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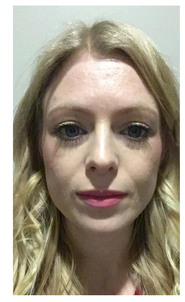
Institute of Biomedical Science IBMS CONGRESS 2022
14-17 MARCH - BIRMINGHAM, UK



Time To FRET? Verification of the Technofluor FXIII Assay

Beth Brannan, Ellie Foxton, Aine McCormick

Viapath Analytics, Diagnostic Haemostasis & Thrombosis, Guy's & St. Thomas' NHS Foundation Trust, London, United Kingdom

Introduction & Aim

FXIII deficiency is a rare bleeding disorder. Severe deficiency (FXIII activity <5%) is associated with spontaneous intracranial bleeding and death[1,2]. Testing guidelines recognise that ammonia release assays require a blank step to prevent miss-diagnosis at low levels of FXIII activity.

This verification study aimed to assess the suitability of the Technofluor FXIII activity assay (fluorescence resonance energy transfer, FRET method - blanking not indicated) and compare it to the currently utilised Siemens Berichrom FXIII activity assay (chromogenic ammonia release assay, un-blanked).

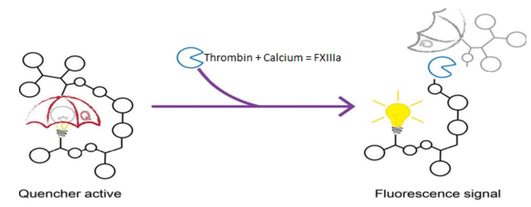


Figure 1 obtained from: https://diapharma.com/product/hemostasis/hemo/coag/technofluor-fxiii-activity/

Figure 1: FRET Method
1. Thrombin + Ca^{2+} = FXIIIa
2. FXIIIa cleaves side-chain of the assay's substrate
3. Releases the dark quencher
4. Light emission from fluorophore
5. Fluorescence is proportional to the FXIII activity

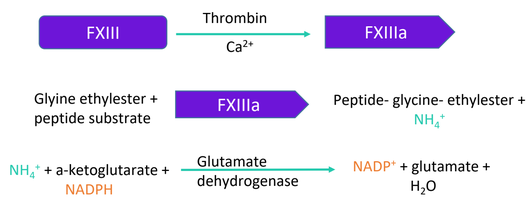


Figure 2: Chromogenic Ammonia Release
1. Thrombin + Ca^{2+} = FXIIIa
2. Glycine- ethylester and peptide substrate reaction is catalysed by FXIIIa=release of ammonia.
3. Ammonia is incorporated into an α-ketoglutarate with NADPH by glutamate dehydrogenase.
4. The decrease of NADPH is measured photometrically

Method

PRECISION
- Normal and abnormal controls
- 6x replicates
- Single Run

REPEATABILITY
- Standardised Plasma
- 8x replicates
- March–May 21

MEASUREMENT TRUENESS
- Standardised Plasma
- Target values: 111, 92, 5.55, 2.77 and 1.38

LLOQ
- Standardised Plasma Dilutions
- 111-1.38IU/dL

STABILITY
- Reagent stability
- Test against fresh QC after 4 weeks
- QC stability
- Frozen v Fresh
- Calibration Stability
- Re-run calibration, compare values and RFUs after 2 months

METHOD COMPARISON
- FRET and ammonia release assay
- Retrospective, observational
- 27 samples
- 1 NEQAS survey

Results

PRECISION

	Calculated CV %	Manu. CV %
CN	1.46	4.2
CA	2.69	3.1

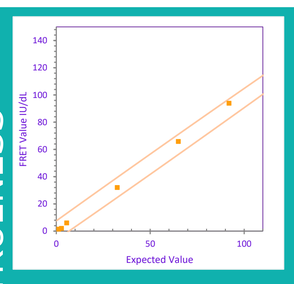
CN: Normal Control
CA: Abnormal Control

REPEATABILITY

	Calculated CV %	Manu. CV %
CN	3.31	6.4
CA	2.78	6.0

CN: Normal Control
CA: Abnormal Control

MEASUREMENT TRUENESS



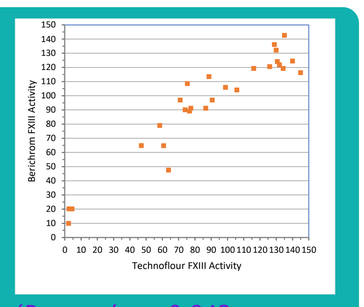
(Pearson's r- 0.999; 95% CI 0.993 – 1.000)

LLOQ

Dil. %	Expected value	FRET Result	CV (%)
1.25	1.38	1.4	1.0
2.5	2.77	2	22.8
5	5.55	4.4	16.4
10	11.1	9	14.8

STABILITY
- Reagent Stability: CV<5% for both CN & CA
- QC stability at 9 weeks: CV<5% for CN & CA
- Repeat calibration = CV acceptable for RFU and values

METHOD COMPARISON



(Pearson's r= 0.943, 95% CI 0.878-0.974, n=27)

The Technofluor FXIII activity assay demonstrated excellent stability, precision, repeatability and accuracy. A LLOQ of 1.4 IU/dL was demonstrated during the validation process. This is a significant improvement on the LLOQ of the currently utilised Siemens Berichrom FXIII activity assay (un-blanked) which is locally defined as 10 IU/dL. This difference in sensitivity at the lower levels is demonstrated in Fig. 3-5.

