# The value of the chromogenic activity assay in diagnosis and therapeutic monitoring of hemophilia

By Dorothy M. Adcock, MD, Stefan Tiefenbacher, PhD, and Rajiv K. Pruthi, MBBS

Hemophilia A and B are X-linked disorders characterized by decreased functional factor VIII (FVIII) and factor IX (FIX) levels, respectively. Disease severity is classified as severe (<1% factor activity), moderate (1%–5%), or mild (6%–<40%).[1] Bleeding risk correlates with level of factor activity, and when levels fall below one percent, spontaneous bleeding occurs. The goal of coagulation factor replacement therapy is to elevate and sustain levels in order to achieve a functional factor concentration of one percent or greater.[2]

Given the importance of accurate measurement of factor activity levels in the diagnosis, classification, and therapeutic monitoring of coagulation factor replacement in hemophilia A and B, accurate assessment of FVIII and FIX activity in the clinical laboratory is critical.[3] The coagulation factor activity assay used by the majority of clinical laboratories, the one-stage assay (OSA), may underestimate or overestimate the true FVIII activity in up to 30 percent of patients with mild or moderate (non-severe) hemophilia A, and this has more recently been described as a potential issue in hemophilia B.[3-5] Furthermore, certain modified recombinant FVIII and FIX replacement products demonstrate variable and clinically significant differences in post-infusion recovery (that is, the amount of factor measured vs. the actual concentration present), based on the activated partial thromboplastin time (APTT) reagent used in the OSA or assay methodology.[6] This article will review when and why discrepancies may occur, as well as what is needed to overcome the potential for inaccurate results in FVIII and FIX measurements, in order to provide optimal patient care.

Approximately 16 percent of patients with mild hemophilia A have a normal FVIII OSA, and the correct diagnosis relies on another less commonly performed activity assay using chromogenic methodology.[3,4] Variations in one-stage activity results using different APTT reagents, as well as differences in activity results when measured in the OSA versus chromogenic FIX activity assay, have recently been described in non-severe hemophilia B patients.[5] Differences in factor activity measurements between the OSA and chromogenic assay (CSA) in non-severe hemophilia is called discrepant hemophilia. This occurs in 30 percent of patients with non-severe hemophilia A and an as-yet unknown percentage of those with hemophilia B.[3,4,7] Such discrepancies may also occur in hemophilia carriers. Although there is no universally accepted definition of what constitutes discrepant hemophilia, the generally accepted criterion is a twofold difference in results between the OSA and CSA. Either OSA results can be greater than CSA or vice versa, depending on the underlying FVIII or FIX gene mutation.[3,4,8] Both possibilities can misclassify hemophilia severity, but the former may result in a missed diagnosis.

## Hemophilia A

Discrepant hemophilia A has a genetic basis, generally due to missense mutations that affect the stability of the activated form of FVIII (FVIIIa) or the ability of FVIII to successfully bind the activated form of FIX (FIXa), von Willebrand factor, or thrombin.[3,8] Missense mutations clustered in the A1-A2-A3 domain interfaces of the FVIII protein cause reduced stability of FVIIIa, which is more apparent in FVIII activity assays where the FVIIIa is generated during a relatively long (for example, two-to-ten-minute) incubation such as the CSA or the infrequently performed two-stage assay.[4,8] In the OSA, FVIII is in the activated form for only a brief period. Missense mutations clustered around thrombin cleavage sites or FIXa binding sites are more readily identified in OSA since the factors are present at physiologic concentrations, unlike the CSA, where factor concentrations are optimized. Also, long incubation times in the CSA may help to overcome mutations that interfere with binding. The underlying mutations and discrepancies between OSA and CSA are consistent within and between discrepant hemophilia A families.

