36 Factor XIII Activity Assay

PRINCIPLE
Fibrinogen is coated on the surface of a microtitre plate. Non-specific binding is prevented by a special blocking agent. FXIII in the sample is activated by thrombin and calcium ions. In the incorporation step, FXIIIa in the test plasma incorporates the substrate (5-biotinamidopentylamin) BAPA into FXIII substrate fibrinogen coated on the plate in the presence of calcium. The amount of incorporated BAPA is proportional to the FXIII activity of the test sample. In the next step, a conjugate Strept-AP (streptavidine-alkaline phosphatase) is bound to the incorporated BAPA. Alkaline phosphatase converts the synthetic substrate pNPP (p-nitro phenyl phosphate) into phosphate and p-nitrophenol, which can be measured at 405 nm.

The reagents for the method described below are commercially available in kit form (Pefakit FXIII incorporation assay, Pentapharm Switzerland). Note that other activity assays are available from other manufacturers, including the Berichrom kit (Dade Behring, Marburg, Germany), which employ different principles of analysis.

REAGENTS
All the required reagents are contained in the commercial kit.

METHOD
Day 1
1. Allow kit components to come up to room temperature for 30 minutes.
2. Reconstitute coating reagent (R2) in distilled water, according to the volume recommended by the manufacturer.
3. Add 100 μl coating reagent per well to empty wells of the microtitre plate strips.
4. Freeze any excess coating reagent for subsequent use. It remains stable for six months at -20°C.
5. Seal strips with provided plastic seal and incubate overnight (14 to 16 hours) at temperatures of 20°C–25°C.
Day 2

6 Dilute 20 x concentrated TBS R1 (Tris buffered saline) 50 ml in 950 ml distilled water or lesser volume, if required.

7 Dilute 3 ml of blocking reagent R3 with 27 ml diluted TBS R1. Freeze excess R3.

8 Discard coating reagent from microtitre plate, invert the strip, and tap on tissue to remove residue.

9 Add 300 μl diluted blocking reagent to each well.

10 Incubate for 1 to 1.5 hours at 37°C in an incubator.

11 Reconstitute calibrator R10 in 0.5 ml distilled water and the three controls, R11, R12, and R13, in 0.2 ml distilled water.

12 Thaw any frozen test plasmas at 37°C for five minutes prior to analysis.

13 Prepare a container with a few hundred ml of ice/water mixture as an ice bath.

14 Make dilutions of all test and control plasmas, 10 μl plasma, and 1 ml diluted TBS R1 buffer (1:101 dilution). Vortex mix.

15 Make calibrator dilutions as follows:
   - Cal 1: 30 μl R10 + 970 μl TBS R1
   - Cal 2: 20 μl R10 + 980 μl TBS R1
   - Cal 3: 75 μl R10 + 25 μl TBS R1
   - Cal 4: 25 μl R10 + 75 μl TBS R1
   - Cal 5: 10 μl R10 + 90 μl TBS R1

   Note: Dilutions 1 and 2 are ready to use. Further dilute 10 μl of calibrator dilutions 3-5 in 1 ml TBS R1.

16 Wash plate three times with 300 μl/well TBS R1. Invert and tap on tissue to remove excess liquid.

17 Reconstitute activator reagent part A (R4) and part B (R5) in 5 ml distilled water each. Keep on melting ice/water ice bath for no more than 30 minutes.

18 Add 25 μl each of calibrator, control, or test plasma into appropriate wells. Include a blank of TBS R1.

19 Mix activator reagents part A and B (R4 and R5) to form the final incorporation reagent.
20 Add 75 μl final incorporation reagent to each well, including blank well.

21 Incubate for 30 minutes at 37°C in incubator.

22 Add 200 μl/well incorporation stopping solution R6. Mix gently for 10 minutes on the plate shaker.

23 Reconstitute detection reagent R7 by adding 12 ml distilled water. Freeze unused diluted R7.

24 Wash plate four times with 300 μl/well TBS. Tap to remove excess liquid.

25 Add 100 μl/well detection reagent R7. Incubate for 15 minutes at 37°C in the incubator.

26 Wash plate four times with 300 μl/well TBS. Tap to remove excess liquid.

27 Make up substrate solution immediately before use:
   - For 96 wells (full plate), add 9 tablets R8b to 22.5 ml diethanolamine buffer R8a
   - For 64 wells (8 strips), add 6 tablets to 15 ml diethanolamine
   - For 32 wells (4 strips), add 3 tablets to 7.5 ml diethanolamine
   - For 24 wells (3 strips), add 2 tablets to 5 ml diethanolamine

28 Add 180 μl/well of substrate solution. Incubate for 11 minutes at 37°C in the incubator.

29 Add 50 μl/well stopping solution (4M NaOH) R9.

30 Read optical densities within 15 minutes at 405 nm in a microtitre plate reader.

**Note:** Several kit reagents can be stored deep-frozen for later use, as described above. However, the substrate, activator reagent parts A and B, calibrators, and controls should not be frozen. Partial reagent kits containing these latter materials can be purchased for use with any partially used reagents that have been frozen. This reduces the cost per test if test samples are analysed in small batches.

**RESULTS CALCULATION**

Calibrator dilutions and control values are supplied with each kit.

Using a suitable data handling software or graph paper, construct a calibration curve by plotting the concentration against the optical density of the calibrator dilutions after subtracting the OD of the blank. Use a linear-linear scale. Subtract the blank OD from the ODs of the test sample/control dilutions, and convert the ODs to FXIII activity, using the calibration curve.