Observations and Conclusions

The clinical significance of the difference in LLOQ between the assays was demonstrated in two separate trough FXIII levels from a known FXIII deficient patient; see Fig. 5. FRET results for the FXIII deficient patient are in keeping with genetic diagnosis (compound heterozygosity) and initial clinical presentation (umbilical stump bleeding and intracranial haemorrhage). As this finding was mirrored in the NEQAS exercise, the robustness of this specific EQA survey appears weak given our history of successful returns with the Berichrom assay. We are unaware of any UK NEQAS participants performing a blanking step, therefore, as EQA target values are determined from median responses, inaccurate reporting is likely. Given that FXIII levels of 11% have been associated with significant bleeding[3] the assay discrepancy is clinically significant - improved accuracy at this clinically important level will aid patient safety.

The results of this comparison study are supported by published literature which states that the Berichrom FXIII assay is affected by endogenous ammonia and elevated fibrinogen levels thereby leading to an overestimation of results[4]. The Technoflour FXIII activity assay is reported not to be affected by endogenous ammonia, elevated fibrinogen levels and it does not require blanking[4]. Additionally, it is specified by the manufacturer that calibration is only required once per lot. From this study it is concluded that the Technofluor FXIII activity assay offers a user- friendly and accurate method for the assessment of FXIII activity.

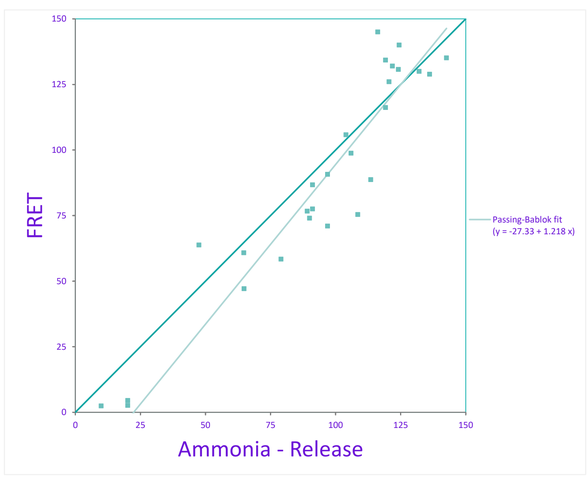


Figure 3: Passing-Bablok regression analysis (n=27) demonstrates greater difference between the two assays at the lower concentrations with an intercept estimate of -27.33

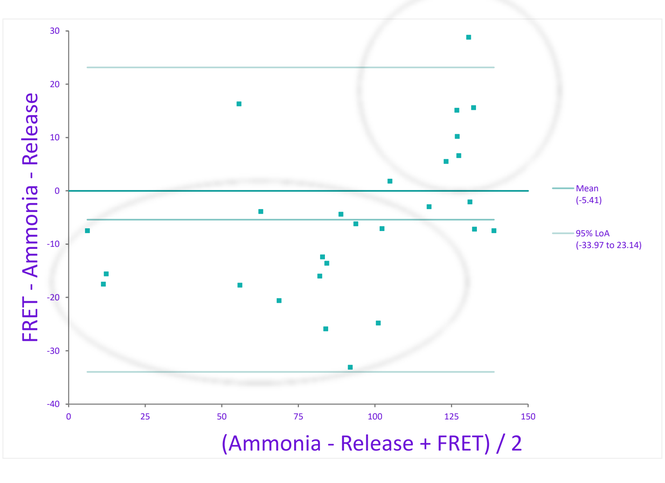


Figure 4: Bland-Altman difference analysis suggests that at lower concentrations, the FRET assay is producing smaller values than the ammonia release assay. However, at higher concentrations, the FRET assay is producing higher values than the ammonia- release assay.

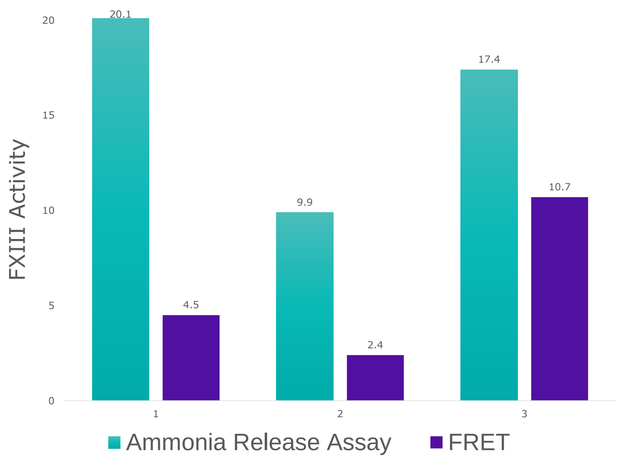


Figure 5 : Disagreement between assays at lower FXIII activity levels.1 and 2 = known FXIII deficient patient (severe). 3= NEQAS survey results

	Result FXIII 21:01	Result FXIII 21:02
Clot solubility test Method:		
Your clot solubility result:		
Assay Method:	Siemens Berichrom FXIII	
Your assay result	70.1	17.9
Interpretation	Normal	Abnormal
Users of your method (n)*	35	34
Kit-specific median (u/dl)	66.30	16.20
% deviation (previous survey)*	5.73 ()	9.15 ()
Performance quantile grade (previous survey)*	B ()	B ()
Z score (previous survey)*	0.40 ()	0.07 ()
Your Performance *	within consensus	within consensus

Figure 6: Supplementary NEQAS returns table demonstrating that our laboratory results for the Siemens Berichrom FXIII assay are in consensus with 35 other centres.

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