PART 9 Laboratory Investigation of Other Coagulation Factors

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TOPICS COVERED

- ✓ Assays Based on PT (One-Stage Assay of FII, V, VII and X)
- ✓ Assays Based on APTT (One-Stage Assay of FXI, FXII, PKK, or HMWK)
- ✓ Factor XIII: Screening, Activity, and Antigen

Assays Based on PT (One-Stage Assay of FII, V, VII, and X): Deficiencies of clotting factors II, V, VII, or X are rare bleeding disorders (Mumford et al, 2014). The assays for factor II, V, VII, or X activity can be performed using a one-stage assay based on PT. The assay compares the ability of dilutions of a standard or reference plasma and test plasma to correct the PT of a plasma known to be totally deficient in the clotting factor being measured. In FV assay, for example (described below), the plasma is deficient in FV but contains normal amounts of all other clotting factors including II, VII, X, and fibrinogen. Clotting factors II, VII, and X may be assayed in a similar way, substituting FV-deficient plasma in the example given below with the corresponding deficient plasma, and using a reference (standard) plasma with a known concentration of the factor being assayed (Baker et al, 2020).

Reagents:

- ✓ FV-deficient plasma. This may be congenitally deficient or artificially deficient in FV (aged plasma).
- ✓ Owren's veronal buffer (OVB).
- ✓ Platelet-poor citrated test plasma.
- ✓ For the standard, a commercial reference (standard) plasma is preferred or if unavailable use a 20-donor platelet poor normal plasma pool (kept at -70°C or below).
- ✓ Internal quality control plasma-either commercial or locally sourced (CLSI, 2016).
- ✓ Thromboplastin reagent which should contain calcium chloride. It is best practice to use the same thromboplastin as used in the PT test but an alternative thromboplastin may be used for diagnosis of unusual patients.

Method: Decant sufficient thromboplastin reagent into a 75 x 12 mm glass tube. Allow to warm to 37°C for 5 minutes. If the thromboplastin reagent does not contain calcium, this needs to be added separately. Decant sufficient M/40 $CaCl_2$ into a 75 x 12 mm glass tube. Allow to warm to 37°C for 5 minutes. For both tests, QC, and standard plasmas, prepare dilutions in plastic tubes, as shown in Table 32.

Table 32. Preparation of test, QC, and standard plasma dilutions for one-stage assays of FII, FV, FVII, and FX

Dilution	Plasma (ml)	OVB (ml)
1/5	0.1	0.4
1/10	0.1	0.9
1/20	0.5 (1/10 dilution)	0.5
1/40	0.5 (1/20 dilution)	0.5

Note: Mix the 1/10 dilution well before using it to prepare the 1/20 dilution. Mix the 1/20 dilution well before using it to prepare the 1/40 dilution. Plasma dilutions should be tested immediately after preparation. If room temperature exceeds 25°C, it may be necessary to keep dilutions on melting ice prior to analysis.

Test each dilution of reference (standard/calibrator) plasma as follows:

✓ Pipette 0.1 ml of 1/10 dilution into a 75 x 10 mm glass tube.
✓ Add 0.1 ml FV-deficient plasma.
✓ Warm to 37°C for 2 minutes.
✓ Add 0.2 ml pre-warmed thromboplastin reagent.
✓ Start stopwatch and mix.

Note: If the thromboplastin reagent does not contain calcium, 0.1 ml of thromboplastin is added to the mixture of dilution and deficient plasma. After a 1- to 2-minute delay for warming to 37°C, the mixture is clotted with 0.1 ml pre-warmed (to 37°C) calcium.

Record clotting time:

- Repeat the 1/10 dilution then test 1/20 and 1/40 dilutions in duplicate.
- Repeat for dilutions of test plasma and QC. Test in duplicate.
- For test plasmas expected to be normal, test 1/10, 1/20, and 1/40 dilutions. For test plasmas expected to have reduced levels, test 1/5, 1/10, and 1/20 dilutions.
- A "blank" should also be tested as follows:
  - 0.1 ml OVB
  - 0.1 ml FV-deficient plasma
  - 0.2 ml thromboplastin/calcium reagent
- The clotting time of the "blank" reflects the quality of the deficient plasma and should be equivalent to less than 1 IU/dl (<0.01 IU/ml).

