Principles of Coagulation Testing

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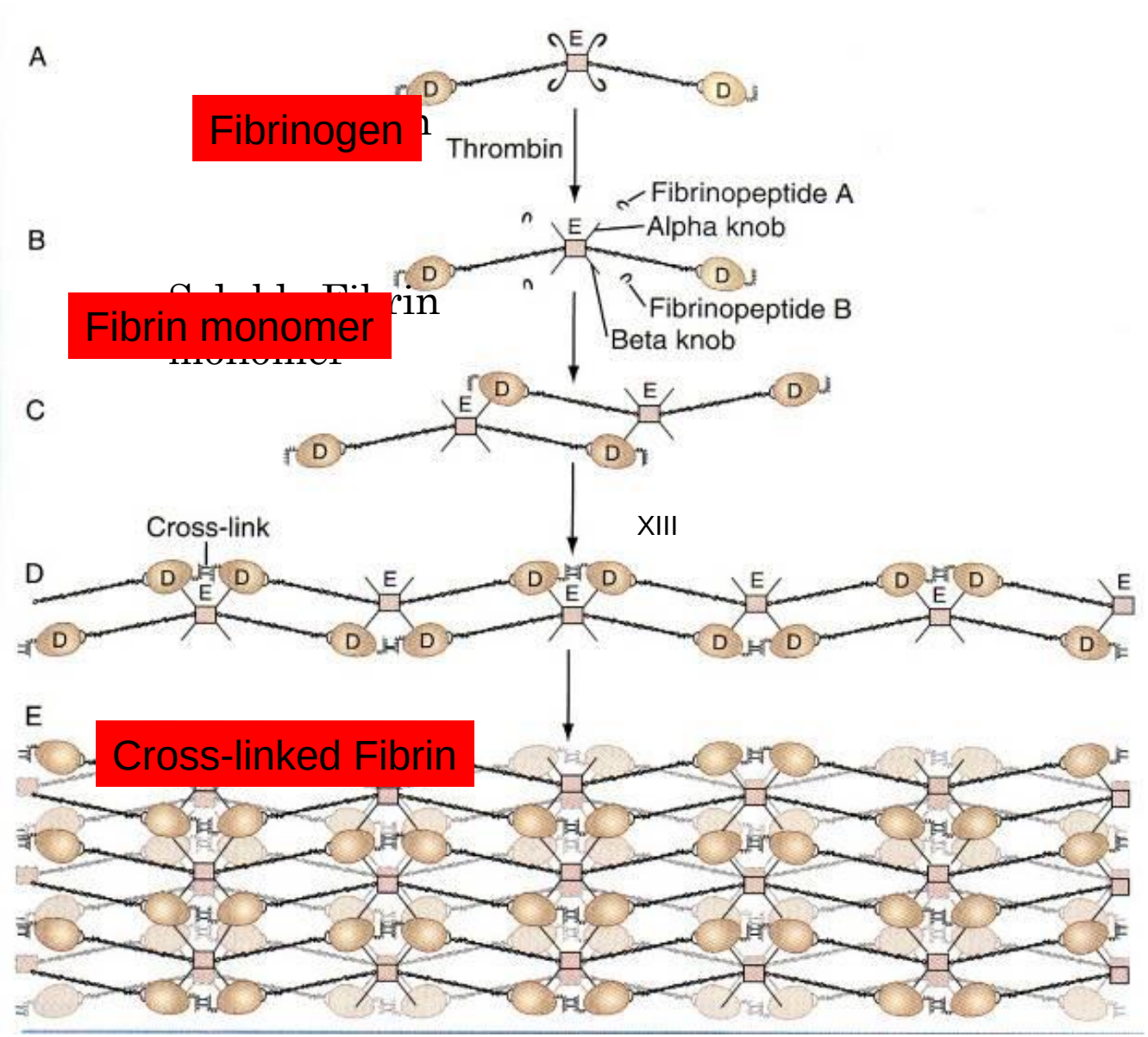
FSP and D-Dimer

