Directions for use

ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT

Isopeptidase-activity based FXIII assay
(Fluorogenic: λ_{ex} = 330 nm; λ_{em} = 418 nm)

Art.-No. F001

For Research & Development Only

Revision Number: 5.0 **Release Date: 2023-07-12**

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1. Introduction

Factor XIII (FXIII) protransglutaminase circulates in plasma as A_2B_2 tetramer, at a plasmatic concentration from 14 to 28 mg/L, the A subunit being the functional form. When activated by thrombin and calcium to FXIIIa, it acts in the last step of the coagulation cascade and contributes to fibrin crosslinking and clot stiffness [1].

2. Assay principle

ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT (F001) is an easy to handle, robust and precise fluorogenic method for in vitro quantitative determination of Factor XIIIa (FXIIIa) activity in citrated plasma based on the isopeptidase activity of transglutaminases [2]. The kit consists of thrombin as ACTIVATOR REAGENT and an aggregation inhibiting peptide to prevent fibrin clotting during the measurement of plasma samples. Factor XIIIa cleaves a dark quenching molecule (2,4-dinitrophenyl) from the side chain of a modified peptide incorporating glycine methyl ester. Subsequently, the fluorescence of an N-terminal coupled fluorophore (2-amino benzoic acid, 2-Abz) increases and can be monitored online (excitation wavelength 313-330 nm; emission wavelength 418 nm). Basically, the isopeptidase activity of Factor XIIIa was described by Parameswaran et al. [3], the modified peptide used is intellectual property of Zedira [4].

3. Intended use

Ready to use assay kit for fluorescent measurement of transglutaminase activity, optimized for FXIII, in plasma samples. Further, the assay principle is suitable for the screening of compound libraries and drug development.

4. Test sample

Blood (9 vol.) must be collected on 0.109 M citrate anticoagulant (1 vol.);

plasma supernatant is decanted following a 10 min. centrifugation at 2,500 g;

citrated plasma should be tested within 8 hours or stored frozen at -20 °C or below for up to 6 months.

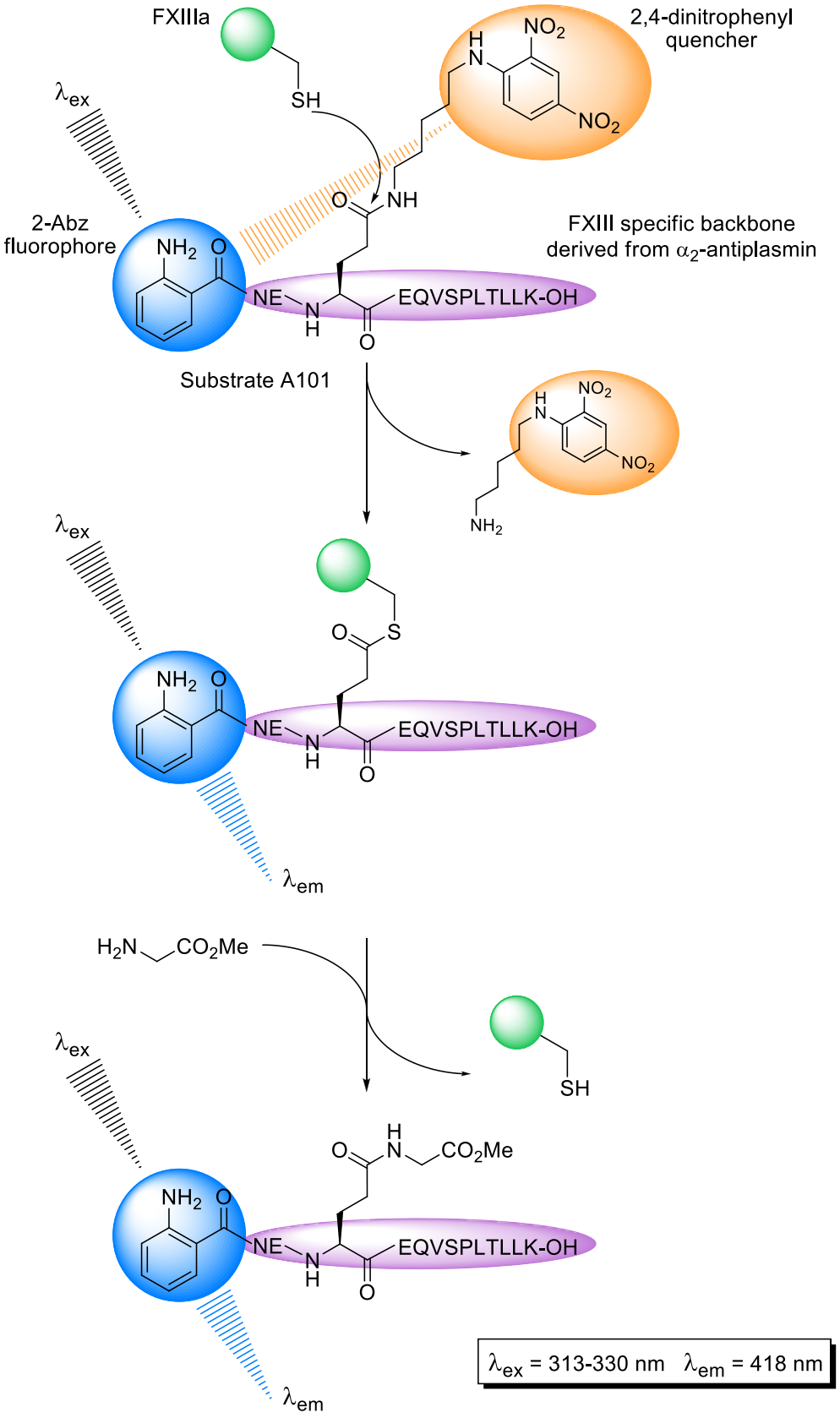


Figure 1: Cleavage of a carboxamide bond in the side chain of a specific peptidic backbone is catalyzed by FXIIIa. The release of the dark quencher is followed by incorporation of glycine methyl ester. After the dark quencher is released, the fluorescence of the fluorophore increases.

