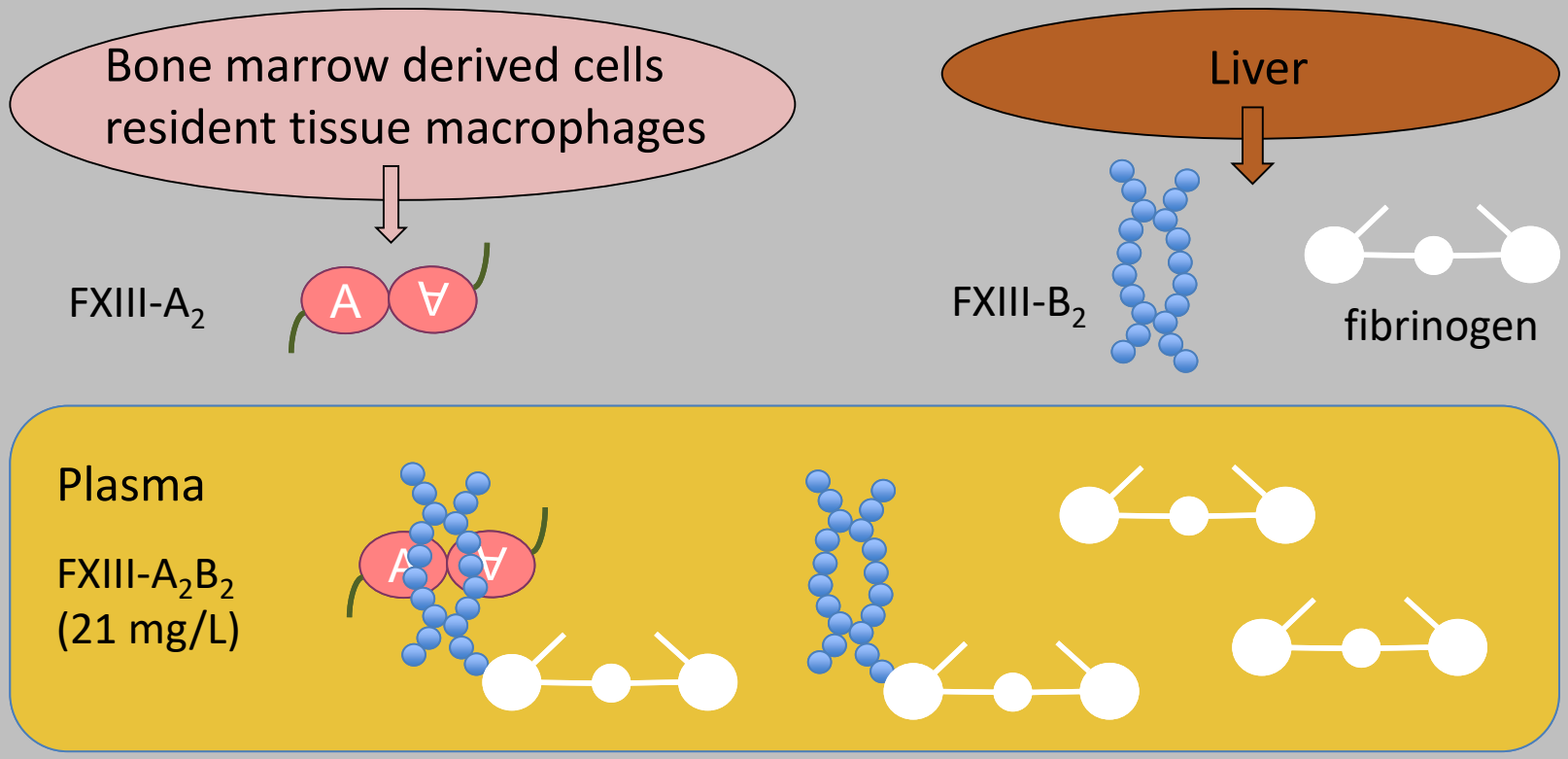
Inherited Factor XIII deficiency: laboratory diagnosis