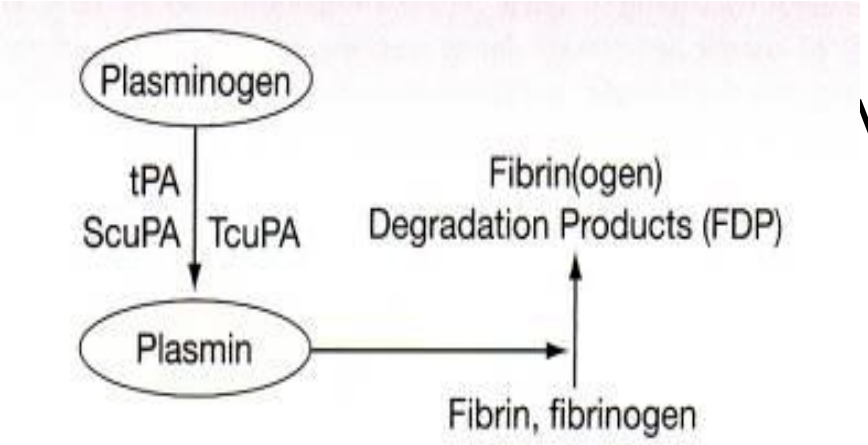
FDP vs. D-DIMER

- Fibrin is formed as the end result of coagulation cascade activation
- Fibrinolysis causes cleavage of fibrinogen, fibrin, and fibrin clot, yields FSP (FDP)
- Only cleavage of fibrin clot (cross-linked fibrin) yields D-dimer -> D-dimer is more specific for DIC

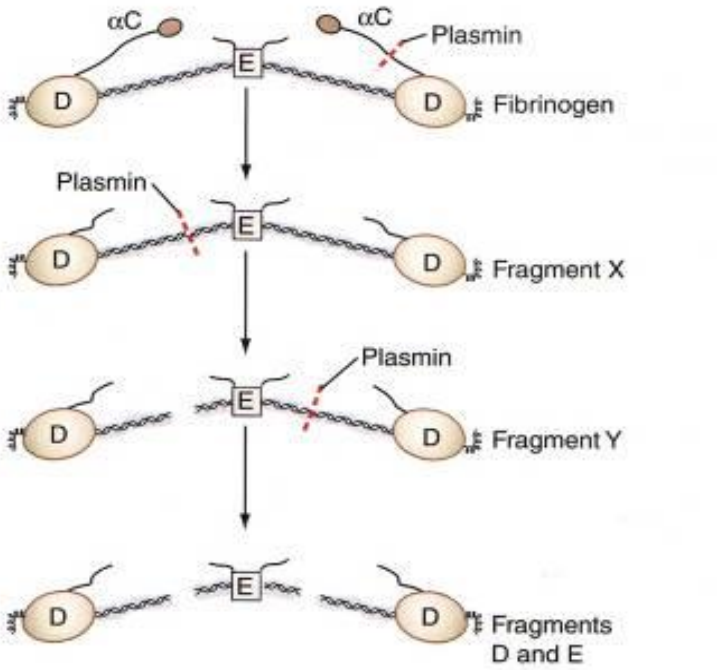
FORMATION OF FIBRIN



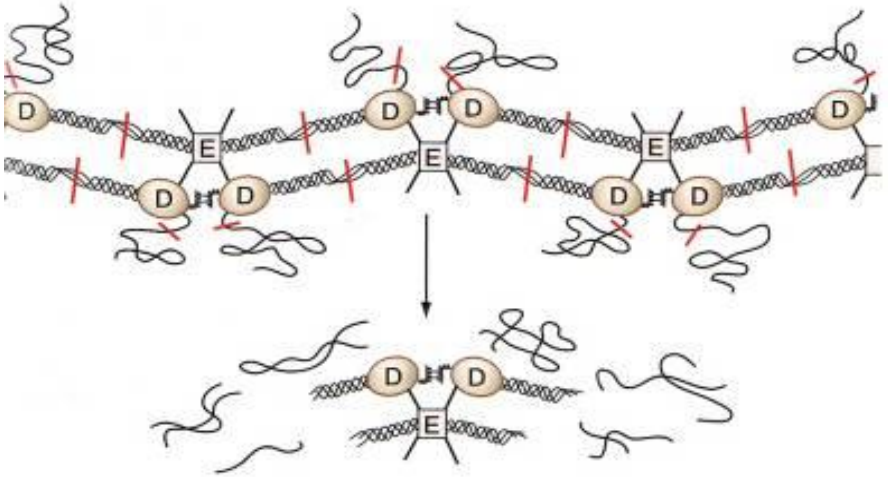
D-dimer



V



- Activators:
  - Tissue plasminogen activator (tPA)
  - Urokinase plasminogen activator (uPA)
- Inhibitors:
  - Plasminogen activator inhibitor-1 (PAI-1)
  - α2-antiplamin

