APPLICATION NOTE

Quantification of ADAMTS13 activity by FRET assay and kinetic data analysis using MyAssays desktop on the VICTOR Nivo.

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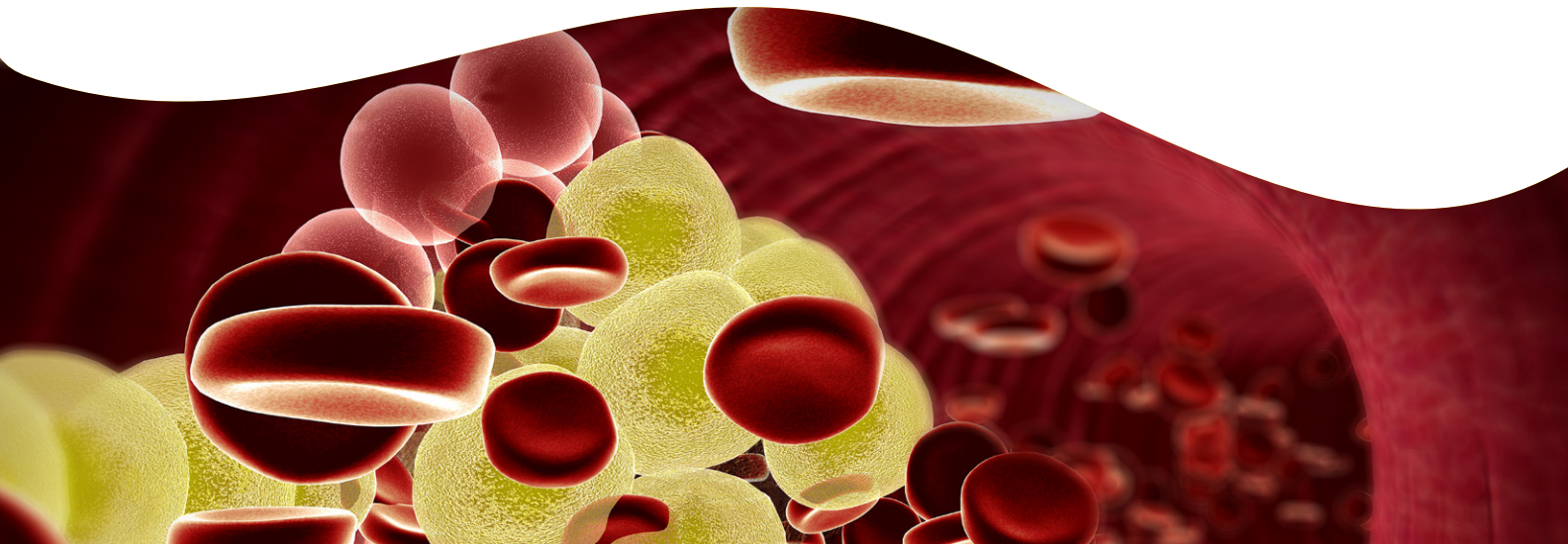
Introduction

ADAMTS13 is a metalloproteinase, also known as von Willebrand factor-cleaving protease (UniProt ID ATS13_HUMAN). This enzyme cleaves von Willebrand factor (vWf), a large protein involved in primary and secondary hemostasis, between Tyr842 and Met843. Deficiency in ADAMTS13 (congenital or acquired, due to the presence of autoantibodies) is reported in plasma samples from patients with thrombotic thrombocytopenic purpura (TTP)[1], a rare disease that can be controlled by plasma exchange. The thought is that plasma exchange can supply ADAMTS13 to patients and/or to remove neutralizing autoantibodies against ADAMTS13.

The FRETS-VWF73 assay is a rapid method (1-hour kinetics assay) to quantify ADAMTS13 activity in plasma samples.[2-3] It is a fluorescence resonance energy transfer (FRET) assay, where a synthetic 73-amino-acid peptide, called FRETS-VWF73, can be cleaved by ADAMTS13, relieving the fluorescence quenching present in the intact peptide. Incubation of FRETS-VWF73 with normal human plasma quantitatively increases fluorescence during the kinetics measurement, while ADAMTS13-deficient plasma has no effect.[2]

The VICTOR® Nivo™ is a compact and user-friendly multimode plate reader. It easily fits on every laboratory bench and provides superior sensitivity for fluorescence measurements due to specific bandpass filters and dichroic mirrors. Its data analysis software, MyAssays® Desktop, is ideal for the analysis of assays in multi-well plate format, both in endpoint and kinetics modes. In MyAssays® Desktop, complex analysis workflows can be saved and applied to new plates for a straightforward, yet robust data analysis.



