A Standardized Kit for a Chromogenic Modified Nijmegen-Bethesda Assay

Repeatability, Reproducibility, and Analytical Sensitivity

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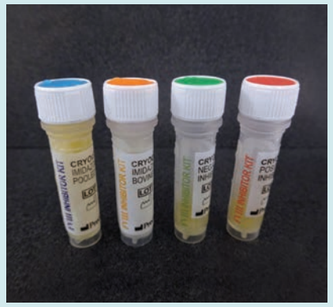
Background

- Antibody-based inhibitor response to Factor VIII replacement therapy is one of the most common complications in the treatment of persons with hemophilia A (PwHA).
- International proficiency studies on FVIII inhibitor testing revealed coefficients of variation as high as 50% between laboratories.[1]
- Quantification of FVIII inhibitors is not standardized. Laboratory-developed tests (LDTs) often vary between laboratories in:
  - **processing** of the patient plasma before testing
  - **reagents** used as the diluent and FVIII source in the assay
  - FVIII activity measurement at the assay **endpoint**
- Different LDTs have different specificities. Some methods fail to detect FVIII inhibitor in the presence of emicizumab (HEMLIBRA®).[2]
- This work describes the characterization of a chromogenic modified Nijmegen-Bethesda assay (MNBA) using a standardized set of reagents.

Materials and Methods

- The CRYO*check*™ FVIII Inhibitor Kit used to perform the assay consists of a standardized set of reagents:



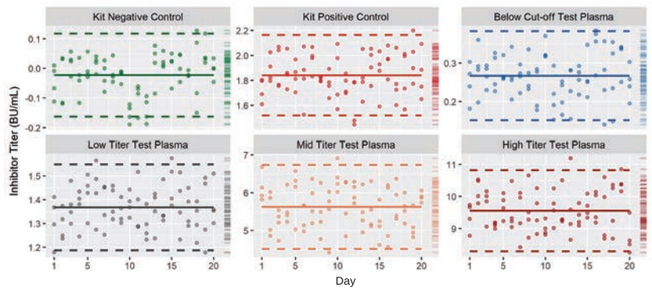
  - 2 × 1.5 mL of imidazole-buffered pooled normal plasma (IB-PNP)
  - 2 × 1.5 mL of imidazole-buffered 4% bovine serum albumin solution (IB-BSA)
  - 1 × 0.5 mL of inhibitor-positive plasma, used as a positive control
  - 1 × 0.5 mL of normal plasma, used as a negative control
- Performing the FVIII inhibitor assay with a CRYO*check* FVIII Inhibitor Kit consists of five steps, which can be completed using as little as 500 µL of 3.2% citrated human plasma.
  1) **Heat Inactivation:** The patient plasma and controls are incubated in a 56 °C water bath for 30 minutes to minimize residual FVIII activity in the samples.
  2) **Centrifugation:** The inactivated plasma is spun at 2700 × g for 5 minutes to remove precipitate from the solution.
  3) **Dilution:** Serial twofold dilutions of the supernatant are made with IB-BSA.
  4) **Mixing:** The neat plasma and dilutions are mixed with an equal volume of IB-PNP. The resulting mixes are incubated at 37 °C for two hours in a water bath. After two hours, the reaction is halted by placing the samples in an ice bath for 10 minutes.
  5) **Measuring:** The FVIII activity of the dilutions are measured, and the inhibitor titer is calculated relative to a control mix containing equal volumes of IB-PNP and IB-BSA.
- In this study, all endpoint FVIII measurements were performed on a Siemens BCS® XP analyzer, using the bovine-based Siemens FVIII Chromogenic Assay.

Conclusions

- In this study, a standardized chromogenic MNBA showed excellent within-laboratory and across-site precision.
  - Within-laboratory precision was < 10% for inhibitor-positive patient samples, and ≤ 0.1 BU/mL for inhibitor-negative samples.
  - Reproducibility of the assay was < 15% for inhibitor-positive patient samples, and ≤ 0.1 BU/mL for inhibitor-negative samples.
- The limit of quantification (LoQ) of the assay was found to be 0.2 BU/mL, well below the medical decision level of 0.6 BU/mL.[3]
- The CRYO*check* FVIII Inhibitor Kit shows potential for labs seeking repeatable and reproducible FVIII inhibitor measurement that can otherwise vary significantly within or between labs.
- Standardization of reagents and protocol yields consistent results and is suitable for multi-center clinical studies of PwHA.