The assay continuously monitors FXIIIa activity.

5. Reagents in the kit

(1) SUBSTRATE REAGENT (**SR**): 2 x 23 µL FXIII specific assay peptide A101 (DMSO solution).

(2) BUFFER REAGENT (**BR**): 2 x 18 mL TRIS buffer pH 7.5 containing NaCl, $CaCl_2$, PEG 8000, Hexadimethrinbromide, NaN_3, Glycinmethylesther, and GPRP-NH_2 (lyophilizate).

(3) ACTIVATOR REAGENT (**AR**): 2 x 100 NIH Units Thrombin (lyophilizate).

Optionally available at Zedira (not included in the kit):

- T027 FXIII REFERENCE, human FXIII-A_2, recombinantly produced in insect cells
- A168 ABZ-PEPTIDE-CALIBRATOR, reaction product formed for calibration purposes
- T087 FXIII-inhibitor, rec. in E. coli, gene derived from *Haementeria ghilianii*
- T101 1,3,4,5-Tetramethyl-2[(2-oxo-propyl)thio] imidazolium chloride
- A108 ZED1301, Ac(D)-Asp-MA-Nle-Nle-Leu-Pro-Trp-Pro-OH [5]

6. Reagent preparation, storage, and stability

ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT (BUFFER REAGENT and ACTIVATOR REAGENT lyophilizates and SUBSTRATE REAGENT) must be stored at 2-8°C (shipment at ambient temperature is possible). The unopened reagents are stable according to the retest date printed on the box.

Table 1: Reconstitution of Kit components

Component	Preparation	Storage
BUFFER REAGENT (**BR**) 2 x 18 mL TRIS buffer lyophilizate (TRIS buffer pH 7.5 containing NaCl, $CaCl_2$, PEG 8000, NaN_3, Hexadimethrinbromide, Glycinmethylesther, GPRP-NH_2)	Add 18 mL of deionized water per vial and mix carefully	Consume within one day or store frozen at -20°C for at least 2 months
SUBSTRATE REAGENT (**SR**) 2 x 23 µL MTG specific assay peptide (DMSO solution)	Ready-to-use DMSO solution Bring to ambient temperature	Store at 2-8°C Stable for at least 12 months

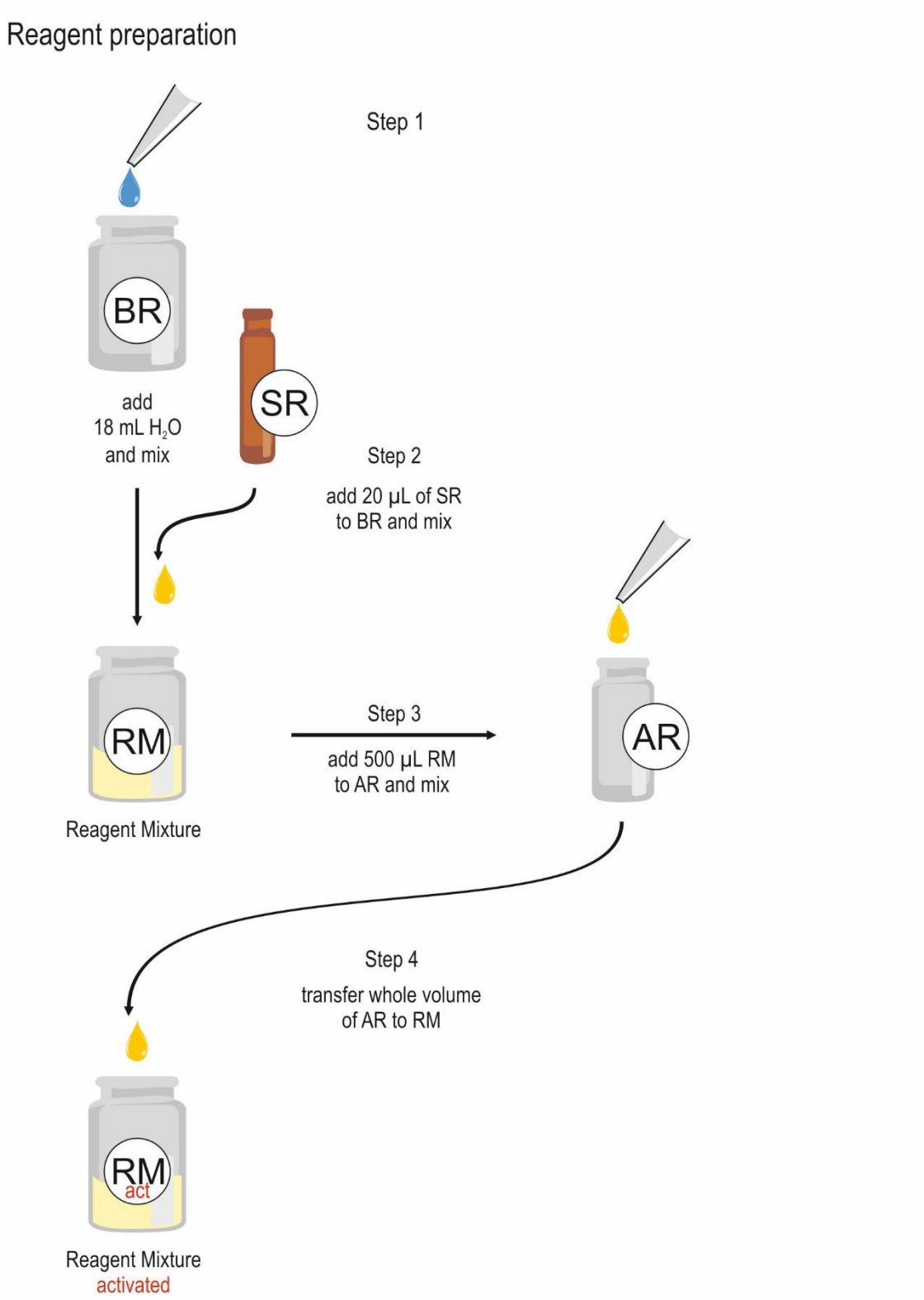
Table 2: Preparation of REAGENT MIXTURE (**RM**)