Éva Katona

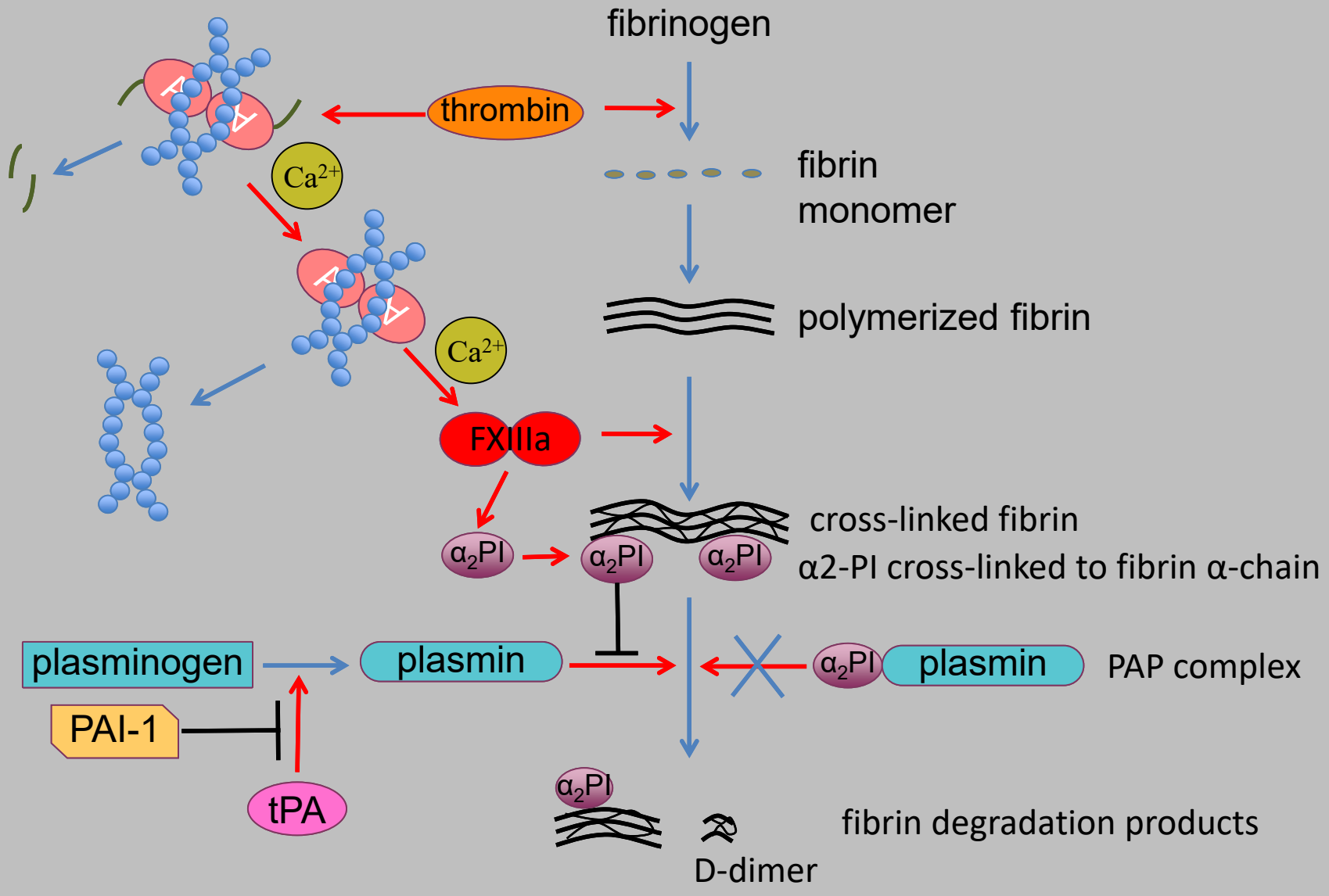
Division of Clinical Laboratory Science,
Department of Laboratory Medicine
University of Debrecen,
Debrecen, Hungary

Introduction



- **Factor XIII (FXIII) circulates in the plasma as a hetero-tetramer of A and B subunits (FXIII-A_2B_2).**
- **Complex formation is important for the stability of plasma FXIII, FXIII-B considerably prolongs the half-life of FXIII-A in the circulation.**
- **The formation of the complex is very rapid; high affinity (K_d = 4.17 x 10^{-10} M).**
- **In the plasma FXIII is bound to fibrinogen.**

Role of FXIII in blood coagulation



General features of FXIII-ID

Rare, autosomal and recessive bleeding disorder

FXIII-A-ID:

1 in 1-3 millions, more frequent in countries where consanguineous marriages are common

Patients with homozygous or compound heterozygote FXIII deficiency have a bleeding tendency that is usually severe.

FXIII-B-ID:

Even more rare (or more rarely diagnosed)

Usually mild bleeding symptoms

General features of FXIII-ID

FXIII activity (%)	Severity of bleeding
<1	very severe (requires life long substitution)
1-5	severe to moderate (requires life long substitution)
5-30	mild, but might contribute to bleeding in hemostatic stress situations
30-60	?, might contribute to bleeding in hemostatic stress situations

Bleeding symptoms in severe FXIII-D	% of cases
Delayed umbilical	80
Superficial bruising	60
Subcutaneous and soft tissue	55
Mouth and gums	30
Intracranial	30
Intramuscular	27
Lacerations	26
After surgery	17
Peritoneal	13
Epistaxis, genital, renal	<10
Poor wound healing	30
Recurrent spontaneous miscarriages	≈100

Classification of FXIII deficiencies

I. Inherited FXIII deficiency (FXIII-ID)

FXIII-A deficiency

Type I (quantitative)

Type II (qualitative) (rare)