Results: Take an average of each duplicate result. The duplicate times must be within 10% of each other to be acceptable. On 3 cycle x 2 cycle logarithmic paper, plot clotting times of reference, test, and QC plasmas against percentage concentration of FV. The 1/10 dilution is arbitrarily assigned a value of 100%, thus the1/20 is equivalent to 50%, 1/40 to 25%, and 1/5 dilution to 200%. Alternatively, plot concentration on a logarithmic scale and clotting time on a linear scale. The relative amount of FV in the test plasma compared with the reference plasma is extrapolated from the graphs. An example of this is shown in the section on APTT-based assays. The clotting time equivalent to 100% test (the place where the test line passes through the 100% activity) is read from the standard line (therefore, the concentration of standard that could give that particular clotting time). This gives the concentration of the test in percentage of standard. This percentage is multiplied by the value of concentration of clotting factor in the standard plasma (in IU/dl) to give the concentration in the test (in IU/dl).

Notes: Low levels of FII, FV, FVII, or FX activity can be measured in patients with liver disease (Kujovich et al, 2015). The adult reference range for each of these clotting factors should be determined locally, but often has a lower limit of 50–70 IU/dl for FV, FVII, and FX. The lower reference limit for FII is higher (Appel et al, 2012; Wakeman et al, 2005). FVII activity can increase during pregnancy (Fu et al, 2022). Individuals with a reduced level of FV should also have a FVIII activity assay performed to exclude combined FV and FVIII deficiency (Zheng et al, 2013). The vitamin K-dependent factors II, VII, and X may naturally be reduced at birth, rising throughout childhood to reach adult levels. Pediatric reference ranges can be established locally or taken from the literature taking account of reagent variation (Toulon et al, 2016; Attard et al, 2013; Di Felice et al, 2022). In some cases of FVII deficiency (FVII Padua, FVII Nagoya, FVII Tondabayashi/Shinjo), there may be a discrepancy between the levels of FVII:C obtained, depending on the source of thromboplastin. The use of human thromboplastin is therefore advisable on the basis that the results are more likely to reflect the in vivo activity. In some rare cases, the result may be very low if rabbit thromboplastin is used, but higher or normal if the assay utilizes human or ox brain thromboplastin. This may be a reason why some cases of apparent severe FVII deficiency do not have bleeding symptoms (Balluet et al, 2020; Sevenet et al, 2017).

Assays Based on APTT (One-Stage Assay of FXI, FXII, PKK, or HMWK): FXI deficiency is a rare bleeding disorder (Mumford et al, 2014), whereas deficiencies of the contact factors FXII, PKK, and HMWK are not associated with bleeding (Maas et al, 2018), but do cause a significantly prolonged APTT. The one-stage APTT-based clotting assay for FXI is described in this section. The assay is based on a comparison of the ability of dilutions of standard and test plasmas to correct the APTT of a plasma known to be totally deficient in FXI but containing all other factors required for normal clotting. For factors FXII, PKK, and HMWK, the assay is essentially the same as that of the one-stage FXI but is performed by substituting the relevant deficient plasma for FXI-deficient plasma, and the selection of the appropriate reference plasma. The APTT reagent used for assay of PKK cannot use ellagic acid as an activator.

Reagents:

- ✓ Platelet-poor citrated test plasma.
- ✓ Standard (reference) plasma.
- ✓ The standard (reference/calibrator) plasma used should be either a locally prepared plasma pool kept at -40°C or lower, or a commercial standard plasma. In either case, this reference plasma must be calibrated for clotting FXI assay against the current international standard for FXI in plasma. It is not acceptable to assume that a pooled normal plasma has 100 IU/dl FXI activity.