Component	Preparation	Storage
SUBSTRATE REAGENT (**SR**) BUFFER REAGENT (**BR**)	Add 20 µL **SR** to 18 mL prepared **BR** and mix	Consume within 2 hours Protect from light
ACTIVATOR REAGENT (**AR**) 2 x 100 NIH Units Thrombin lyophilizate	Add 500 µL of **RM** per vial and mix carefully. Retransfer the whole volume of reconstituted **AR** (thrombin) promptly to **RM** to get $\mathbf{RM_{act}}$ Do not vortex!	Consume within one day or store frozen at -20°C for at least 2 months Prepare **RM** directly before use Store at ambient temperature until usage

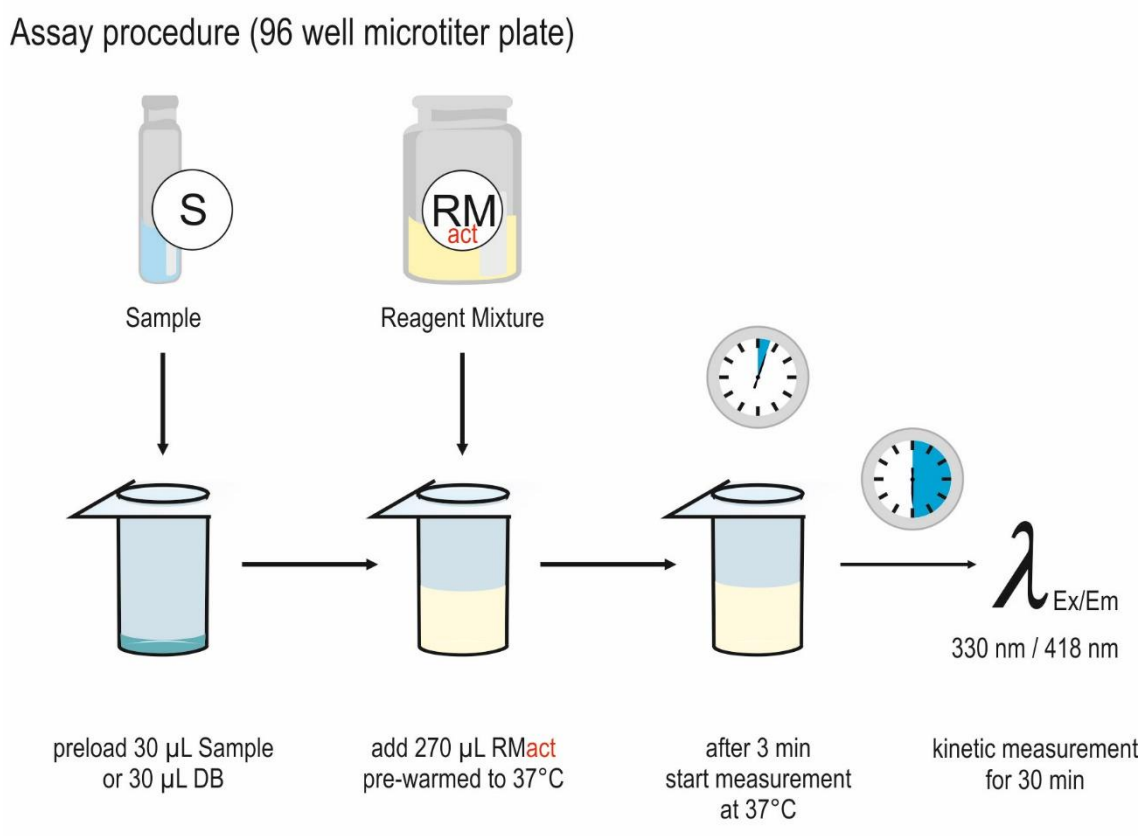
Table 3: Components optionally available at Zedira (not included in the kit)

Component	Preparation	Storage
T027 FXIII Reference, human FXIII-A2, recombinantly produced in insect cells	Add the volume of H_2O as indicated in the respective CoA. Rotate vial gently until solid dissolves Serial dilution 400-6.3 nM (32-0.5 µg/mL) in phosphate-buffered saline [PBS, pH 7.4] Final assay concentration will be one-tenth	Prepare enzyme diluent directly before use Do not vortex! Store diluent on ice
A168 ABZ-PEPTIDE-CALIBRATOR reaction product formed for calibration purposes	Prepare a DMSO stock solution or serial dilution according to your desired assay concentration as indicated in the respective PDS	DMSO stock solutions can be stored at -20°C for at least 6 months
T101 FXIII-inhibitor	Prepare a DMSO stock solution or serial dilution according to your desired assay concentration as indicated in the respective PDS	DMSO stock solutions can be stored at -20°C for at least 6 months