FXIII-B deficiency

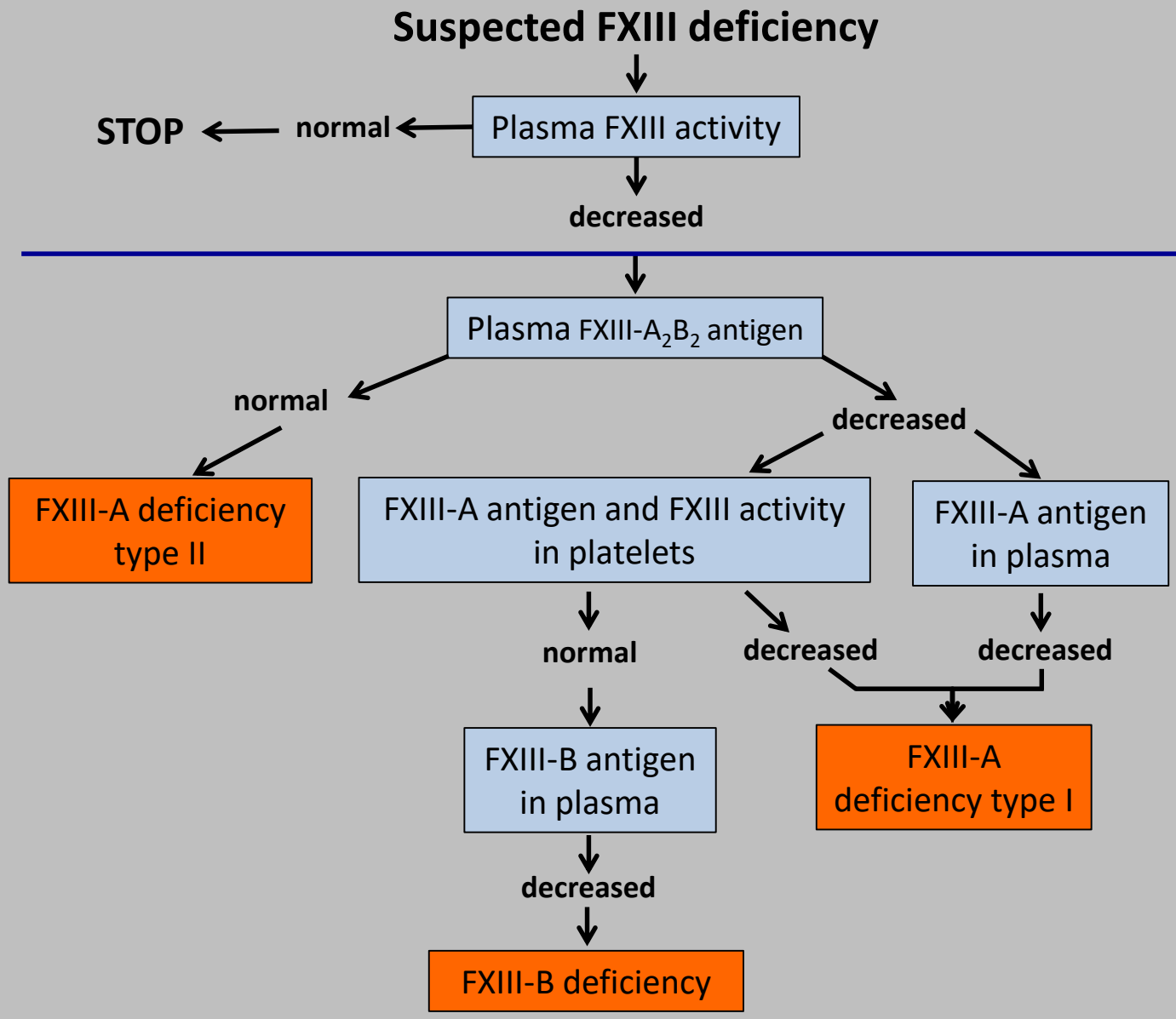
Type I (quantitative)

Type II (qualitative) (have not been detected)

II. Acquired FXIII deficiency

Auto-antibody against FXIII-A:
(Neutralizing or non-neutralizing antibody)
Auto-antibody against FXIII-B
Consumption, decreased synthesis

Diagnostic algorithm for FXIII-ID



1.
Quantitative FXIII activity assay for screening

2.
FXIII antigen determinations:

- **FXIII-A2B2**
- **FXIII-A subunit**
- **FXIII-B subunit**

to discriminate quantitative vs. qualitative defects and to determine which subunit is deficient

Functional FXIII assays

Determination of the end products of the transglutaminase reaction

1. **Qualitative assays (evaluation of fibrin cross-linking)**
   - **Clot solubility test**
   - **Fibrin clot analysis by SDS-PAGE**

2. **Quantitative assays**
   - **Amin incorporation assays**
   - **Ammonia release assays**

Clot solubility test (CST)

- The plasma samples [patient and control] are clotted by the addition of calcium with or without thrombin and incubated at 37° C for 30 minutes.
- The clot is placed in
  - 5M or 8M urea
  - 1% or 2% monochloroacetic acid
  - 2% acetic acid

  and incubated for 24 hours at either 37° C or room temperature and inspected for presence of clot at regular intervals.
- In severe FXIII-ID the clot is completely dissolved in one hour.