In the one-stage FVIII activity assay, patient sample is mixed with FVIII-deficient plasma and APTT reagent, and this mixture is allowed to incubate at 37°C for three to five minutes.[7] This allows contact activation but does not activate the FVIII that is present in the mixture. The mixture is recalcified, and the time to clot formation is measured in seconds and compared to a standard curve (**Figure 1**). In the CSA, patient sample is mixed with purified FIXa and FX, with or without added prothrombin or thrombin. The concentrations of these factors are optimized and are not necessarily physiologic. This mixture is incubated for five to seven minutes in

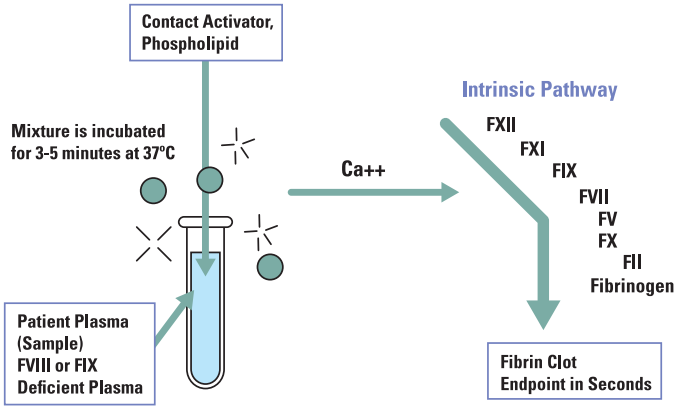


**Figure 1.** One-stage FVIII/FIX activity assay
*Image courtesy of Dot Adcock, MD, and Rajiv Pruthi, MBBS*

*continued on page 10*

## Continuing Education

To earn CEUs, see test on page 15 or online at www.mlo-online.com under the CE Tests tab.

### LEARNING OBJECTIVES

**Upon completion of this article, the reader will be able to:**

1. **Describe the pathogenicity of hemophilia.**
2. **Discuss different assays currently used to diagnose and monitor hemophilia.**
3. **Discuss the factors that contribute to inaccuracies in different factor activity test methodologies.**
4. **Describe procedures and ideas to overcome the problematic reporting of inaccurate results in patients with hemophilia.**

*continued from page 8*

order to generate FXa, and the amount of FXa produced is dependent on the amount of FVIIIa generated during this incubation.[7,9] In the second stage of the assay, the amount of FXa generated is determined using a chromogenic substrate, and this is compared to a standard curve in order to determine the FVIII activity present in the sample (**Figure 2**).

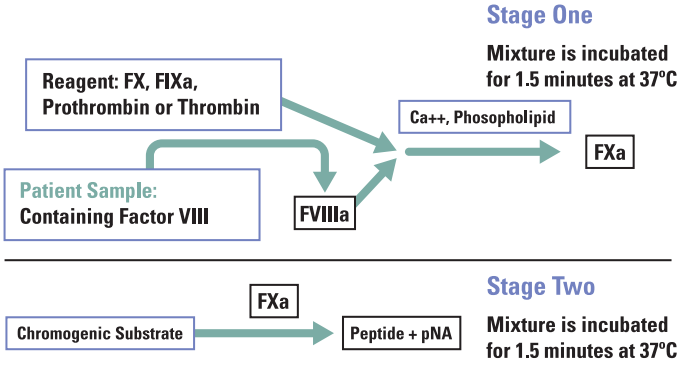


**Figure 2.** Chromogenic FVIII activity assay
pNA, p-nitroanilide
*Image courtesy of Dot Adcock, MD, and Rajiv Pruthi, MBBS*

Leaders in the field of hemophilia, as well as the International Society on Thrombosis and Hemostasis and the World Federation of Haemophilia, agree that there is an advantage to performing both the OSA and the CSA in the initial screen and evaluation for non-severe hemophilia A.[3,4,8,9] Even in those patients already diagnosed with mild or moderate hemophilia A, evaluation of both assays should be considered, as this may alter disease classification from mild to moderate hemophilia or, less commonly, from moderate to mild disease.

