Performance of a New Kit for a Modified Nijmegen-Bethesda Assay: Comparison of a Chromogenic Versus a Clot-based Factor VIII Inhibitor Assay in Plasma from Persons with Hemophilia A (PwHA)

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Background/Objectives

- International proficiency studies on FVIII inhibitor titer revealed coefficients of variation as high as 50% between laboratories.[1]
- Different reagents and methods used across labs (e.g. plasma sources, absence of or variations in heat deactivation procedures, use of buffered vs. non-buffered plasma) may contribute to the high variability.
- Chromogenic endpoint assays have better specificity than one-stage clot-based assays since the latter depends on fibrin clot formation, which is impacted by the presence of heparin, lupus anticoagulants and inhibitors of coagulation factors other than FVIII.
- Thus, there is a need for standardization and improvement of the FVIII inhibitor assay.

Conclusions

- In this study, the Chromogenic MNBA showed superior reproducibility compared to the one-stage clot-based assay.
- The MNBA kit shows promise for laboratories seeking a standardized inhibitor assay suitable for clinical management or multi-center clinical studies of PwHA.

References

[1] Favaloro E.J. *et al.* Laboratory testing for factor inhibitors. Haemophilia 2014; 20(S4):94-98.

[2] Miller C.H. *et al.* Validation of Nijmegen-Bethesda assay modifications to allow inhibitor measurement during replacement therapy and facilitate inhibitor surveillance. Thromb Haemostasis 2012; 10:1055-1061.

[3] Blanchette V.S. *et al.* Definitions in hemophilia: communication from the SSC of the ISTH. Thromb Haemostasis 2014; 12:1935-1939.

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Methods

- To eliminate FVIII depleted plasma as a potential source of variation and in order to standardize inhibitor titer measurement, a kit for Modified Nijmegen-Bethesda Assay (MNBA) was developed with these components:
  - IB-PNP: Imidazole Buffered Pooled Normal Plasma (pH = 7.4, 100 mM Imidazole, FVIII 95-105%).
  - IB-BSA: Imidazole Buffered Bovine Serum Albumin (pH = 7.4, 4% w/v BSA in 50 mM Imidazole) to replace FVIII depleted plasma in the Nijmegen assay.
  - POS-Ctrl: Positive FVIII inhibitor control (~1 BU/mL, polyclonal anti-human FVIII antibody in a buffered human FVIII depleted plasma).
  - NEG-Ctrl: FVIII inhibitor-free human plasma (buffered).
- The MNBA kit components were frozen and stored at < -70 °C until use **(Figure 1)**.
- A total of 22 frozen plasma samples from PwHA with a history of FVIII inhibitors and 30 frozen plasma samples from normal donors were thawed, heat deactivated, and centrifuged. The supernatant was drawn off and stored at < -70 °C until testing **(Figure 2)**.
- A heat deactivation step was incorporated in the MNBA to prevent the likelihood of false negative FVIII inhibitor results due to remaining FVIII activity in plasma samples from PwHA.[2]
- After thawing the heat deactivated plasma samples and FVIII Inhibitor Kit controls (Test Samples), a 1:1 mixture of IB-PNP, with either undiluted or IB-BSA pre-diluted Test Samples, were prepared (Test Mix, 400 µL).
- A Control Mix was prepared: 1:1 mixture of IB-PNP:IB-BSA (400 µL).
- Both the Test Mix and Control Mix were incubated for 2h at 37 °C in a water bath followed by a 10 min. incubation on ice.
- FVIII activities were determined on a Siemens BCS® XP analyzer using Siemens Factor VIII Chromogenic Assay and a one-stage clot-based assay using Pathromtin® SL aPTT reagent.
- The closest residual FVIII activity to 50% was used to calculate the FVIII inhibitor titer in Bethesda Units **(Figure 3)**.

Figure 1

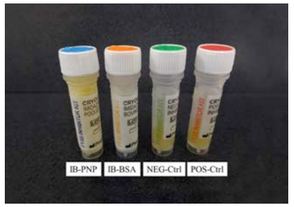


Figure 2

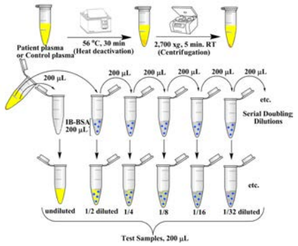
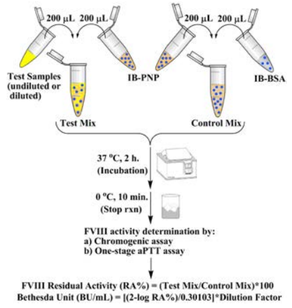


Figure 3



Results

- A strong correlation in FVIII inhibitor titer was observed between the chromogenic and clot-based MNBA **(Figure 4)**, indicating that the new MNBA kit has potential for both assay systems. However, it is possible that additional discrepant results between chromogenic and clot-based MNBA may be seen in a clinical setting where the presence of interfering conditions is more likely for the clot-based MNBA.
- Inhibitor titers obtained by the clot-based MNBA were mostly higher than those determined by the chromogenic assay **(Figure 5, Figure 6)**.
- Application of a cut off ≥0.6 BU/mL, a consensus recommendation by ISTH-SSC,[3] showed only one discrepant result out of 22 samples. This result was borderline positive by the one-stage clot-based assay, i.e. 0.6 BU/mL compared to 0.1 BU/mL by chromogenic MNBA **(Table 1)**.
- Analysis of plasma samples from normal donors (N=30) and kit controls revealed that variation of results was 2-3 times smaller for the chromogenic MNBA as compared to the clot-based MNBA **(Figure 6)**.

Table 1

Diagnostic agreement* between chromogenic and clot-based MNBA

FVIII Inhibitor detection in plasma samples from PwHA (n=22)		Clot-based MNBA	
		Positive	Negative
Chromogenic MNBA	Positive	14	0
	Negative	1	7
Agreement		Percent	95% CI
Positive Percent Agreement (PPA)		93%	68-100%
Negative Percent Agreement (NPA)		100%	59-100%
Total Percent Agreement (TPA)		96%	77-100%

* ≥0.6 BU/mL cutoff

Figure 4

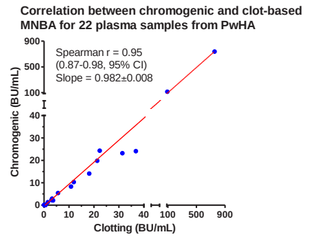


Figure 5

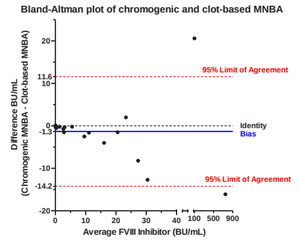


Figure 6