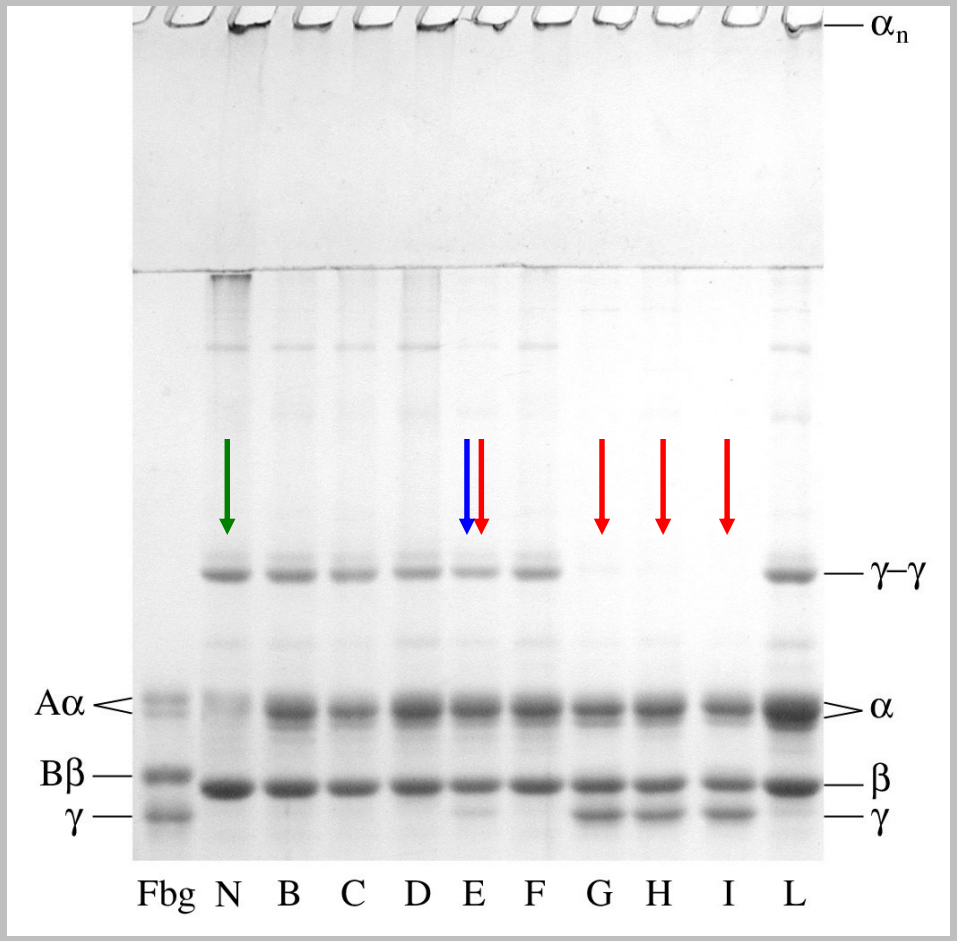
Problems with the clot solubility tests

1. Poorly standardized, the concentration of thrombin/Ca, the clotting time, type and concentration of the solubilizing agent, the concentration of fibrinogen are influence the sensitivity of CST
2. It detects only the most severe FXIII deficiencies (< 0.5-5%)

   ECAT surveys: it detect in only 16% of samples with < 2% FXIII activity. Hsu P et al. Semin Thromb Haemost 2014;40:232-238.
3. Because of the low pH of acetic acid the pepsinogen in the sample is activated and the clots are digested, simulating the dispersion of the fibrin clot which occurs in the absence of FXIII.
4. It cannot be used for monitoring prophylactic treatment and substitution therapy
5. It cannot detect FXIII-B-ID
6. It cannot be used in most acquired deficiencies

Clot solubility tests should not be used as screening tests! However, still a significant number of laboratories (17-19%) are using CST according to the results from the ECAT external quality assessment program.

SDS PAGE analysis of recovered clot



Samples B,C,D,F and L are from patients receiving substitution therapy

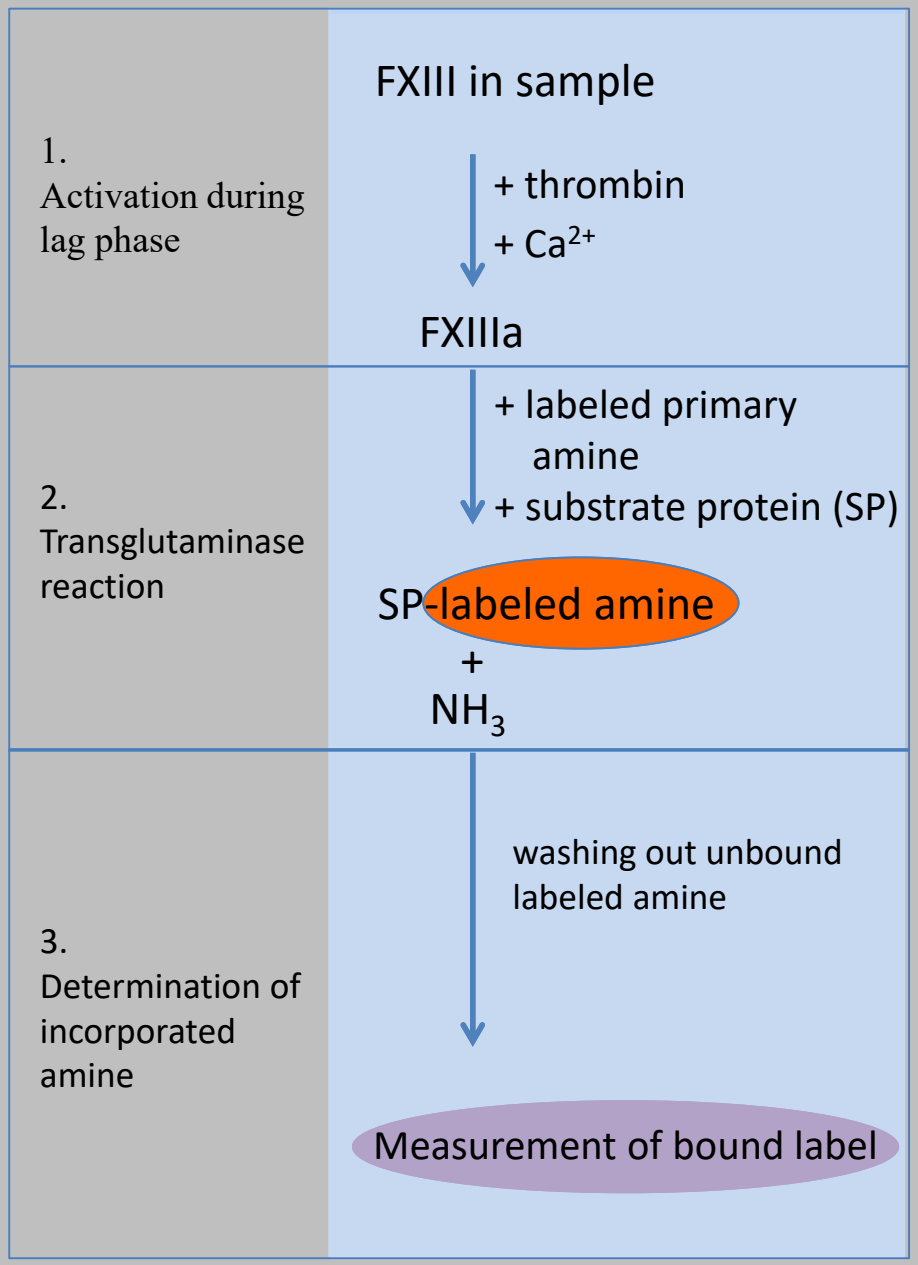
FXIII-A-ID without substitution

normal

FXIII activity and antigen level <1%

Peyvandi et al. Human Mutation 2004;23:98.

