A Factor VIII Chromogenic Assay System for Measuring Emicizumab and Factor VIII Inhibitors in Plasma

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Assays for Measuring FVIII and FVIII-like Activities

• Recent technological and protein engineering developments have introduced improved substitutive FVIII replacement therapies: recombinant proteins, extended half-life products, engineered antibodies with FVIII-like activity (Emicizumab: injected subcutaneously, insensitive to FVIII Inhibitors).

• Emicizumab is a bispecific antibody, binding to FIXa and FX, which expresses a FVIII-like response.

• One-stage clotting assays present variable recoveries for all these products, and a unique reference material cannot be used. A much better response homogeneity is obtained with chromogenic assays, designed with human or bovine purified proteins (mainly FIXa and FX).

• FVIII assays are used not only for testing FVIII activity but also for measuring titrating FVIII inhibitors in patients with FVIII neutralizing antibodies.

• A combined chromogenic FVIII assay system is presented for measuring Emicizumab or FVIII inhibitors in treated patients’ plasmas.
Reagents and Material

• Normal citrated plasma pool (Cryocheck, PBI)
• Emicizumab plasma calibrator (100 µg/ml Emicizumab in FVIII deficient plasma), lyophilized (R² Diagnostics)
• Factor VIII deficient plasma (HYPHEN BioMed)
• Anti-Factor VIII IgGs (~ 128 BU/mg)
• APTT assay: Cephen (HYPHEN BioMed)
• Biophen FVIII: chromogenic assay for FVIII:C activity, designed with human FIXa, human FX, human thrombin and synthetic phospholipids
• Biophen Factor VIII variant assay: same assay designed by replacing human FX with bovine FX
• Automated coagulation analyzer: CS-5100 Sysmex
Two FVIII chromogenic assays proposed

Assay principle based on FX generation induced by FIXa (constant), FVIII (to be tested = limiting), phospholipids, thrombin (traces) and calcium, then measurement of FXa with a specific chromogenic substrate

- First assay designed with **human** Factor X (**FVIII-HFX**)
- Second assay designed with **bovine** Factor X (**FVIII-BFX**)

**FVIII-HFX** measures both FVIII and Emicizumab FVIII-equivalent activity in tested plasmas. If used for testing FVIII inhibitors (neutralizing antibodies), presence of Emicizumab interferes in the assay and inhibitor potency is underestimated.

**FVIII-BFX** is insensitive to Emicizumab; presence of human FX in the tested plasma does not interfere in the assay: it can be used for titrating FVIII inhibitors.
Dose-response curves of FVIII in a normal plasma pool (blue curve) or Emicizumab (100 µg/ml) in FVIII-Deficient Plasma (red curve) as tested with FVIII-HFX.

- 100 µg/ml Emicizumab generates a FVIII « equivalent » activity of about 0.67 IU/ml (i.e. ~ 6.7 IU/mg Emicizumab)

Dose-response curves of Plasma FVIII (blue curve) or Emicizumab in FVIII deficient plasma (red curve) tested with FVIII-BFX:
- No reactivity of Emicizumab with FVIII-BFX, whether is the concentration tested.
- High reactivity of human plasma FVIII.
With the one-stage FVIII clotting assay, Emicizumab has a much higher FVIII equivalent activity than with the chromogenic assay, and than that of FVIII (same plasma dilution). About 5µg/ml Emicizumab generate a 1,00 IU/ml (100%) FVIII:C equivalent clotting activity (i.e. ~ 200 IU/mg Emicizumab)
Reactivity of FVIII with Emicizumab using Biophen FVIII (HFX or BFX)

A normal plasma pool is mixed (vol/vol) with FVIII deficient plasma containing Emicizumab at 100 µg/ml, then dilutions in FVIII deficient plasma are tested with Biophen FVIII HFX (blue curve) or BFX (red curve)

Emicizumab strongly interferes in the FVIII measurement with HFX (measures both FVIII and Emicizumab activities), but not in that with BFX (only measures FVIII:C activity).
Reactivity of Emicizumab with Biophen FVIII (HFX or BFX)

Emicizumab plasma calibrator dilutions in FVIII deficient plasma are tested with Biophen FVIII HFX (blue curves) or BFX (red curves).

Emicizumab is measured with FVIII-HFX, but not with FVIII-BFX.
Recovery of FVIII in presence of Emicizumab (FX = 1 IU/ml) with FVIII-BFX

When FVIII:C is measured in dilutions of a normal plasma pool with a FVIII: DP containing 100 µg/ml Emicizumab with FVIII-BFX, there is no interference in FVIII:C recoveries.
Matrix Effect (FVIII-HFX)

BIOPHEN™ FVIII:C (Human FX)
BIOPHEN™ Plasma Calibrator diluted with FVIII:C Deficient plasma or assay buffer

- BIOPHEN™ FVIII:c (Human FX)
  - Low range of BIOPHEN™ Plasma Calibrator in Buffer

- BIOPHEN™ FVIII:c (Human FX)
  - Low range of BIOPHEN™ Plasma Calibrator in FVIII:c Deficient plasma

R² = 0.9924
R² = 0.997
Matrix Effect FVIII-BFX

BIOPHEN™ FVIII:C (Bovine FX)

BIOPHEN™ Plasma Calibrator diluted with FVIII:C Deficient plasma or assay buffer

- OD at 405nm
- FVIII:C Concentration (%)

BIOPHEN™ FVIII:c Variant (Bovine FX)
Low range of BIOPHEN™ Plasma Calibrator in Buffer

BIOPHEN™ FVIII:c Variant (Bovine FX)
Low range of BIOPHEN™ Plasma Calibrator in FVIII:c Deficient plasma

R² = 0.9697
R² = 0.9936
No Interference of FVIII Inhibitors in Emicizumab Measurement with FVIII-HFX

The same experiment with BFX gives no signal, as expected.
Titration of Plasma with FVIII Inhibitors using FVIII-HFX

**Titration of FVIII Inhibitors with the Bethesda Method:**

- When FVIII-HFX chromogenic assay is used, Emicizumab interferes in the titration, especially at low plasma dilutions

- Inhibition titer estimated at about 100 BU/ml (underestimation much higher for low titers)
Titration of Plasma with FVIII Inhibitors using FVIII-BFX

Titration of FVIII Inhibitors with the Bethesda Method:

• When FVIII-BFX chromogenic assay is used, Emicizumab has no incidence for the inhibitors’ titration

• Inhibition titer estimated at about 150 BU/ml
Discussion

The combined use of FVIII chromogenic assays designed with human or bovine FX are convenient and practical laboratory tools for testing FVIII and Emicizumab activities, or titrating FVIII inhibitors.

• FVIII and Emicizumab are measured with the HFX FVIII chromogenic assay (100 µg Emicizumab generates a FVIII activity of about 0.65 IU FVIII eq., whilst the one-stage clotting assay gives > 10 IU/ml).

• No interference of Emicizumab or endogenous FX in the FVIII chromogenic assay with BFX.

• Titration of FVIII antibodies (Bethesda method) possible with the FVIII chromogenic assay using BFX.
Conclusions

Proposed FVIII:C Chromogenic assay system, combining use of Human FX (sensitive to Emicizumab) or Bovine FX (insensitive to Emicizumab):

• Assays appropriate for measuring FVIII activity of the various new FVIII substitutive products available.

• Assays fully automatable on all coagulation instruments, especially the major platforms: CS, BCS: XP/Atellica, ACL-Top and STA-R.

• Measurement of Emicizumab in treated hemophiliacs (use of FVIII-HFX), and of Anti-FVIII inhibitors in treated patients (use of bovine FVIII-BFX).