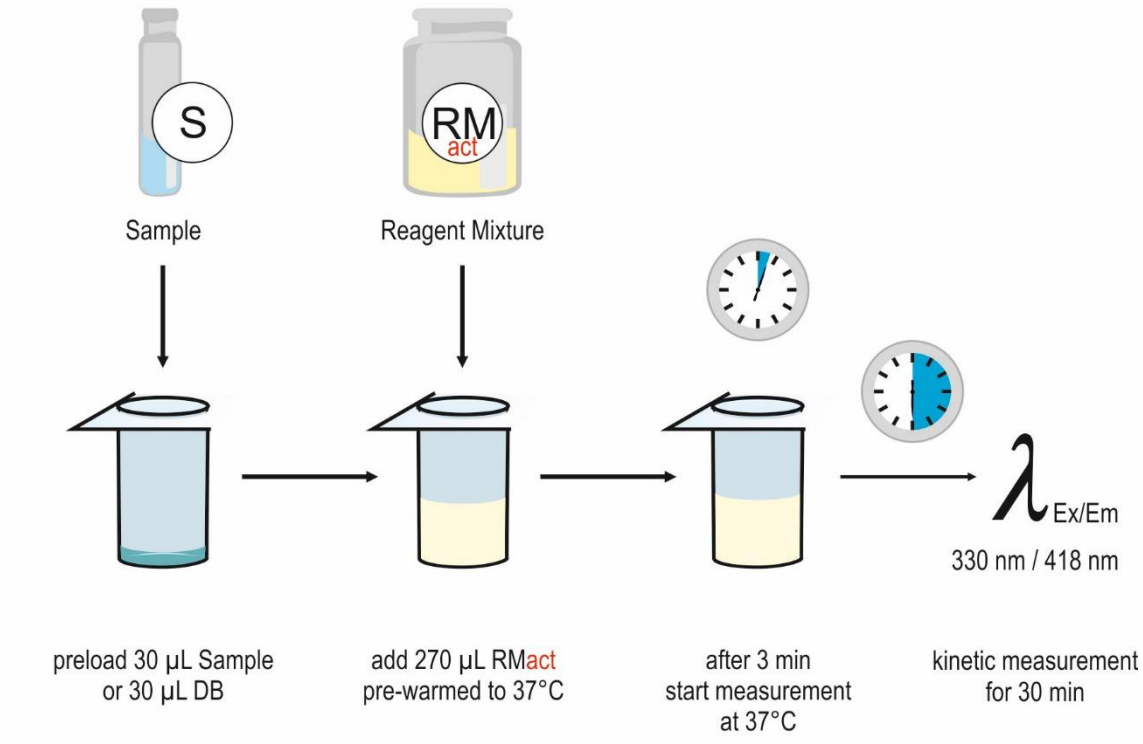
7. Schematic assay overview



Assay procedure (96 well microtiter plate)



Assay procedure (96 well microtiter plate)



8. Procedure and Equipment

Set the fluorescence spectrophotometers temperature to 37°C, if applicable. Data shown in the manual are obtained at this temperature. However, the kit may also be run at ambient temperature.

The ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT (F001) can be used in fluorescence plate readers using microplates as well as in standard fluorescence spectrophotometers with cuvettes. Refer to the instructions of the manufacturer.

Add SAMPLE (**S**) and REAGENT MIXTURE (**RM**) depending on your assay format:

Microtiter plate (96 well, 300 µL): Select a microplate that is rated for fluorescence-based assays and exhibits little or no autofluorescence in the emission range of the reagent you wish to use. Black plates are typically recommended.

Preload the wells with 30 µL of your SAMPLE. Start the reaction by adding 270 µL of prewarmed REAGENT MIXTURE (**RM**) to SAMPLE (**S**) and mix thoroughly.

Fluorescence cuvette (1 mL): Start the reaction by adding 100 µL of SAMPLE (**S**) to 900 µL of REAGENT MIXTURE (**RM**), mix thoroughly.

Start the kinetic measurement 3 minutes after starting the reaction using the instrument settings shown in table 4.

Table 4: Instrument settings for fluorescence spectrophotometer.

Excitation wavelength	313-330 nm
Emission wavelength	418 nm
Assay time (min)	30

Determine the slope of fluorescence increase over a reaction time of 20 min. Use the linear part of slope for assessment.

Unlike absorbance, fluorescence is not an absolute measurement. The RFU scale cannot be standardized. Accordingly, absolute counts cannot be compared between readers of different manufacturers. The intensity of a fluorescent signal is usually relative to other measurements, to a reference measurement, or to the gain settings [6].

Adjust fluorescence gain of the sample with the expected highest signal output (e.g. your positive control) to avoid saturation but still cover a good assay dynamic range.

NOTE: A FXIII REFERENCE is optionally available (see section 4) for quantification. You may also consider using Zedira product A168 ("ABZ-PEPTIDE-CALIBRATOR"), the reaction product formed, for calibration purposes.

9. Number of sample measurements

The assay reagents per kit are sufficient for 130 measurements in microtiter plates and 38 measurements in cuvettes.

10. Results

Fluorescence increase is proportional to the FXIII activity. The correlating FXIII amount can be calculated with a reference curve. Figures 2A and B show typical plots.