AMIN INCORPORATION ASSAYS



Commercial amine incorporation assay currently not available, successfully used in research and specialist diagnostic laboratories.

amine substrate: fluorescent, radiolabelled or biotinylated amine

Q-protein: derivatized casein or fibrinogen adsorbed to a microtiter plate,

detection: streptavidin linked to an enzyme in case of biotinylated amine substrate

Measurement by spectrofluorimeter, β scintillation, or by spectrophotometer, depending on the label of primary amine

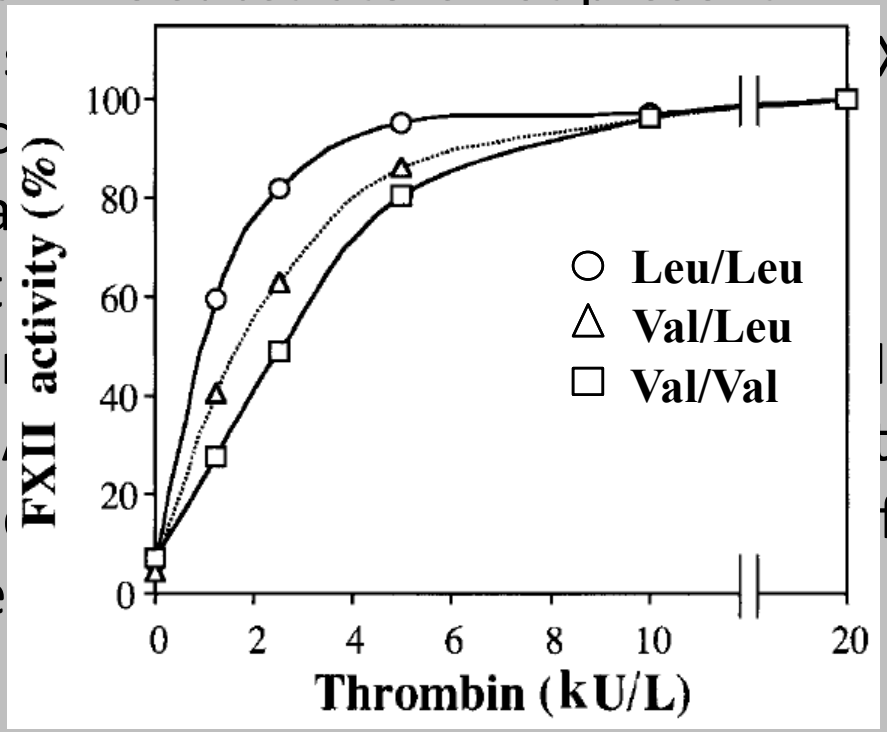
Advantages and disadvantages of amine incorporation assays

Advantage:

High sensitivity even in the low activity range

Disadvantages:

1. Not a kinetic assay, the glutamine substrate is not present in saturating concentration, re… XIII concentration. Multi-point c…
2. Relatively time-consuming a…
3. As low thrombin concentrat… activation does not go to co… III activation depends on FXIII-… tivation of FXIII-A Leu34 is faster ➔ … fferent FXIII-A Val34Leu genotype…



Kárpáti et al. Clin Chem 2000; 46: 1946-55

AMMONIA-RELEASE ASSAYS

1. Activation during lag phase

FXIII in sample

↓ + thrombin + Ca^{2+} + GPRP(A)