In this application note, VICTOR Nivo was used to perform a FRETS-VWF73 kinetic assay, to generate a standard curve with standard plasma. MyAssays® Desktop Software was used for the analysis of kinetic data obtained by VICTOR Nivo.

Material and methods

Material

Substrate - Peptide Institute Inc., #FRETS-VWF73 3224-s
Human plasma (with citrate) from Sigma-Aldrich, #P9523.

Assay protocol

The protocol provided by the vendor (Peptide Institute Inc.) was followed, with the exception of the sample volumes pipetted in the microplate that were scaled down by half. Briefly, the FRET substrate was reconstituted with 35 µL DMSO followed by 105 µL pure water, to obtain a stock solutions of 100 µM. The stock solution was then diluted 25-fold in reaction buffer (5 mM Bis-Tris pH 6.0, 25 mM $CaCl_2$, 0.005% Tween-20).

Plasma was diluted in the reaction buffer to obtain the following standard curves: 1, 2, 3, 4, 5 and 6 µL plasma/100 µL reaction buffer (referred to as Standards 1-6). When analyzing 4 µL of unknown samples, Standards 1-6 correspond to 24.75, 49.02, 72.82, 96.15, 119.05 and 141.51% enzyme activities. Next, 50 µL of diluted plasma was pipetted in half-area black Optiplate™ (Revvity, #6052260), in triplicate wells. 50 µL of substrate solution was added to each well and the measurement protocol was immediately started. Final volume/well was 100 µL.

Measurement protocol

The VICTOR Nivo multimode plate reader (Figure 1) was used to acquire FRET data using FI-Kinetics operation, according to the settings detailed in Table 1. Plate kinetics was set to acquire the signal every five minutes, for a total of 13 measurements. The plate was automatically shaken in between measurements, using orbital shaking at medium speed (600 rpm).

Figure 1. VICTOR Nivo multimode plate reader.

Results

Raw fluorescence intensity data shows high reproducibility, with CV% values for blanks and standard samples comprised between 0.21% and 6.70%. Table 2 reports examples of raw data for the 12th repeat.

Raw fluorescence intensity data were exported from VICTOR Nivo as ".xlsx" file and imported in MyAssays® Desktop Pro from MyAssays Ltd., v4. The plate scheme was configured to select blank samples and standard samples, visualized in yellow and red colors in Figure 2 respectively.

Table 1. Settings used for acquisition of FRET data using the FI-Kinetics operation on the VICTOR Nivo multimode plate reader.

Specification	Setting
Temperature	37 °C
Excitation Filter	355/40 nm
Emission Filter	465/20 nm
Dichroic Mirror	D400
Focus Height	6 mm
Measurement Time	250 ms
Reading	Top

Table 2. Example of fluorescence intensity (raw) data for measurement #12.

	Replicate 1	Replicate 2	Replicate 3	CV%
blank	86314	85004	86084	0.82%
Standard 1	159850	159493	162956	1.18%
Standard 2	212100	215103	217212	1.20%
Standard 3	285217	266157	272794	3.52%
Standard 4	324614	322910	329422	1.04%
Standard 5	378935	369470	379452	1.49%
Standard 6	419900	411318	412215	1.14%