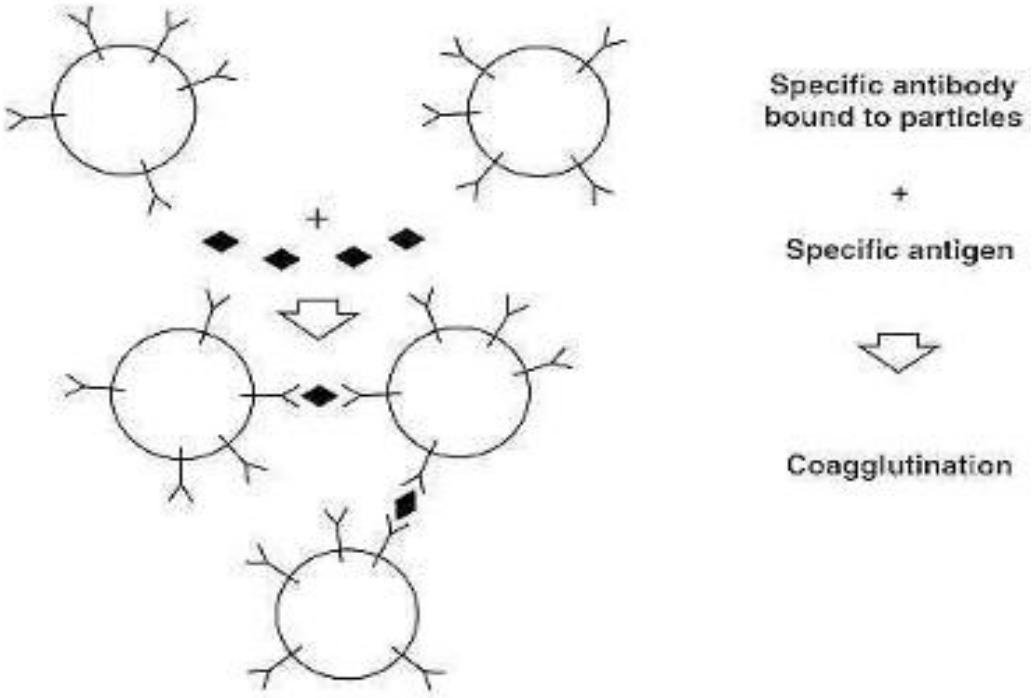


Testing: FSP and D-dimer

- Semi-quantitative FSP
- Qualitative D-dimer
- Semi-quantitative D-dimer
- Quantitative D-dimer

SEMI-QUANTITATIVE FSP

- The first test developed (in the early 70's)
- Latex agglutination, FSP antibodies are bound on latex beads, if sample contains FSP, agglutination can be detected



SEMI-QUANTITATIVE FSP

- Semi-quantitation:
  - Serial dilution of sample (1: 20 through 1:640)
  - A positive result at 1:20 corresponds to 20 µg/mL of fibrinogen equivalent units (FEU)

SEMI-QUANTITATIVE FSP

- Early generation FSP polyclonal antibodies cross-react with fibrinogen
  - Must use serum or plasma in tubes with bovine thrombin (consumes fibrinogen)
- Current FSP monoclonal antibodies do not cross react with fibrinogen
  - Can use plasma or serum
  - False-positive result with rheumatoid factor
- Clinical application: DIC, hyperfibrinolysis

QUALITATIVE D-DIMER

- Monoclonal antibodies directed against D-dimer domain
  - More specific for in-vivo fibrin clot formation
- Manual latex agglutination technique (as for FSP), plasma or serum sample:
  - Cut-off value: 0.5 µg/mL FEU
  - Semi-quantitative format: dilutions 1:2 through 1:16
- Abnormal result in DIC
- Normal result in primary fibrinolysis
- False-positive result by rheumatoid factor