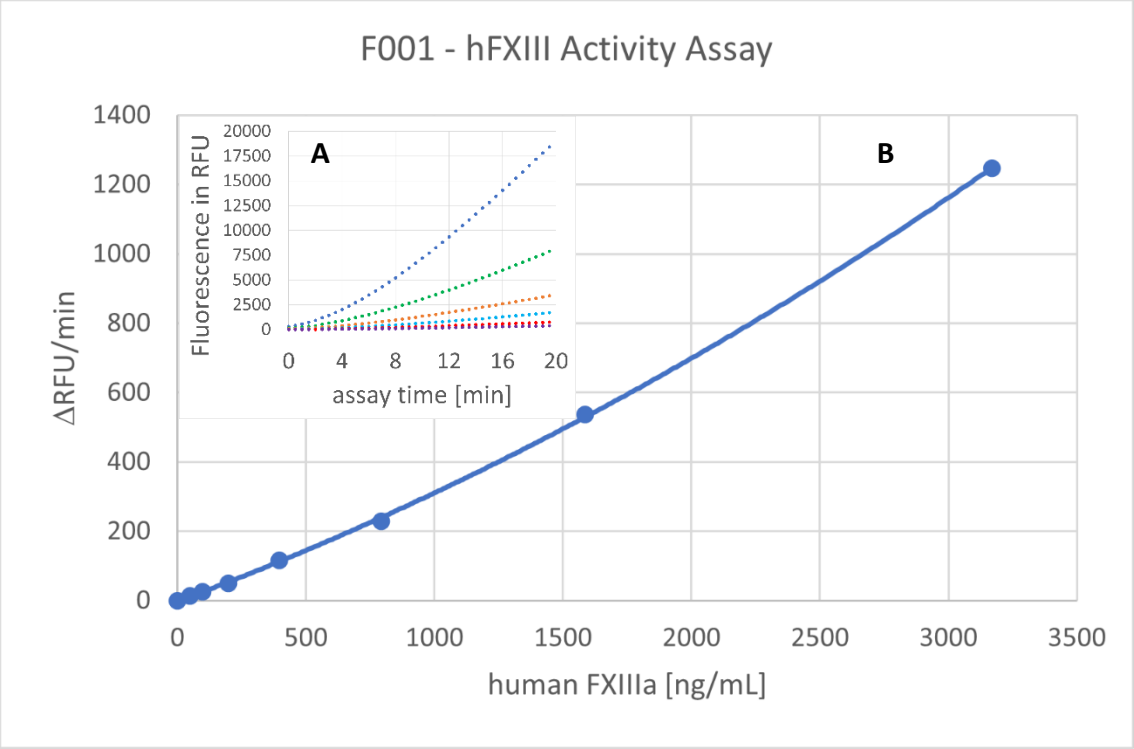


Figure 2A: **Recombinant human FXIII** (T027, hFXIII REFERENCE) dependent increase in fluorescence emission (RFU: relative fluorescence units) for a serial dilution from 3,200 to 50 ng/mL (40 to 0.63 nM) over 20 min determined in ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT. The increase in fluorescence was found to be linear between 15 and 20 min. The control without hFXIII did not yield any increase in signal intensity (not shown). The graph represents the average curves of quadruplicate measurements (error bars are not shown due to readability).

Figure 2B: Plotting ΔRFU/min (taken from figure 2A, assessed between 15 and 20 min) against the indicated hFXIII concentration, a non-linear regression fit (2nd polynomial fit, $R^2 > 0.99$) was obtained. The lower limit of quantitation of the assay was found to be 200 ng/mL (2.5 nM) hFXIII.

11. Calibration of Enzyme Units, FXIII (%) or International Units (IU)

Since the determination of a FXIII concentration (in ng/mL or nM) might be not applicable, the enzyme activity can be calculated to **Enzyme Units** using Zedira product A168 ("ABZ-PEPTIDE-CALIBRATOR").

The standard definition for 1 unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute. However, in most applications, the conversion of 1 µmol of substrate is not feasible and other definitions may be preferred.

In this assay the common non-standard definition of 1 U = 1 nmol/min is used.

The conversion of enzyme concentration to units requires the extinction coefficient, determined by the measurement of the fluorescence (RFU) of a serial dilution of A168 ("ABZ-PEPTIDE-CALIBRATOR") as shown in figure 3. The micromolar extinction coefficient ε [µM]^{300µL}, measured with 300 µL assay volume in a microtiter plate, equals to the slope of the linear regression and was determined to

$$\varepsilon\,[\mu M]^{300\,\mu L} = 10{,}227 \quad \left[\frac{RFU \times mL}{nmol}\right]$$

Note: Only valid for the individual Fluorescence Microplate Reader; cannot be translated to any other reader. Determination of ε is required on each individual reader.