Internal quality control plasma (Baker et al, 2020):

- ✓ FXI-deficient plasma.
- ✓ This is available commercially or may be collected from a severely deficient donor under the following conditions.
- ✓ FXI level is less than 1 IU/dl, no history of anti-FXI antibodies, received no treatment for 2 weeks including plasma normal liver function tests.
- ✓ Abnormal liver function could lead to reduction in other clotting factors, which affect the specificity of the assay. This plasma can be stored in aliquots at -20°C or lower for approximately 3 months (Zhao et al, 2018; Woodhams et al, 2001).
- ✓ It is preferable to use FXI-deficient plasma produced by immunodepletion of FXI from normal plasma using a monoclonal antibody. This type of material is available commercially and has the advantage of enhanced viral safety compared with plasma sourced from patients who have been treated with plasma-derived products.
- ✓ However, not all immunodepleted plasmas are found to be <1 IU/dl, and care should be taken to check this before use.
- ✓ APTT reagent that is sensitive to factor deficiencies (CLSI, 2016). Note that APTT reagents that are activated by ellagic acid are insensitive to PKK deficiency.
- ✓ Owren's buffered saline (OBS or glyoxaline buffer).
- ✓ 25mM $CaCl_2$ (note that Werfen $CaCl_2$ supplied with SynthASil is 20mM).

Method:

- ✓ For FXI, FXII, and HMWK: Make 1/10 dilutions of standard, QC, and test plasma in buffered saline in plastic tubes. (If the test plasma is expected to have a very low level of factor, start at a 1/5 dilution.) Using 0.2 ml volumes, make doubling dilutions in OBS of standard, QC, and test plasma from 1/10 to 1/40 in plastic tubes. (Mix each dilution well before transferring to next tube.) Plasma dilutions should be tested immediately after preparation. If room temperature exceeds 25°C, it may be necessary to keep dilutions on wet ice prior to testing.
- ✓ For PKK only, higher dilutions of 1/100, 1/200 and 1/400 are usually required. Test the standard plasma first. Pipette 0.1 ml of each dilution of standard into a 75 x 10 mm glass tube. Add 0.1 ml of FXI-deficient plasma, mix and transfer to 37°C water bath. Add 0.1 ml of APTT reagent, mix and incubate for 2-5 minutes depending on the recommended incubation time of the APTT reagent. At the end of the incubation time add 0.1 ml $CaCl_2$, mix tilt tube until a clot is visible.

Record the clotting time. Repeat steps 4-7 using QC then test plasma. A "blank" should also be set up using 0.1 ml of OBS in place of test plasma. The clotting time of the blank should be longer than the time of 1% FXI activity of standard read from the calibration graph. If the time is shorter, this indicates that the deficient plasma is not totally deficient in FXI and thus is not a suitable substrate plasma.

Results: Plotting of results is the same as for assays based on PT (described above), requiring double logarithmic or logarithmic/linear scale graph paper. For FXI, FXII, and HMWK, the 1/10 dilution is arbitrarily assigned a value of 100%, the 1/20 dilution a value of 50%, and the 1/40 dilution a value of 25%. If used, a 1/5 has a value of 200%. For PKK, the 1/100 dilution is arbitrarily assigned a value of 100%, the 1/200 dilution a value of 50%, and the 1/400 dilution a value of 25%. If used, a 1/50 has a value of 200%. Straight lines, parallel to each other, should be obtained. Read off concentration of QC and test sample as for assays based on PT (described above). In this example, the FXI concentration in the test sample is 7% of that in the standard. If the standard has a concentration of 85 IU/dl, the calculation is 85 IU/dl x 7% = the test sample has a concentration 6 IU/dl. If the lines are not parallel, the assay should be repeated. Check the clotting times of the test sample. If very long, then test a 1/5 (or 1/50 for PKK) dilution. Non-parallel lines may occur due to technical error. If technical error has been eliminated, it may be due to the presence of an inhibitor, which may act specifically against FXI or may be of the "lupus type", showing a converging pattern. Diverging lines are typical of an activated sample (Baker et al, 2020).

Notes: Deficiencies of FXII, PKK, or HMWK are not associated with an increased bleeding risk. Ellagic acid-activated APTT reagents are insensitive to PKK deficiency. If the test plasma FXI, FXII, PKK, or HMWK concentration is close to zero (i.e. the clotting times of all dilutions are similar to the blank), non-parallel lines may occur. The normal reference range should be established locally but often has a lower limit of 50–70 IU/dl for each of these factors. International Units have now been established for FXI and FXII in plasma, but there are no plans to establish international units for PKK or HMWK.