QUANTITATIVE D-DIMER

- Automated ELISA, immuno-turbidimetry
- Increased in DIC (>0.66 µg/mL)

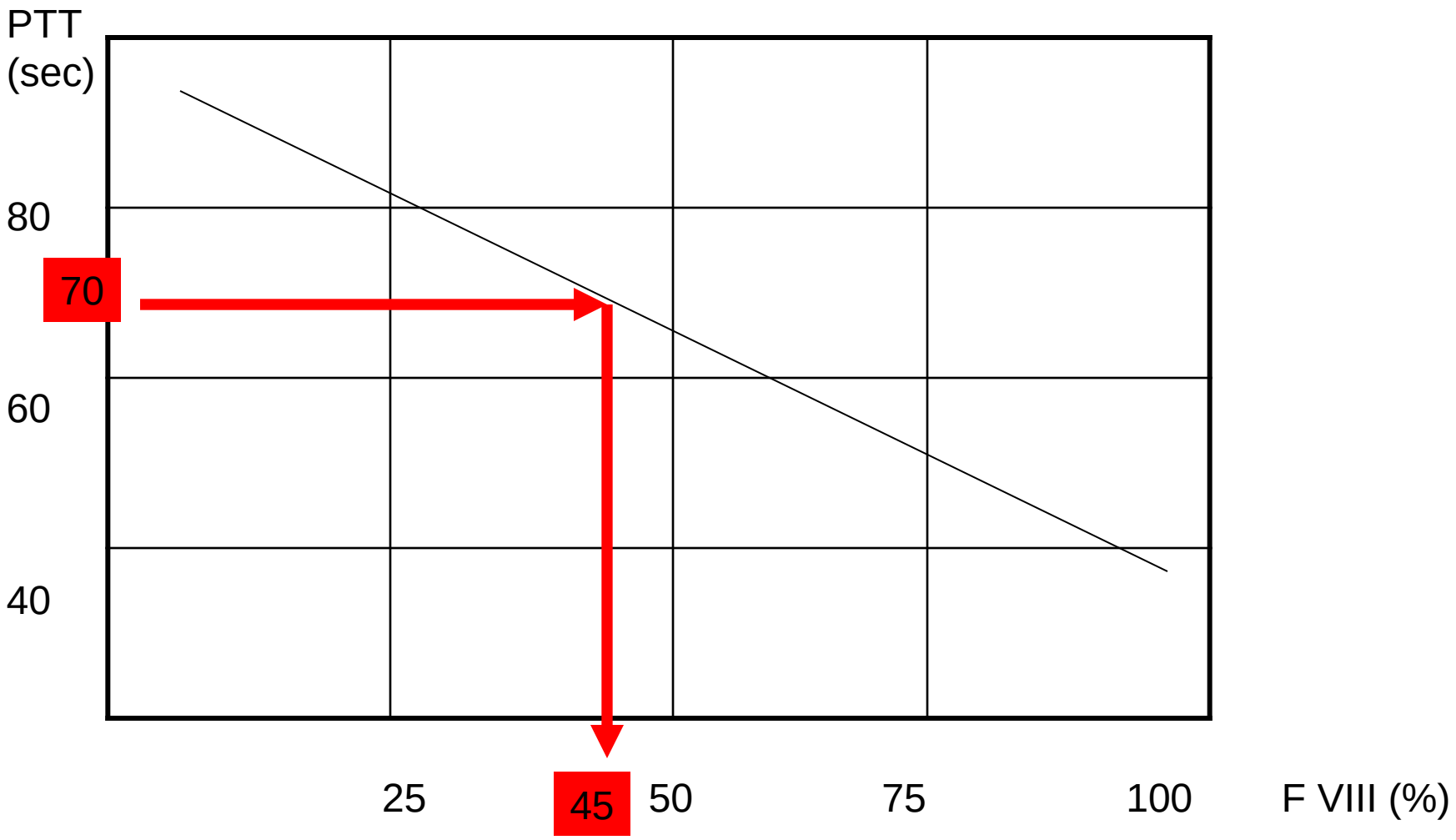
- Quantitative D-dimer also has high negative predictive value for venous thromboembolism (VTE including DVT, PE):
  - <0.4 µg/mL, VTE can be ruled out
  - Very sensitive but not specific: high Negative Predictive Value / low Positive Predictive Value