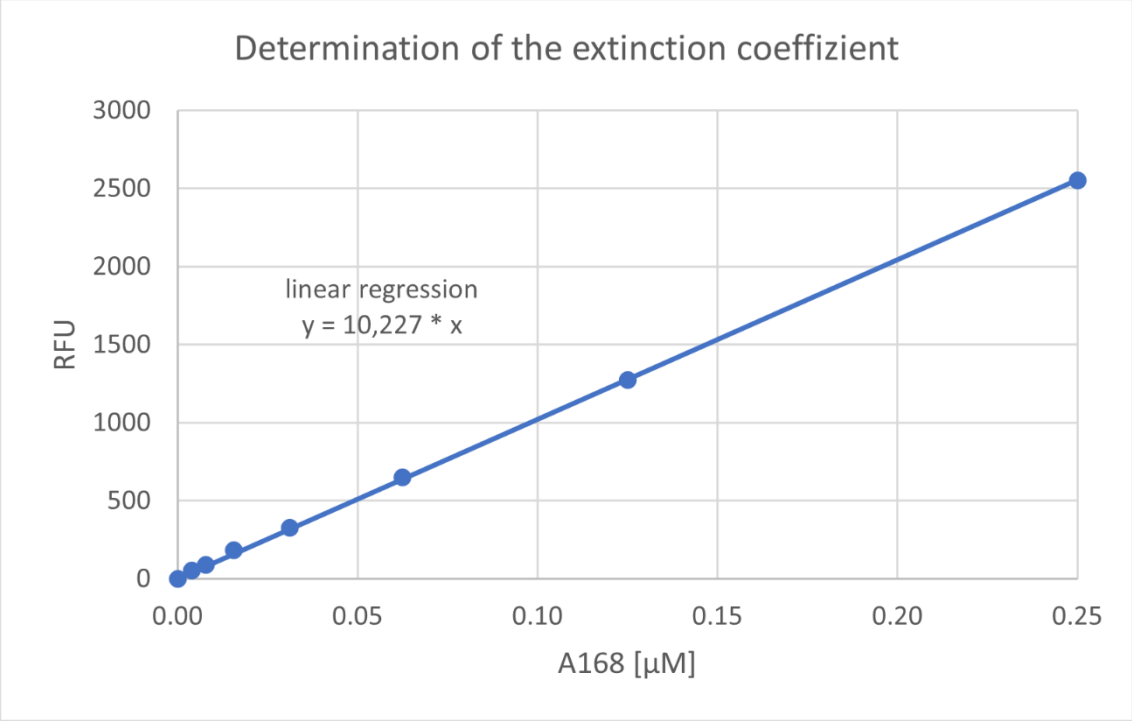


Figure 3: Determination of the extinction coefficient ε [µM]^{300µL} by plotting the fluorescence (RFU) of a serial dilution of A168 ("ABZ-PEPTIDE-CALIBRATOR"). The extinction coefficient equals to the slope of the linear regression.

For the determination of **FXIII (%) or International Units (IU)**, the usage of specific commercially available calibrators or controls with known FXIII titration, traceable to the International Standard for FXIII in plasma (not included in this kit) is obligatory [7]. Please note: Calibration plots and samples must be performed under the same gain settings.

Reconstitute the calibrators as indicated in the specific instructions of the manufacturer and prepare a calibration plot (stepwise dilution). Plot the increase in fluorescence emission (ΔRFU/min) of the dilutions against FXIII (%) or IU.

Calibration plots with Non WHO Reference Material SSC/ISTH Secondary Coagulation Standard Lot#5 (NIBSC, South Mimms, Potters Bar, UK) and Coagulation Control N (Art. No. 5020040, Technoclone) are shown in figures 4 and 5.

Figure 6 shows the determination FXIII activity of a normal donor plasma sample (Citrated Human Plasma, Art. No.HPNC-100-170217-01, Biotrend) in IU.