Repeatability

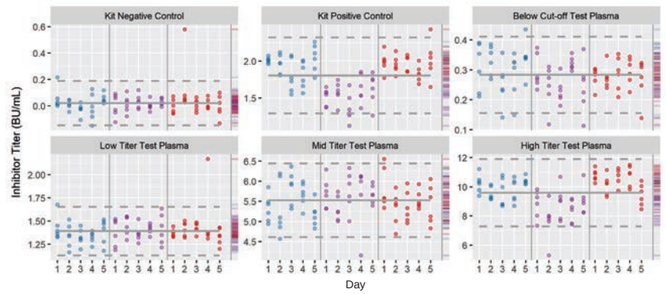
- Inhibitor-negative and inhibitor-positive plasma from PwHA were combined to yield a panel of test plasmas at four different levels of inhibitor.
- A **3 lot × 20 day × 2 run × 2 repeat** precision study was performed using the standardized MNBA to determine the repeatability and within laboratory imprecision.
- Below, the mean measured titer is shown as a solid line, and the 2σ limit as a dotted line.



Sample	N	Mean Value (BU/mL)	Repeatability		Between-Run		Between-Day		Within Lab	
			SD	%CV	SD	%CV	SD	%CV	SD	%CV
Kit Negative Control	80	0	0.0	–	0.0	–	0.0	–	0.1	–
Kit Positive Control	80	1.6	0.1	8.8%	0.1	3.5%	0.1	5.5%	0.2	11%
Below Cut-off Test Plasma	80	0.3	0.1	–	0.0	–	0.0	–	0.1	–
Low Titer Test Plasma	80	1.2	0.1	6.4%	0.0	3.8%	0.0	2.3%	0.1	7.8%
Mid Titer Test Plasma	80	5.3	0.3	5.9%	0.2	3.9%	0.1	1.3%	0.4	7.2%
High Titer Test Plasma	80	8.6	0.6	7.6%	0.4	4.6%	0	0%	0.7	8.9%

Reproducibility

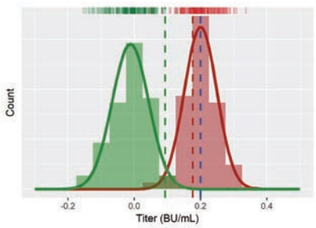
- The same panel of inhibitor positive plasmas were subjected to a **3 site × 5 day × 2 run × 3 repeat** precision study using the standardized MNBA to gauge the reproducibility and across-site imprecision.
- The testing was performed on three different analyzers with three different operators and a single lot of chromogenic FVIII kit.
- Below, the mean measured titer is shown as a solid line, and the 2σ limit as a dotted line.



Sample	N	Mean Value (BU/mL)	Repeatability		Between-Run		Between-Day		Between Site		Reproducibility	
			SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
Kit Negative Control	90	0	0.1	–	0	–	0	–	0	–	0.1	–
Kit Positive Control	90	1.8	0.2	8.8%	0.1	3.4%	0.1	3.4%	0.2	12.4%	0.3	15.9%
Below Cut-off Test Plasma	90	0.3	0.1	–	0	–	0.0	–	0.0	–	0.1	–
Low Titer Test Plasma	90	1.4	0.1	10.1%	0	0%	0.0	1.9%	0.0	1.1%	0.1	10.3%
Mid Titer Test Plasma	90	5.5	0.4	7.8%	0.1	2.2%	0.1	2.0%	0	0%	0.5	8.3%
High Titer Test Plasma	89	9.6	0.7	7.8%	0.4	4.1%	0.3	3.3%	0.9	9.2%	1.3	13.1%

Lower Limits of the Assay

- The titer of four plasmas from healthy individuals and four plasmas from PwHA with low levels of inhibitor were measured in a **3 lot × 5 day × 3 repeat** study to gauge the limits of the assay.



- The **limit of blank (LoB)** of the assay was determined to be less than 0.1 BU/mL.
- The **limit of detection (LoD)** of the assay was determined to be less than 0.2 BU/mL.
- The **limit of quantification (LoQ)** of the assay was determined to be 0.2 BU/mL.

References

[1] Favaloro EJ, Verbruggen B, Miller CH. Laboratory testing for factor inhibitors. Haemophilia. 2014;20(S4):94-98. (DOI: 10.1111/hae.12408)

[2] Nogami K, Soeda T, Matsumoto T, Kawabe Y, Kitazawa T, Shima M. Routine measurements of factor VIII activity and inhibitor titer in the presence of emicizumab utilizing anti-idiotype monoclonal antibodies. J Thromb Haemost. 2018;16(7):1383-1390. (DOI: 10.1111/jth.14135)

[3] Blanchette VS, Key NS, Ljung LR, Manco-Johnson MJ, van den Berg HM, Srivastava A. Definitions in hemophilia: communication from the SSC of the ISTH. J Thromb Haemost. 2014;12(11):1935-1939. (DOI: 10.1111/jth.12672)

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