FXIIIa

2. Transglutaminase reaction

↓ + Gly-OEt + Q-peptide

Q-peptide-Gly-OEt

+

NH_3

3. Monitoring the release of ammonia

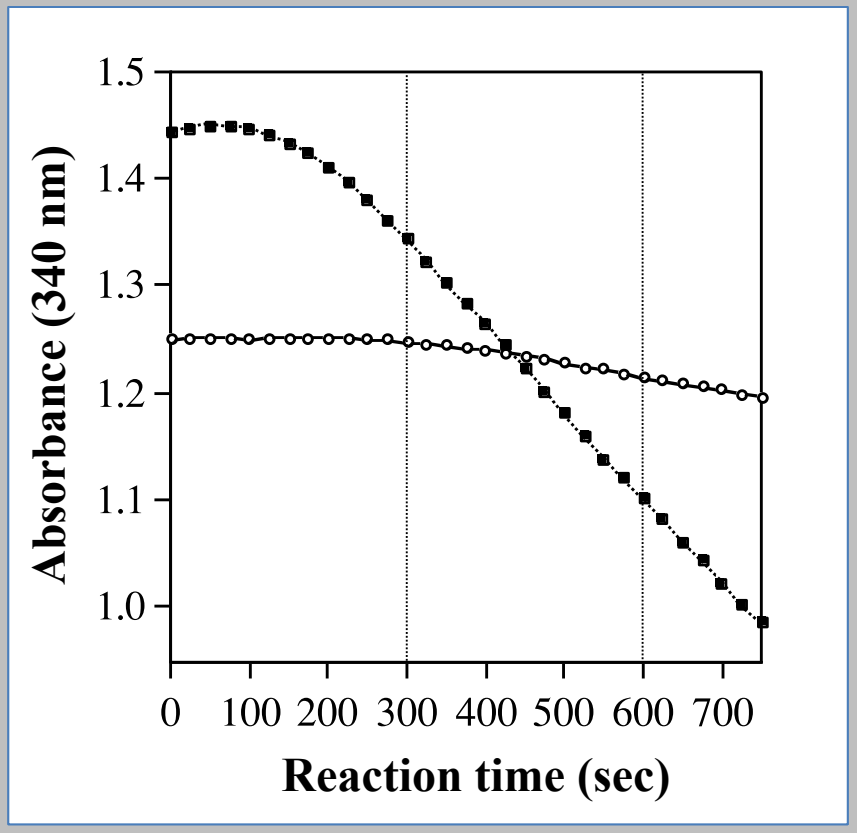
↓ + α-ketoglutarate + NAD(P)H + GIDH

glutamate

+

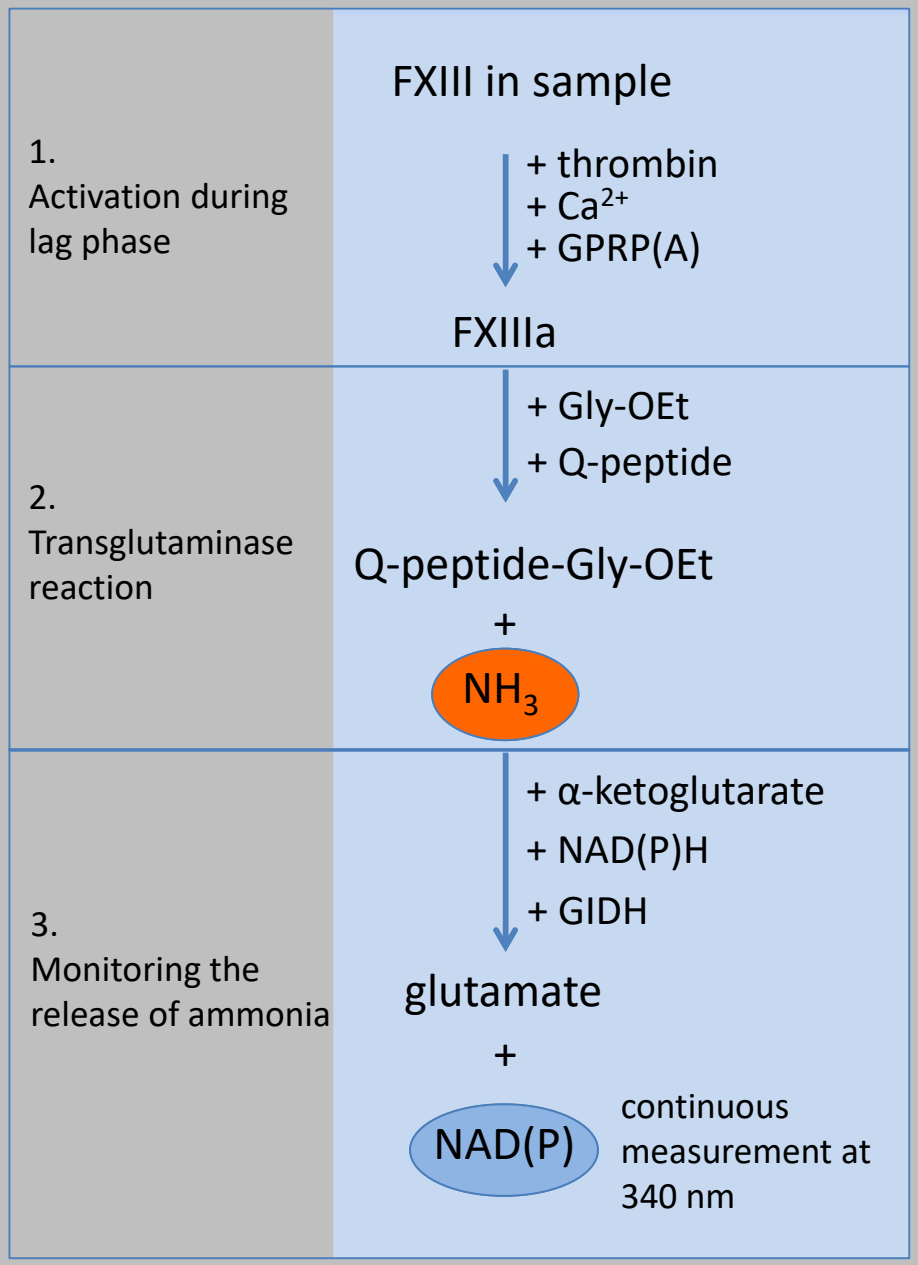
NAD(P)

continuous measurement at 340 nm



Kárpáti et al. Clin Chem 2000; 46: 1946-55.

AMMONIA-RELEASE ASSAY



Commercial ammonia-release assays

Berichrom (Dade Behring): Fickenscher et al, Thromb Haemost 1999; 65: 530-40.

TECHNOCHROM (Technoclone), REA-chrom (Reanal): Kárpáti et al. Clin Chem 2000; 46: 1946-55.