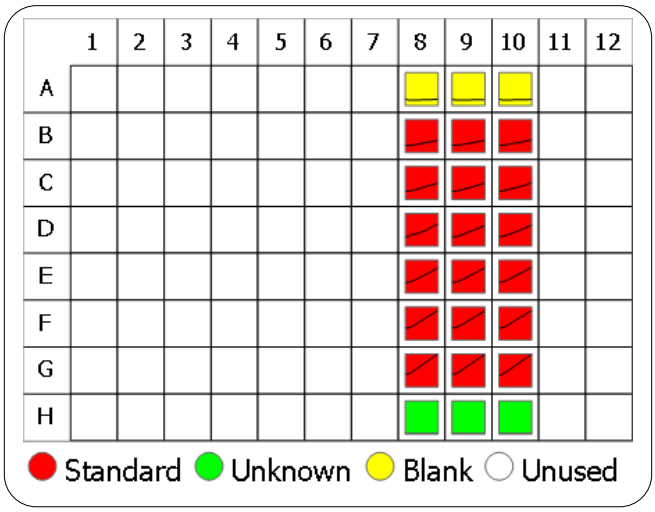


Figure 2. Raw kinetic data (FRET signal intensities), as visualized in MyAssays® Desktop Software. Plate scheme: A8-A10 reaction buffer only; B8-B10 Standard 1 (1 µL plasma in 100 µL reaction buffer); C8-C10 Standard 2 (2 µL plasma in 100 µL reaction buffer); D8-D10 Standard 3 (3 µL plasma in 100 µL reaction buffer); E8-E10 Standard 4 (4 µL plasma in 100 µL reaction buffer); F8-F10 Standard 5 (5 µL plasma in 100 µL reaction buffer); G8-G10 standard 6 (6 µL plasma in 100 µL reaction buffer); H8-H10 empty wells.

Kinetic data were analyzed as suggested by the vendor. To do so, a new protocol for data analysis was created (Figure 3). Blank signal was subtracted from each standard measurement, and data were interpolated by linear regression from the second kinetic point to the last one (13th), as shown in Figure 4. The slopes obtained for each well are shown in Figure 5.

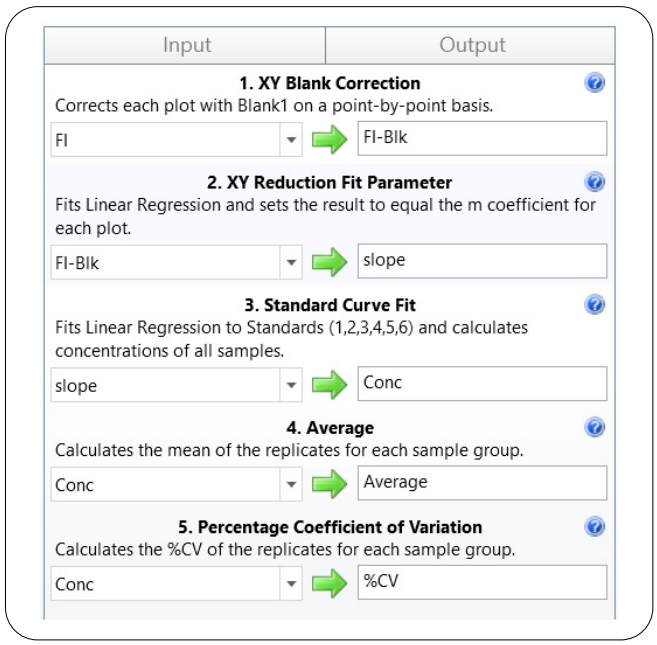


Figure 3. Protocol for the analysis of kinetic data.

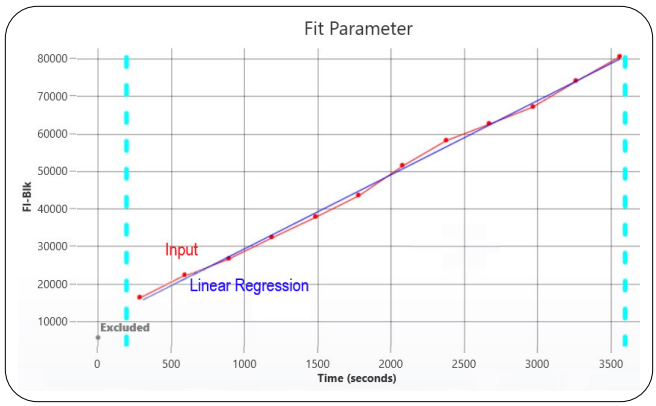


Figure 4. Slope calculation. Slope of well B8 calculated from the second kinetic point to the last kinetic point (13th).

The slopes obtained for the standard samples were plotted against plasma amounts added to 100 µL of reaction buffer (1-6 µL). The resulting dose-response graph is shown in Figure 6 and it represents the standard curve for the interpolation of unknown samples, to quantify ADAMTS13 activity.