Accuracy of the OSA in measuring FVIII activity for post-infusion FVIII replacement therapy monitoring can also be a significant issue. Variation in recovery may occur with several of the modified recombinant FVIII (rFVIII) products, especially pegylated rFVIII factors.[10] This variation can be 50 percent greater or lower than the true value, depending on the factor replacement product and the APTT reagent used in the FVIII activity assay.[10,11] Available data to date suggest that chromogenic assays may measure these modified products more accurately, although additional study is needed.

## Hemophilia B

In a recently studied small cohort of hemophilia B patients who were cross-reacting material positive (CRM+; discrepancy between factor activity and antigen values), factor activity with the CSA was at least two times greater than that determined with the OSA.[5] Bleeding phenotype in this cohort appeared to correlate more closely with CSA results. This discrepancy was demonstrated to occur with a mutation at the N-terminal site of the activation peptide at Arg191. In another population of mild CRM+ hemophilia B patients, different FIX activity results have been described for one-stage assays using different APTT reagents.[12]

## Hemophilia A and B replacement therapies

Available information on performance characteristics of various one-stage and chromogenic factor activity assays in the measurement of modified rFVIII and rFIX products is limited, but data suggest that significant inaccuracies can exist, depending on the assay system and the specific product. Examples of modified FVIII and FIX replacement products are listed in **Table 1** and **Table 2 (page 12)**, respectively. Some information can be found in the few published field studies in full manuscript form,[13-15] selected reviews and replacement product–specific articles,[16-18] abstracts,[11,19-22] and data presented in a workshop organized by the European Medicines Agency and the European Directorate for the Quality of Medicines and HealthCare.[10]

Hemophilia centers and laboratories involved in the care of patients with hemophilia A and B must be aware of the potential for APTT reagent–dependent over- or under-recovery in the OSA, as these discrepancies can significantly impact patient care. Overestimation of post-infusion plasma factor activity can lead to underdosing of the replacement factor and an increased risk of bleeding. Conversely, underestimation of factor activity in the post-infusion sample may lead to overdosing of the replacement factor, which not only has cost implications but also places the patient at risk for thrombosis. At present, there are no simple answers as to how to overcome these discrepancies in recovery in the OSA with the different modified rFVIII and rFIX products and various APTT reagents. Discrepant recovery based on the APTT reagent used in the factor activity assay appears to be more significant with the modified rFIX products and assays compared to rFVIII products and assays. Not all modified recombinant factors demonstrate clinically significant discrepancy in recovery between APTT reagents.[13-15]

All laboratories involved in the care of patients with hemophilia A and B must be informed as to what specific coagulation factor concentrate the patient has received, and they should know how this particular factor replacement product behaves in the local laboratory OSA and CSA prior to reporting patient results. In order to correct for the variable recovery that may occur in the OSA, a number of strategies have been proposed.[6] These include use of a calibration curve in the OSA or CSA assay that consists of the particular recombinant factor product being measured in the plasma (an approach currently utilized in selected countries for a B-domain-deleted rFVIII).[23] However, this is not a favored strategy, since laboratories would need to have multiple FVIII and FIX calibrators on hand and would consistently need information on the coagulation factor product being used in each patient so the correct calibrator is used.

| Name | Modification for Half-Life Extension |
|---|---|
| Bax 855 (FL)[a] | 20-kDa branched PEG |
| rFVIII-Fc (BDD)[a] | Fusion to Fc domain of IgG1 |
| NB-GP (BDtrunc PEGylated) | 40-kDa glycoPEGylation |
| CSL 627 (BDD)[a] | Single-chain |
| BAY 94-9027 (BDD) | Site-specific 60-kDa PEG |

