Two-Stage Clotting Assay for Factor VIII:C

**PRINCIPLE**

This assay was first described in 1955 as a modification of the thromboplastin dilution test. The two-stage FVIII:C assay is based on the principle that the amount of FVIII present in the system is rate-limiting during clotting of a test mixture containing FX, activated FIX, phospholipid, calcium, and FV in excess. Adsorption of plasma by aluminium hydroxide removes activated factors and vitamin K–dependant factors. This is necessary to remove prothrombin so that none is present in the initial incubation mixture. Without this step, the mixture would contain all the components required for fibrin to form, and the mixture would clot.

The dilutions of adsorbed standard and test plasma are incubated with the combined reagent in the 1st stage. This generates FXa. A source of prothrombin and fibrinogen from pooled normal plasma is added in the 2nd stage, which allows a clot to form and for which the resulting clotting time is dependent on the initial amount of factor VIII:C.

**REAGENTS**

- Combined reagent (see “Production of Combined Reagent” on page 73)
- Owren’s Veronal buffer
- Pooled normal plasma (substrate plasma): store deep frozen
- Standard or reference plasma
- Internal quality control (IQC)
- 0.0125M CaCl₂ (for example, 1 in 2 dilution of 0.025M CaCl₂ as used in APTT testing)
- Alumina hydroxide suspension (e.g. SIGMA catalogue code A8222): store at room temperature

**SAMPLE REQUIREMENTS**

Citrated plasma, which can be stored frozen prior to testing, if required.
METHOD

1. Reconstitute standard plasma with distilled water 10 minutes before use, and combined reagent at least 15 minutes before use with 3 ml of 0.0125M CaCl₂.

2. Prepare IQC plasma and substrate plasma. If any are frozen, thaw at 37°C for 5 minutes before use.

3. Label sufficient disposable plastic tubes for each of the samples, IQC, and standard.

4. Place 0.45 ml of standard, IQC, and patient plasma into each plastic tube.

5. Add 50 μl of well-shaken alumina to each tube and mix well.

6. For small volumes of plasma, use 0.225 ml plasma and 25 μl alumina.

7. Incubate at 37°C for 3 minutes, then spin at 5000 g for 2 minutes in a suitable centrifuge.

8. Immediately transfer the supernatant plasma to plastic cups or tubes compatible with the analyser being used. Take care not to disturb the sedimented alumina.

Note: The following steps are based on use of a Sysmex CA series analyser. The test can be run on instrumentation from a number of other manufacturers. One co-author of this manual has successfully run assays on analysers from Instrumentation Laboratory, and most likely the method would be compatible with other types of analyser. Therefore, this specific method is included as an illustrative example.

9. Load combined reagent, assay buffer, and pooled normal plasma/substrate onto autoanlyser.

10. Adsorbed plasmas are presented for analysis in the following order: standard, IQC, patient plasmas, and lastly, a second standard. Samples with normal concentrations of FVIII are normally assayed using three different dilutions in the range of 1/50–1/400.

For low levels of FVIII (< 0.05 IU/dl) dilutions of 1/10, 1/20, and 1/50 might be needed. For raised levels (>1.5 IU/dl), dilutions of 1/800–1/3200 might be needed. Otherwise, patient dose response lines may not be parallel to the standard line.

RESULTS

Plotting of results and calculations of FVIII activity are as described in the one-stage assay (Section 23).
25.1 Production of Combined Reagent for Two-Stage Factor FVIII:C Assay

Obtain sufficient FV and phospholipid to produce a 5:1:1 ratio with diluted aged serum (i.e. 5 parts serum to 1 part FV and 1 part phospholipid). For example, 240 ml diluted serum, 48 ml FV, and 48 ml phospholipid (or 180 ml:36 ml:36 ml).

**REAGENTS**
- Aged serum
  1. 10 ml blood taken from at least 6 normal donors in plain glass tubes; no additives.
  2. Incubate for 4 hours at 37°C, then overnight at 4°C.
  3. Spin at 3000 rpm for 10 minutes.
  4. Separate and mix supernatant in non-siliconized glass (can be stored frozen).
- Bovine FV (Diagnostic Reagents Ltd., Thame, Oxfordshire, England) Reconstitute each with 1 ml glyoxaline buffer.
- Phospholipid (Bell and Alton, Diagnostic Reagents Ltd., Oxford England) Reconstitute each with 1 ml glyoxaline buffer (i.e. 5x concentration).
- Glyoxaline buffer
- HEPES Acid (Sigma H3375)

**METHOD**
1. Dilute aged serum 1/10 with glyoxaline buffer.
2. Activate diluted serum for one hour by adding one small glass ball (ballotini ball) per ml, and place on rotary mixer for one hour with lab film over the top. (Note: 100 ballotini balls weigh 3.7 g).
3. Reconstitute FV plasma in 1 ml glyoxaline buffer.
4. Reconstitute phospholipid in 1 ml glyoxaline buffer.
5. Mix diluted activated serum, FV, and phospholipid in a 5:1:1 ratio.

Note: for steps 3 to 8, work as quickly as possible.
6 Mix well and add HEPES powder, giving a concentration of 1% to stabilize the pH at 7.0 (e.g. if 300 ml fluid, add 3 g HEPES).

7 Dispense into 0.5 ml amounts.

8 These can be lyophilized (see Section 40 on lyophilization).

9 Test new reagent for optimum conditions, as described below.

ASSESSMENT OF FXA GENERATION PLATEAU

The optimum sample dilution, incubation time, total volume in the assay, volume of 0.0125M CaCl₂ for reconstitution, length of reconstitution, and stability of the reconstituted combined reagent need to be systematically assessed to obtain a FXa generation plateau.

The order and extent that the following occur is not definitive. They are subject to alteration, depending on the batch. A good starting point is to use the same conditions that the current batch uses, then change one parameter at a time.

- Reconstitution volume with 0.0125M CaCl₂: 0.5 ml, 1 ml, 1.5 ml, 2 ml, 2.5 ml, 3 ml, 3.5 ml.
- Length of reconstitution prior to use/stability after reconstitution: Immediately before use, 5 minutes, 10 minutes, 30 minutes, 60 minutes, 120 minutes.
- Incubation time: 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes.
- Sample dilution: 1/20, 1/50, 1/80, 1/100.
- Volume of combined reagent in assay: 20 μl, 30 μl, 40 μl, 50 μl, etc.

For each assay run, plot all results on log-log graph paper, as per the example in Figure 25.1. Select dilutions and conditions that have the most points on the linear part of the curve. Ideal conditions have clotting times around 20 to 30 seconds for the first dilution.

NOTES

- Pooled normal plasma/substrate is pooled from residual normal plasma from normal clotting screens or from healthy normal subjects.
- Constituent plasmas should have normal PT and normal APTT.
- Pooled normal plasma can be stored in pools of 3 ml or 5 ml in plastic vials at -80°C for at least six months.
Figure 25.1. Example of FXa generation plateau