Clotting Factor Assay

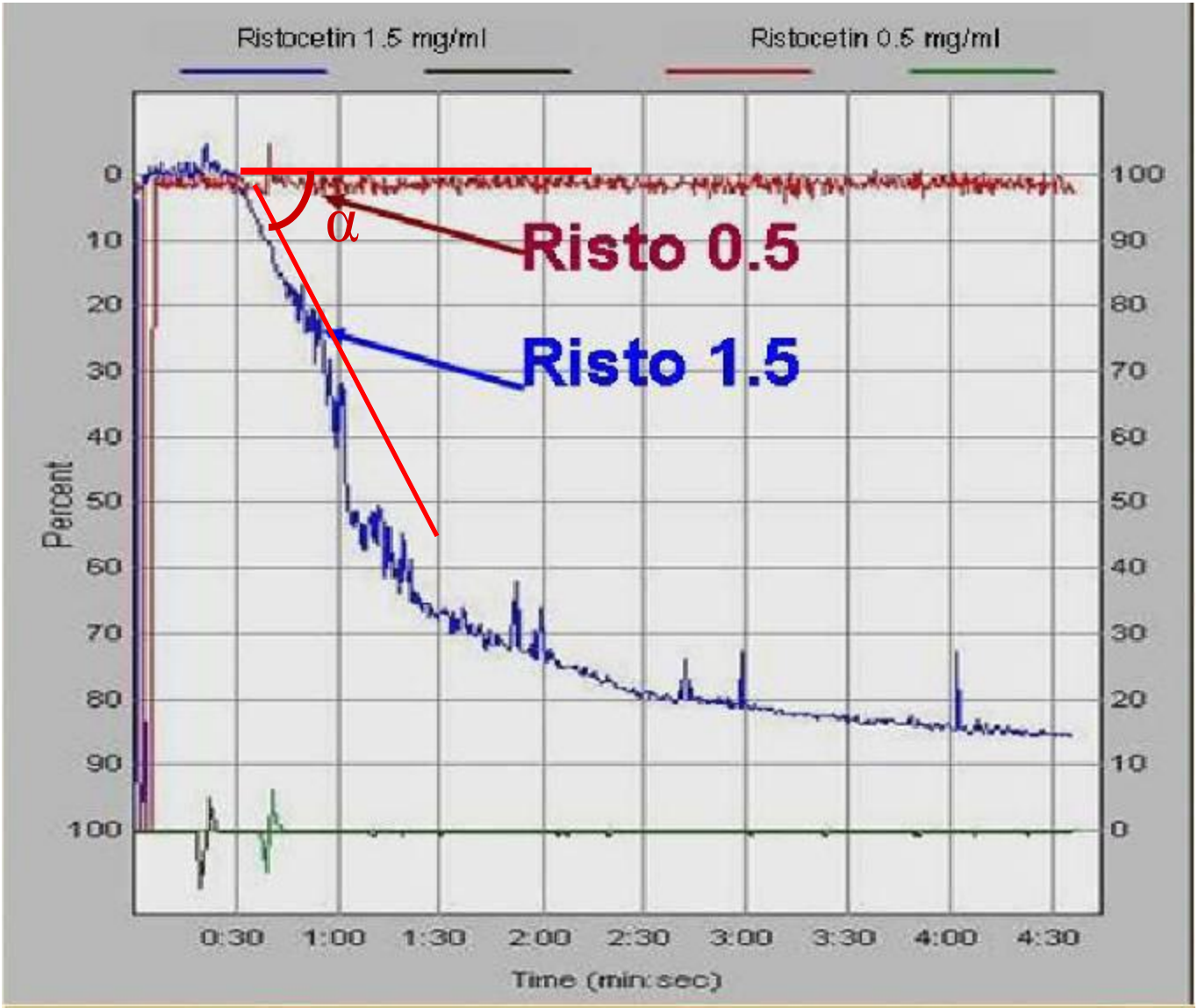
Factor VIII assay

- Factor VIII level is inversely proportional to PTT
- A standard curve (PTT vs F VIII) is first set up using commercial assayed samples
- Mutiple dilutations of patient's sample (using F VIII-deficient substrate) are tested for PTT.
- These PTT's are plotted on the standard curve to intrapolate for F VIII
- Each F VIII is multiplied by the dilutation factor to obtain the actual F VIII before dilution
- F VIII level is the mean of F VIII's from multiple dilutions