Berichrom Q-peptide:
Leu-Gly-Pro-Gly-Gln-Ser-Lys-Val-Ile-Gly

TECHNOCHROM, REA-chrom Q-peptide:
Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Leu-Leu-Lys

Berichrom: NADH

TECHNOCHROM, REA-chrom: NADPH

Quantitative ammonia release assays

Advantages:

1. True kinetic assays, both amine and glutamine substrates can be used at saturating concentration.
   Single point calibration in the measuring range.
2. Quick, one-step reactions, easily automated, ideal screening tests
3. Reference interval established according to CLSI guidelines is available (Kárpáti et al. Clin Chem 2000; 46: 1946-55)
4. The commercially available assays are not influenced by the FXIII-A Val34Leu polymorphism

Disadvantages:

1. Relatively insensitive in the lowest activity range: 0-5%
   New modification increases its sensitivity to 1%.
   Katona et al et al. Clin Chem Lab Med 2012; 50: 1191-202
2. Without plasma blank measurement there is an overestimation of FXIII activity.

Overestimation of FXIII activity without plasma blank subtraction

Sample	FXIII activity %	
	Without blank	With blank
1	3.9	0
2	9.6	4.3
3	12.6	6.6
4	13.7	6.7
5	134.6	108.0
6	141.9	120.2

Ajzner and Muszbek J Thromb Haemost 2004; 2:2075-7

Sample	FXIII activity %	
	Without blank	With blank
1	5.6	<1.0
2	1.7	<1.0
3	9.4	4.9
4	8.2	4.9
5	12.5	9.4
6	28.1	23.9
7	70.2	68.9

Lawrie et. al. J Thromb Haemosts 2010; 8: 2478-82

For samples with less than 2% FXIII activity Berichrom assay without blank correction gives results in the range of 8-14% activity. Lim et al. J Thromb Haemost 2004;2:1017-19.

Overestimation of FXIII activity by the Berichrom assay in the ECAT surveys:

Meijer P. Clin Chem Lab Med 2011;49:1753-4 and Meijer P. ECAT Special Issue 2017.
Hsu P et al. Semin Thromb Haemost 2014;40:232-238.

Why plasma blank measurement is needed in ammonia release FXIII assays?

FXIIIa-independent decrease of OD at 340 nm result in the overestimation of FXIII activity:

1. Ammonia producing reaction (deamidation of glutamine by γ-glutamyl transferase)
2. NADH consuming reactions (the effect of LDH on pyruvate present in the plasma
   NADPH does not participate in this reaction

Blank correction:

Blank measurement in the presence of a FXIIIa inhibitor (iodoacetamide) and subtraction of blank results from the test results.

Blank reagent is included in the Technochrom and REA-chrom kits. In the case of Berichrom assay the user has to prepare it. In ECAT surveys only ≈15% of the laboratories using the Berichrom assay perform blank corrections.

FXIII antigen measurements

Traditional tests:

- electroimmunoassays
- radioimmunoassays

Recent methods:

- *Latex enhanced immunoturbidimetric assays (LIA)* for the measurement of FXIII-A antigen using polyclonal anti-FXIII-A antibody coated latex particles

  HemosIL FXIII (Instrumentation Laboratory, Milan, Italy)

  STAGO/K-assay FXIII (Kamiya Biomedical Company, Seattle, USA)

  HEXAMATE Factor XIII (Medical & Biological Laboratories Co., Nagano, Japan)

- *ELISA assays*

✧ one-step sandwich type ELISAs for FXIII-A_2B_2, FXIII-A and FXIII-B; monoclonal antibodies, evaluated according to CLSI guidelines (R-ELISA FXIII, Reanal ker, Budapest, Hungary and TECHNOZYM FXIII ag, TECHNOZYM FXIII-B SUB and TECHNOZYM FXIII-A SUB) but currently not available commercially

✧ ZYMUTEST FXIII-A ELISA, one-step sandwich ELISA, polyclonal antibodies, research use only (Hyphen BioMed, Neuville sur Oise, France)

✧ In house ELISAs using matched pair anti-FXIII antibody set (Affinity biologicals), majority of them without proper evaluation.

FXIII antigen measurements

Advantage:

High sensitivity, well below 1% of the normal average for the ELISA type assays and 0.025 IU/mL for HemosIL FXIII-A LIA

LIAs are quick, automated assays

Disadvantages:

ELISA methods are time consuming, usually not automated

Results in ECAT surveys suggested, that HemosIL has substantial inter-laboratory variability (57% CV) at levels below 0.1 IU/mL FXIII-A

Notes:

- FXIII-A antigen measurement may not reflect FXIII-A_2B_2 level in the plasma at pathological conditions.
- Antibodies used for individual FXIII subunit determination should react with the free and complexed form of the respective subunit equally well.
- The assay should not be influenced by fibrinogen concentration.
- FXIII autoantibodies may interfere with the binding of antibodies in the reagent kit.

FXIII measurement from platelets

In FXIII-A-ID FXIII activity and antigen are decreased both in plasma and in the platelet lysates.
In FXIII-B-ID and in the case of acquired FXIII deficiency (anti-FXIII autoantibody) platelet FXIII activity and antigen remain normal.

Preparation of the sample:

1. Platelets are prepared by differential centrifugation:
   - other blood cells are removed at low speed (PRP)
   - platelets are pelleted at high speed
     (Pelleted platelets (without buffer) can be shipped frozen to a specialized laboratory.)
2. Platelet pellets are lysed in a buffer containing 1% Triton X-100 detergent.
3. FXIII activity and antigen are measured in the lysate, using the same method as for plasma FXIII.