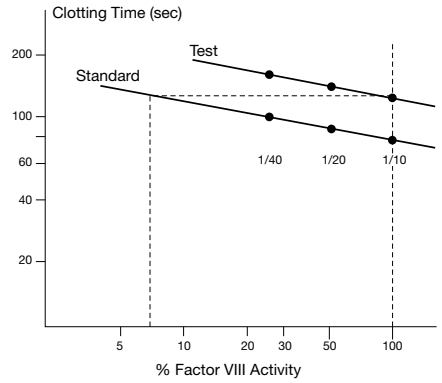

Figure 20. Graph of FXI assay

Factor XIII (FXIII): Screening, Activity, and Antigen

FXIII Screen (clot solubility tests): FXIII mediates cross linking of α and γ chains of fibrin. FXIII deficiency is classified as a rare bleeding disorder and may be caused by mutations in either FXIII-A catalytic subunits or FXIII-B carrier subunits (Karimi et al, 2018). Fibrin clot solubility qualitative tests are not currently recommended for use due to lack of standardization and poor sensitivity. It is recommended that FXIII deficiency is diagnosed using a functional FXIII activity assay rather than clot solubility (Mumford et al, 2014; Palla et al, 2015; Kohler et al, 2011) but in some areas of the world only clot solubility assays are available. Clot solubility tests containing 5M urea are more specific than those containing acetic acid, however, hypofibrinogenemia and dysfibrinogenemia can cause false positive results in the urea-based test (Dorgalaleh et al, 2016). Clot solubility tests containing 1% monochloroacetic acid (MCA) are more

sensitive and faster than urea-based tests (Dorgalaleh et al, 2016). It has been suggested that both urea- and acetic acid-based tests should be performed in parallel to optimize diagnosis (Dorgalaleh et al, 2016). A positive result will only be observed in severe FXIII deficiency, at FXIII activity of <5-10 IU/dl, depending on the method used.

Method: Thrombin and calcium are required to activate FXIII such that it will cross-link fibrin into a stable form. In this method, despite using citrated plasma, sufficient calcium ions are still available for FXIII activation. A normal ethylenediaminetetraacetic acid (EDTA) anticoagulated plasma is used for a control. In this plasma, EDTA results in a complete chelation of calcium ions, which means that the FXIII is not able to crosslink fibrin. Addition of 2% acetic acid or 5M urea results in the lysis of non-cross-linked clots, whereas citrated plasma with >10 U/dl of FXIII activity has an insoluble clot. The test is generally more sensitive if acetic acid (rather than urea) is employed, since the clot will dissolve at higher levels of FXIII in the presence of acetic acid (Jennings et al, 2003).

Materials/reagents:

- ✓ 75 x 10 mm glass tubes
- ✓ 0.9% saline
- ✓ 30 U/ml thrombin
- ✓ Normal EDTA plasma
- ✓ 2% acetic acid

Method: Add 0.2 ml test citrated plasma to 0.2 ml 0.9% saline in a glass tube. For positive control, repeat with 0.2 ml EDTA plasma. For a negative control, repeat with 0.2 ml normal citrated plasma. Add 0.1 ml of 30 U/ml thrombin, mix. Leave for 30 minutes at 37°C. Flick tubes to loosen clots from sides. Add 5 ml 2% acetic acid and stopper the tube. Leave at room temperature for 12 hours.

Results: EDTA plasma should have no visible clot. Normal citrated plasma should have an intact, visible clot. If clot is not visible, the subject has FXIII deficiency.

Normal range: Normal subjects have a visible clot after 12 hours in 2% acetic acid.

Notes: 5M urea can be used in place of 2% acetic acid. The incubation time for clot dissolution is then 18 hours. This method is less sensitive but more specific than acetic acid (described above). Clotting with calcium and lysis with urea produces abnormal results only when levels of FXIII are below 5 U/dl. By comparison, clotting with 30 U/ml thrombin followed by lysis with 2% acetic acid produces abnormal results at levels below 10 U/dl (Jennings et al, 2003). Occasionally, patients with FXIII levels above 5 U/dl may bleed (see Bolton-Maggs et al, 2004 for review). Patients with hypofibrinogenemia or afibrinogenemia must be excluded from testing by these tests.