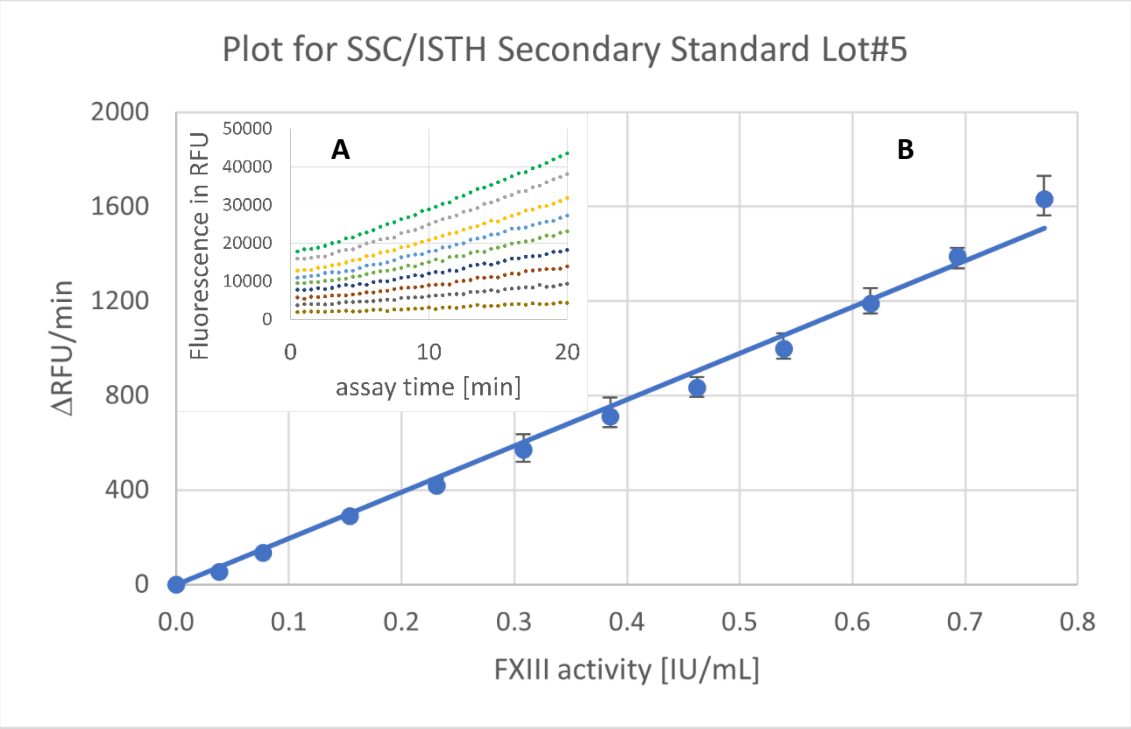


Figure 4A: FXIII dependent increase in fluorescence emission (RFU) for a stepwise dilution of SSC/ISTH Secondary Coagulation Standard Lot#5 from 0.69-0.08 IU/mL in FXIII-deficient plasma over 20 min determined in ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT (F001). The increase of fluorescence was found to be linear between 10 and 20 min. The control (FXIII-deficient plasma) did not yield any increase in signal intensity (data not shown). The graph represents the average curves of triplicate measurements (error bars are not shown due to readability).

Figure 4B: Linearity plots (correlation coefficient > 0.99) for Non WHO Reference Material SSC/ISTH Secondary Coagulation Standard Lot#5 (NIBSC, South Mimms, Potters Bar, UK) with a calibrated FXIII activity value of 0.77 IU/mL (77%).

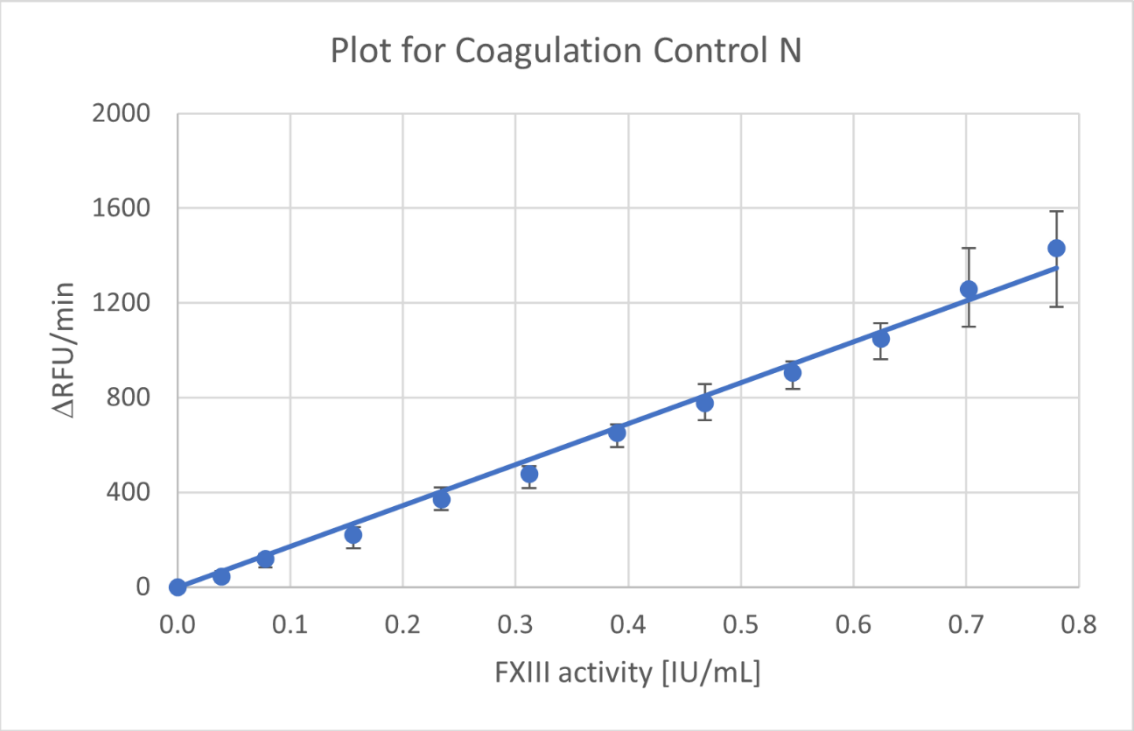


Figure 5: Plot for commercially available Coagulation Control N (Technoclone), traceable to the international standard with 0.78 IU/mL, Std. IS 02/206.