FXIII genotyping

F13A1 chromosome 6 (6p24-25), 15 exons producing a 3.9-kb mRNA
F13B chromosome 1 (1q31-32.1), 12 exons producing a 2-kb mRNA

Over 95% of severe cases are due to FXIII-A deficiency
More than 153 mutations in the *F13A1* gene have been published, most of them missense or nonsense mutations
FXIII-B-ID has been described in fewer than 20 families (6 family with severe def.)

No mutational hotspots in either genes ➔ sequencing the coding regions and exon-intron boundaries
For the detection of large deletions ➔ multiplex ligation-dependent probe amplification (MLPA analysis)

In geographic regions with high incidence of consanguinity, persistence of founder mutations increases the prevalence of FXIII deficiency. In these regions ➔ targeted genetic analysis, such as combination of polymerase chain reaction with restriction fragment length polymorphism detection (PCR RFLP) or sequencing one or two exons containing the most prevalent mutations (FXIII-A p.Arg77His and p.Trp187Arg in Southeast Iran) might provide advantages.

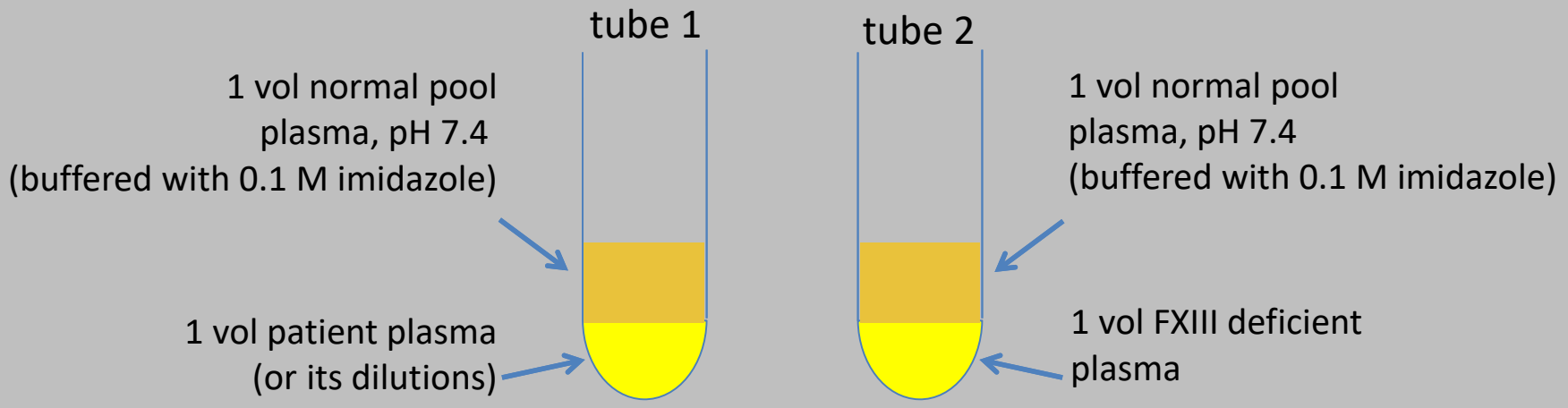
Take home messages:

1. **Accurate diagnosis of FXIII-ID is challenging due to both the rarity of the condition and the limitations of the currently available FXIII specific assays.**
2. **The ISTH SSC guideline for the laboratory diagnosis of FXIII-ID has not been uniformly followed in clinical laboratories.**
3. **A quantitative functional assay should be the first-line test for screening FXIII-ID.**
   Neither clot solubility test nor FXIII antigen assay is recommended for screening.
2. **In the case of ammonia release assays a plasma blank is to be measured and subtracted from the results, otherwise FXIII activity is overestimated especially in the low activity range.**
3. **If FXIII is not fully activated, FXIII-A Val34Leu polymorphism might influence the results of functional assays.**
4. **Reference plasma should be calibrated against WHO-ISTH FXIII plasma standard by the manufacturer.**

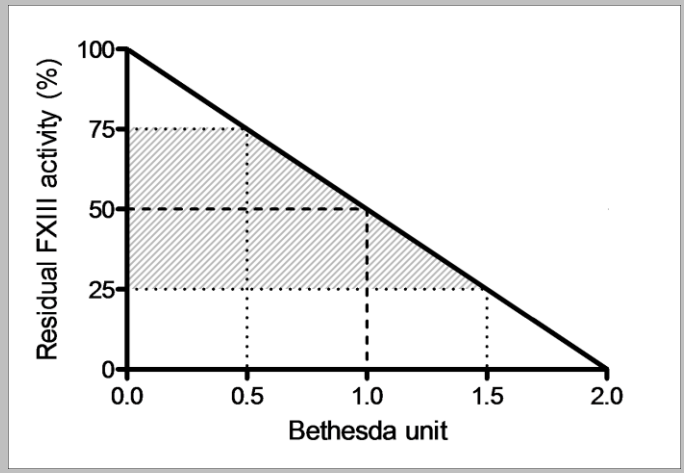
e-mail: ekatona@med.unideb.hu

FXIII inhibitor assay

(Nijmegen modification of Bethesda assay)



1. Mix and incubate both tubes at 37 °C for 2 hr
2. Measure FXIII activity
3. Subtract half of the intrinsic FXIII activity value of patient plasma from the result of tube 1
4. Calculate residual FXIII activity (%)
5. Read Bethesda unit in the range of 25-75% residual FXIII activity
6. Multiply the result by the dilution factor



Köhler et al. J Thromb Haemost 2012; 9: 1404-6, Supporting information

$$\text{Residual FXIII activity \%} = \frac{\text{FXIII activity (corrected) in tube 1}}{\text{FXIII activity in tube 2}} \times 100$$