FXIII activity assay: FXIII mediates cross linking of α and γ chains of fibrin. FXIII deficiency is classified as a rare bleeding disorder and may be caused by mutations in either FXIII-A catalytic subunits or FXIII-B carrier subunits (Karimi et al, 2018; Mumford et al, 2014). The currently available methods for clinical laboratory diagnoses of FXIII deficiency include clot-solubility assays, quantitative FXIIIa activity assays, FXIII antigen assays specific for the FXIII-A_2B_2 complex, FXIII-A_2, or FXIII-B_2, and genetic testing (Palla et al, 2015). It is recommended that FXIII deficiency is diagnosed using a functional FXIII activity assay rather than clot solubility (Mumford et al, 2014; Kohler et al, 2011; Dorgalaleh et al, 2016). Most FXIII activity assays are only sensitive to deficiencies of the FXIII-A subunit. Fibrinogen is coated on the surface of a microtiter 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 FXIII kit (Siemens, Marburg, Germany) ammonia release, and Technoflur FXIII activity fluorogenic assay (Technoclone, Vienna, Austria)

Reagents: All the required reagents are contained in the commercial kit.

Method:

Day 1

- ✓ Allow kit components to come up to room temperature for 30 minutes.
- ✓ Reconstitute coating reagent (R2) in distilled water, according to the volume recommended by the manufacturer.
- ✓ Add 100 µl coating reagent per well to empty wells of the microtiter plate strips.
- ✓ Freeze any excess coating reagent for subsequent use. It remains stable for 6 months at −20°C.
- ✓ Seal strips with provided plastic seal and incubate overnight (14 to 16 hours) at temperatures of 20–25°C.

Day 2

- ✓ Dilute 20x concentrated TBS R1 (tris buffered saline) 50 ml in 950 ml distilled water or lesser volume, if required.
- ✓ Dilute 3 ml of blocking reagent R3 with 27 ml diluted TBS R1. Freeze excess R3.
- ✓ Discard coating reagent from microtiter plate, invert the strip, and tap on tissue to remove residue.
- ✓ Add 300 µl diluted blocking reagent to each well.
- ✓ Incubate for 1–1.5 hours at 37°C in an incubator.
- ✓ Reconstitute calibrator R10 in 0.5 ml distilled water and the three controls, R11, R12, and R13, in 0.2 ml distilled water.
- ✓ Thaw any frozen test plasmas at 37°C for 5 minutes prior to analysis.
- ✓ Prepare a container with a few hundred ml of ice/water mixture as an ice bath.
- ✓ Make dilutions of all test and control plasmas, 10 µl plasma, and 1 ml diluted TBS R1 buffer (1:101 dilution). Vortex mix.
- ✓ 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. Wash plate three times with 300 µl/well TBS R1. Invert and tap on tissue to remove excess liquid. 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. Add 25 µl each of calibrator, control, or test plasma into appropriate wells. Include a blank of TBS R1. Mix activator reagents part A and B (R4 and R5) to form the final incorporation reagent. Add 75 µl final incorporation reagent to each well, including blank well. Incubate for 30 minutes at 37°C in incubator. Add 200 µl/well incorporation stopping solution R6. Mix gently for 10 minutes on the plate shaker. Reconstitute detection reagent R7 by adding 12 ml distilled water. Freeze unused diluted

R7. Wash plate four times with 300 µl/well TBS. Tap to remove excess liquid. Add 100 µl/well detection reagent R7. Incubate for 15 minutes at 37°C in the incubator. Wash plate four times with 300 µl/well TBS. Tap to remove excess liquid. 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

Add 180 µl/well of substrate solution. Incubate for 11 minutes at 37°C in the incubator. Add 50 µl/well stopping solution (4M NaOH) R9. Read optical densities within 15 minutes at 405 nm in a microtiter 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 analyzed in small batches.

Results calculation: Calibrator dilutions and control values are supplied with each kit. Using suitable data handling software or graph paper, construct a calibration curve by plotting the concentration against the optical density (OD) 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. The patient results can be accepted if the control sample values are within the acceptable range.

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