PEG, polyethylene glycol
[a] FDA approved

**Table 1.** Examples of Modified Factor VIII replacement products

Another option is using the standard one-stage laboratory assay and applying a correction factor specific for the recombinant factor product being measured. This, in fact, is the recommendation in the product insert for measurement of one single-chain rFVIII product.[24] Specifically, the insert reads, "If the one-stage clotting assay is used, multiply the result by a conversion factor of 2 to determine the patient's FVIII activity level." Most rFVIII and rFIX products may be accurately measured using a chromogenic assay, even when this is performed with a plasma calibrator rather than a product-specific calibrator. In certain circumstances, laboratories may need to send post-infusion plasma samples to a

*continued on page 12*

*continued from page 10*

| Name | Modification for Half-Life Extension |
|---|---|
| rFIX-Fc[a] | Fusion to Fc domain of IgG1 |
| N9-GP | 40-kDa glycoPEGylation |
| CSL 654[a] | Fusion to albumin |

[a] FDA approved

**Table 2.** Examples of Modified Factor IX replacement products

reference laboratory that performs the appropriate factor activity assay that closely aligns with the assay used to assign the potency to the specific factor replacement product.

In conclusion, preliminary data suggest that the successful development and use of modified recombinant factor replacement products have improved outcomes for patients with hemophilia. A consequence of protein engineering is the potential variable effect such modified factors can have on standard coagulation assays used to measure factor activities. It is important for hemophilia treatment centers and affiliated coagulation laboratories to be aware of these effects and to establish optimal communication to ensure that the most accurate assays are performed for the coagulation factor replacement therapies being used. A challenge for all clinical laboratories is that such information may not be easily transmitted. Going forward, close cooperation among pharmaceutical companies, reagent vendors, and regulatory agencies will be required to identify specific factor assays that are capable of accurately measuring the modified recombinant factor replacement products in the clinical laboratory. Available data to date suggest that FVIII and FIX chromogenic assays may provide more accurate results; however, they are currently available in only a few clinical laboratories. The implementation of chromogenic FVIII and FIX assays in the clinical laboratory also plays an important role in the diagnosis and classification of non-severe hemophilia A and B due to the potential for discrepant results between methodologies, potentially leading to missed diagnosis or misclassification.

## REFERENCES


1. Blanchette VS, Key NS, Ljung LR, Manco-Johnson MJ, van den Berg HM, Srivastava A; Subcommittee on Factor VIII, Factor IX and Rare Coagulation Disorders of the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis. Definitions in hemophilia: communication from the SSC of the ISTH. *J Thromb Haemost.* 2014;12(11):1935-1939.
2. Nilsson IM, Hedner U, Ahlberg A. Haemophilia prophylaxis in Sweden. *Acta Paediatr Scand.* 1976;65(2):129-135.
3. Oldenburg J, Pavlova A. Discrepancy between one-stage and chromogenic factor VIII activity assay results can lead to misdiagnosis of haemophilia A phenotype. *Hemostaseologie.* 2010;30(4):207-211.
4. Potgieter JJ, Damgaard M, Hillarp A. One-stage vs. chromogenic assays in haemophilia A. *Eur J Haematol.* 2015;94(Suppl 77):38-44.
5. Kihlberg K, Strandberg K, Ljung R, Berntorp E, Astermark J. Discrepancies between the one-stage clotting assay and the chromogenic assay in haemophilia B. *Hemophilia.* 2016;22(Suppl 4):70.
6. Dodt J, Hubbard AR, Wicks SJ, et al. Potency determination of factor VIII and factor IX for new product labelling and postinfusion testing: challenges for caregivers and regulators. *Haemophilia.* 2015;21(4):543-549.
7. Peyvandi F, Oldenburg J, Friedman KD. A critical appraisal of one-stage and chromogenic assays of factor VIII activity. *J Thromb Haemost.* 2016;14(2):248-261.
8. Pavlova A, Delev D, Pezeshkpoor B, Muller J, Oldenburg J. Haemophilia A mutations in patients with non-severe phenotype associated with a discrepancy between one-stage and chromogenic factor VIII activity assays. *Thromb Haemost.* 2014;111(5):851-861.


*continued on page 14*