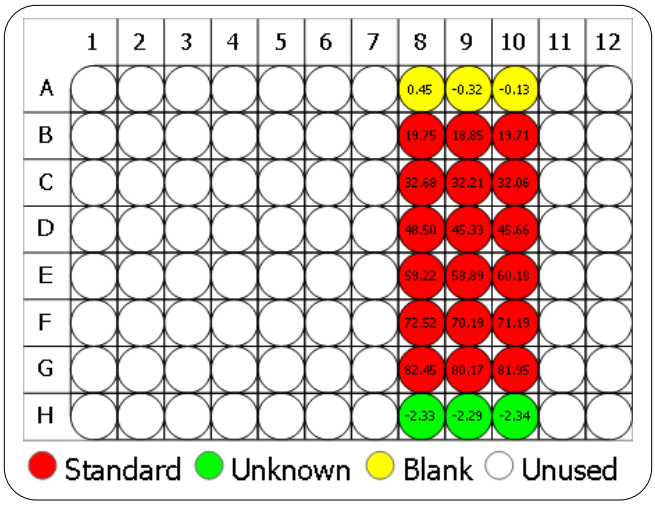


Figure 5. Slopes calculated from each well.

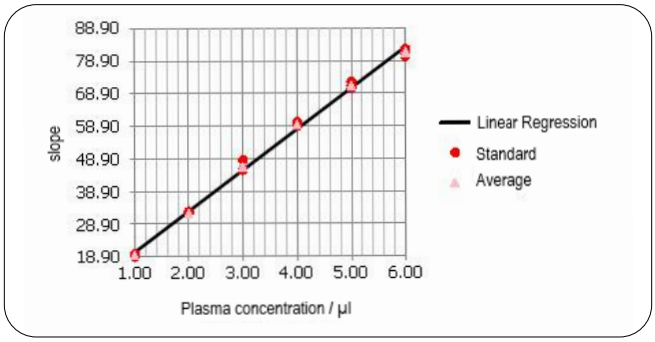


Figure 6. Standard curve for the interpolation of unknown samples. Black line: linear regression; Red dots: individual values; Pink triangle: averaged values.

Table 3. Reproducibility as indicated by coefficient of variation (CV%).

Sample	Positions	FI	FI-Blk	Slope	Conc	Slope Average	%CV
Standard 1	B8			19.75	0.96	0.93	4.31
	B9			18.85	0.89		
	B10			19.71	0.95		
Standard 2	C8			32.68	1.98	1.96	1.30
	C9			32.21	1.95		
	C10			32.06	1.94		
Standard 3	D8			48.50	3.24	3.08	4.49
	D9			45.33	2.99		
	D10			45.66	3.02		
Standard 4	E8			59.22	4.09	4.11	1.29
	E9			58.89	4.07		
	E10			60.18	4.17		
Standard 5	F8			72.52	5.15	5.05	1.84
	F9			70.19	4.97		
	F10			71.19	5.05		
Standard 6	G8			82.45	5.94	5.87	1.62
	G9			80.17	5.76		
	G10			81.95	5.90		
Blank1	A8			0.45	-0.58	-0.61	-5.17
	A9			-0.32	-0.64		
	A10			-0.13	-0.62		

For each standard sample and for the blank control, the slopes were averaged and the percentage of coefficient of variation (CV%) was calculated, to evaluate assay reproducibility. Individual and averaged data are reported in Table 3, which includes the MyAssays® Desktop Software readout.

Conclusions

With this experiment, we have used a simple and precise assay to evaluate ADAMTS13 activity in plasma. The curve obtained by following the vendor's instruction was robust and simple to analyze. Assay volumes were reduced by half, allowing reagent saving while still obtaining robust results, (R^2 = 0.997 for regression in Figure 6, maximum CV% <5% for Standards in Table 3) comparable to what is described by the manufacturer in the original protocol.[2]

The easy-to-use interface of the VICTOR Nivo multimode plate reader enabled quick set up of the measurement protocols and data transfer to MyAssays® Desktop software. With this software, data processing and analysis can be performed in the lab and conveniently at your desk after the data has been acquired.

Acknowledgements

We are grateful to Dr. Vicente Cortina, Hematology Department, University Hospital Vall d´Hebron, Barcelona (Spain) for the collaboration and scientific discussion.

References

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