF:RCo [%]	CV [%]
ACL TOP 500	Manual	92.0–104.7	4.8
	CyBio FeliX	95.8–102.1	2.3
BCS-XP	Manual	83.8–110.2	8.8
	CyBio FeliX	89.9–108.8	7.4
Stago STA compact max	Manual	83.5–115.5	11.5
	CyBio FeliX	83.8–115.0	12.5



**Figure 6. Comparison of two pre-dilution methods for measurement of VWF:RCo on three different devices.** rVWF sample aliquots (n = 6) were pre-diluted manually and by a CyBio FeliX liquid handling system to 1 IU/mL. Three coagulation analyzers were used for the VWF:RCo measurements: ACL TOP (Instrumentation Laboratory, USA), BCS-XP (Siemens, Germany), and Stago STA Compact Max (Stago, France). The VWF:RCo was normalized to the mean separately for both pre-dilution procedures on each device. Normalized data and the graph were created using Minitab® 21.2. n = 1 with six technical replicates on each of the three devices.

## General notes and troubleshooting

The CyBio FeliX script dilutes the VWF samples to 1 IU/mL VWF:RCo. This concentration demonstrated accurate and precise results using several coagulation analyzers [29,17]. In addition, the expected concentration of VWF in the blood is 1 IU/mL, which is routinely measured in the clinic using a variety of instruments [30]. In this study, a drug product of a specific supplier was used. Hence, when measuring other VWF samples, it should be noted that the dilution steps in the CyBio FeliX script may need to be adjusted accordingly. The main difference of the adapted method on BCS-XP, as compared to the pre-installed procedure, lies in the thorough nature of the measurement. Instead of only using four dilutions of the calibrator, this approach evaluates four dilutions from every sample. Furthermore, modifications were made to the programming of the BCS-XP system to ensure that measurements

were exclusively obtained within the linear range of the assay. Consequently, the point-to-point calibration was replaced with a linear regression.

We included a comparison between the two dilution procedures on different coagulation analyzers in the validation section to emphasize that the automated dilution setup can be combined with devices other than BCS-XP. However, we still recommend using the CyBio FeliX system in combination with the adapted VWF:RCO assay on BCS-XP, as described in the protocol. This approach demonstrated reproducible results over six days. Further research is needed for a meaningful assessment of the other coagulation analyzers.

Compared with other more sophisticated LHS such as Tecan Fluent (Tecan, Switzerland), the CyBio FeliX is cheaper and more compact. Despite its smaller size and lower cost, it remains versatile, as described in the background section. Furthermore, the automated dilution setup creates the basis for the full automation of this assay and several other VWF analyses, such as VWF:Ag and VWF:GPIbR, on the CyBio FeliX system.

The CyBio FeliX system is robust and executes the dilution script without errors. However, it is important to double-check for human errors, such as providing too little buffer or too few tips, as the system does not track the liquid level or when there are not enough tips.

Also, there are no common errors related to the BCS-XP device itself. While this system recognizes insufficient volumes, it is still important to provide enough volume to avoid errors due to a disrupted workflow. In addition, ensure the magnetic stirrer is in the reagent flask, as the system will not detect its absence.

Further research is needed to validate the protocol for utilization with VWF-containing samples other than rVWF concentrates. While this protocol is most suitable for use in research labs, it paves the way for high-throughput applications in the pharmaceutical industry and clinical settings.

## Acknowledgments

The authors would like to thank Elisabeth Pum and Christina Dimberger-Elboraei for performing the experiments for the operator comparison.

**Author Contributions:** Conceptualization: Tobias Schachinger, Ann-Katrin Holik, Gerald Schrenk, and Herbert Gritsch; Methodology: Tobias Schachinger, Ann-Katrin Holik, Gerald Schrenk, and Herbert Gritsch; Writing—original draft preparation: Tobias Schachinger; Writing—review and editing: Ann-Katrin Holik, Gerald Schrenk, Herbert Gritsch, Peter Turecek, Paul G. Furtmüller, and Stefan Hofbauer; Supervision: Ann-Katrin Holik, Gerald Schrenk, Herbert Gritsch, Paul G. Furtmüller, and Stefan Hofbauer. All authors have read and agreed to the published version of the manuscript.

## Competing interests

Baxalta Innovations GmbH (part of Takeda) provided funding for this research. Authors Tobias Schachinger, Ann-Katrin Holik, Gerald Schrenk, Herbert Gritsch, and Peter L. Turecek are employed at Baxalta Innovations GmbH, part of Takeda. Gerald Schrenk, Herbert Gritsch, and Peter L. Turecek hold stocks of Takeda. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: March 21, 2024; Accepted: July 22, 2024; Available online: August 08, 2024; Published: September 05, 2024

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## Supplementary information

The following supporting information can be downloaded [here](#):

1. Supplementary information 1: CyBio FeliX script
2. Supplementary information 2: BCS-XP tests
3. Supplementary information 3: Video demonstrations
4. Supplementary information 4: Example of the Microsoft Excel evaluation sheet including formulas