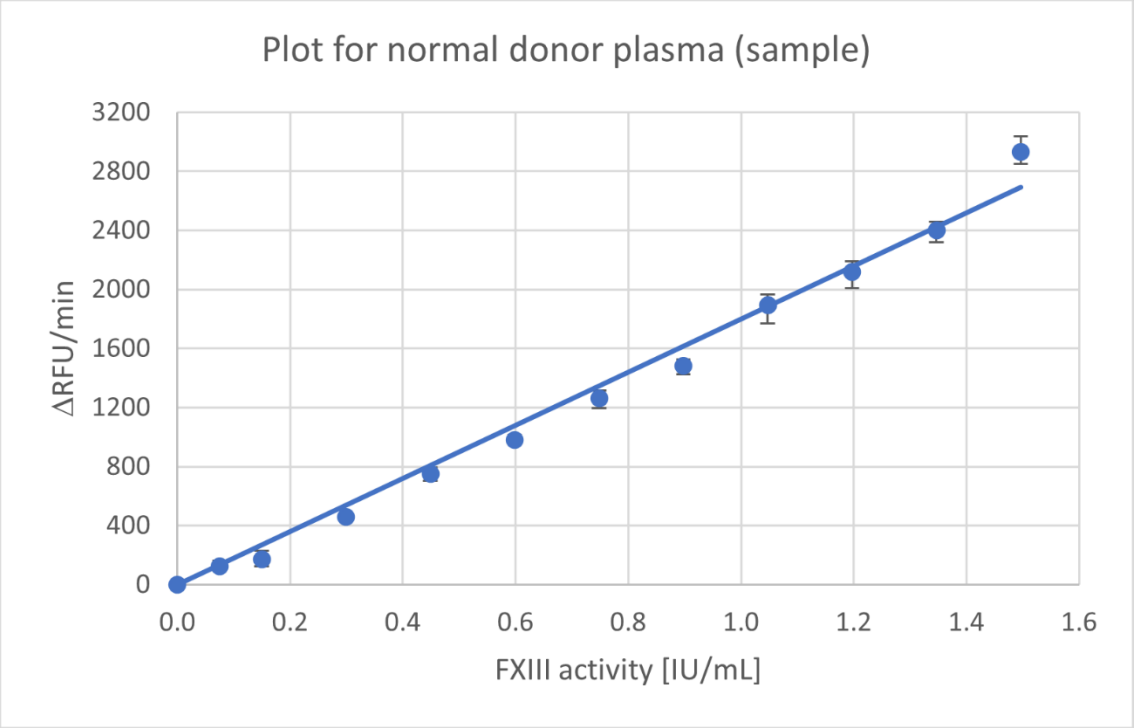


Figure 6: Plot for normal donor plasma sample (Citrated Human Plasma, Biotrend). The FXIII activity was calculated to 1.50 IU/mL according to the calibration with Non WHO Reference Material SSC/ISTH Secondary Coagulation Standard Lot#5 (NIBSC, South Mimms, Potters Bar, UK).

NOTE: All examples shown are only valid for the individual Fluorescence Microplate Reader used for measurement and cannot be translated to any other reader.

Reference measurements or calibration are required on each individual reader. Suitable references and a calibrator are optionally available at Zedira, but not included in the kit.

12. Reference Range

The ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT (F001) is suitable for measurements of 2.5 nM to 40 nM (0.2 µg/mL to 3 µg/mL) of FXIIIa.

The lower limit of quantification (LLOQ) depends on the analytical system used (CLARIOstar, Serial number: 430-0347; BMG LabTech, MARS, Vrsion 4.00 R2) and was found to be 0.2 µg/mL (2.5 nM) FXIIIa.

13. Kinetic parameters

The determination of FACTOR XIII activity is based on the ability of FXIII to cleave isopeptide bonds. The kinetic parameters obtained for FXIII specific peptidic substrate in ZEDI*XCITE* FLUOROGENIC FXIII-ASSAY are summarized in table 5.

Table 5: Kinetic parameters of MTG substrate determined by ZEDI*XCITE* FLUOROGENIC MTG-ASSAY with MTG.

	K_M [µM]	k_{cat} [s^{-1}]	k_{cat}/K_M (M^{-1} s^{-1})
ZEDI*XCITE* substrate peptide	10.5	0.14	13,014

14. Limitations

The ZEDI*XCITE* 330/418 FLUOROGENIC FXIII-ASSAY KIT (F001) is meant for research and development only. The kit has been optimized for the measurement of purified FXIII in buffer as well as FXIII in plasma samples.

Reference measurements or calibration by using suitable control plasmas are required on each individual reader. Basically, the assay can be performed in a 96 well plate reader.

Please consider that very high concentrations of fibrinogen in your plasma sample could lead to measurement bias of the enzymatic activity of Factor XIII.

15. Precision

All performance studies were conducted on CLARIOstar, Serial Number 430-0347; BMG LabTech using MARS Software package, Version 4.00 R2 and black 96 well plates (PS black, Greiner Bio One, Art-Nr.: 655076). Performance was assessed using rec. FXIIIa (T027, lot 4416aT027, Zedira GmbH) from 40-0.63 nM (3.2-0.05 µg/mL).

The intra-assay-variance (coefficient of variation, CV%) determined during kit development was found to be 1.94 % for 40 nM (3.2 µg/mL) recombinant human FXIIIa (T027, hFXIIIa, Lot 4416aT027) and 6,64 % in human plasma (BioTrend, HPNC-100-170217-01, Lot P17032901NC). The day-to-day variance was found to be 3.01 % for 40 nM (3.2 µg/mL) hFXIII and 7.15 % in human plasma. All measurements were performed over a 3-day period, 2 series per day and 4 repetitions within each series.

16. References

1. Komaromi, I., Z. Bagoly, and L. Muszbek, *Factor XIII: novel structural and functional aspects.* J Thromb Haemost, 2011. **9**(1): p. 9-20.
2. Durda, M.A., A.S. Wolberg, and B.A. Kerlin, *State of the art in factor XIII laboratory assessment.* Transfus Apher Sci, 2018. **57**(6): p. 700-704.
3. Parameswaran, K.N., et al., *Hydrolysis of gamma:epsilon isopeptides by cytosolic transglutaminases and by coagulation factor XIIIa.* J Biol Chem, 1997. **272**(15): p. 10311-7.
4. Oertel, K., et al., *A highly sensitive fluorometric assay for determination of human coagulation factor XIII in plasma.* Anal Biochem, 2007. **367**(2): p. 152-8.
5. Stieler, M., et al., *Structure of active coagulation factor XIII triggered by calcium binding: basis for the design of next-generation anticoagulants.* Angew Chem Int Ed Engl, 2013. **52**(45): p. 11930-4.
6. BMG Labtech “How to optimise fluorescence gain”.
7. Leitner, M., et al., *Clinical Validation of an Automated Fluorogenic Factor XIII Activity Assay Based on Isopeptidase Activity.* Int J Mol Sci, 2021. **22**(3).