Factor VIII Standard Curve

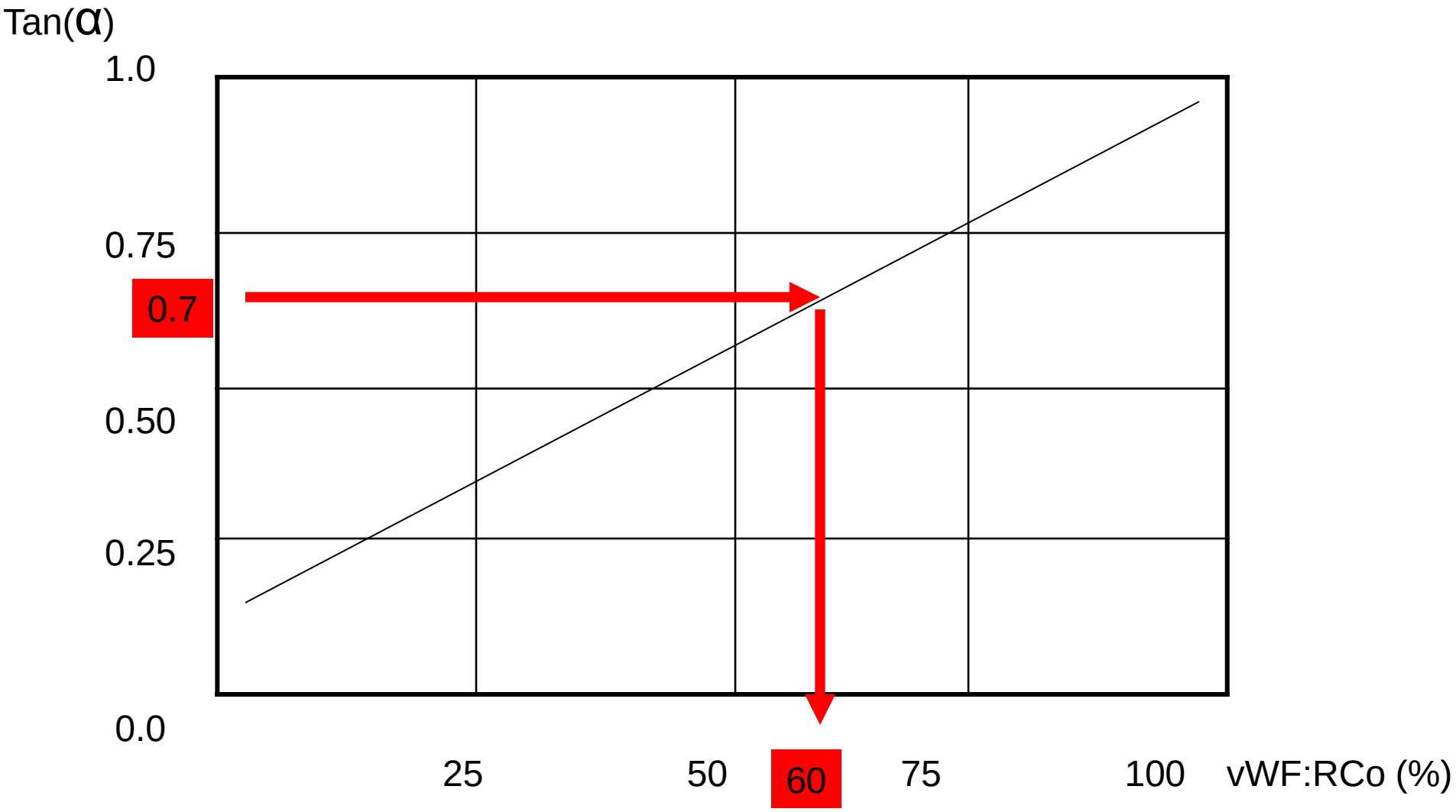


Ristocetin Cofactor



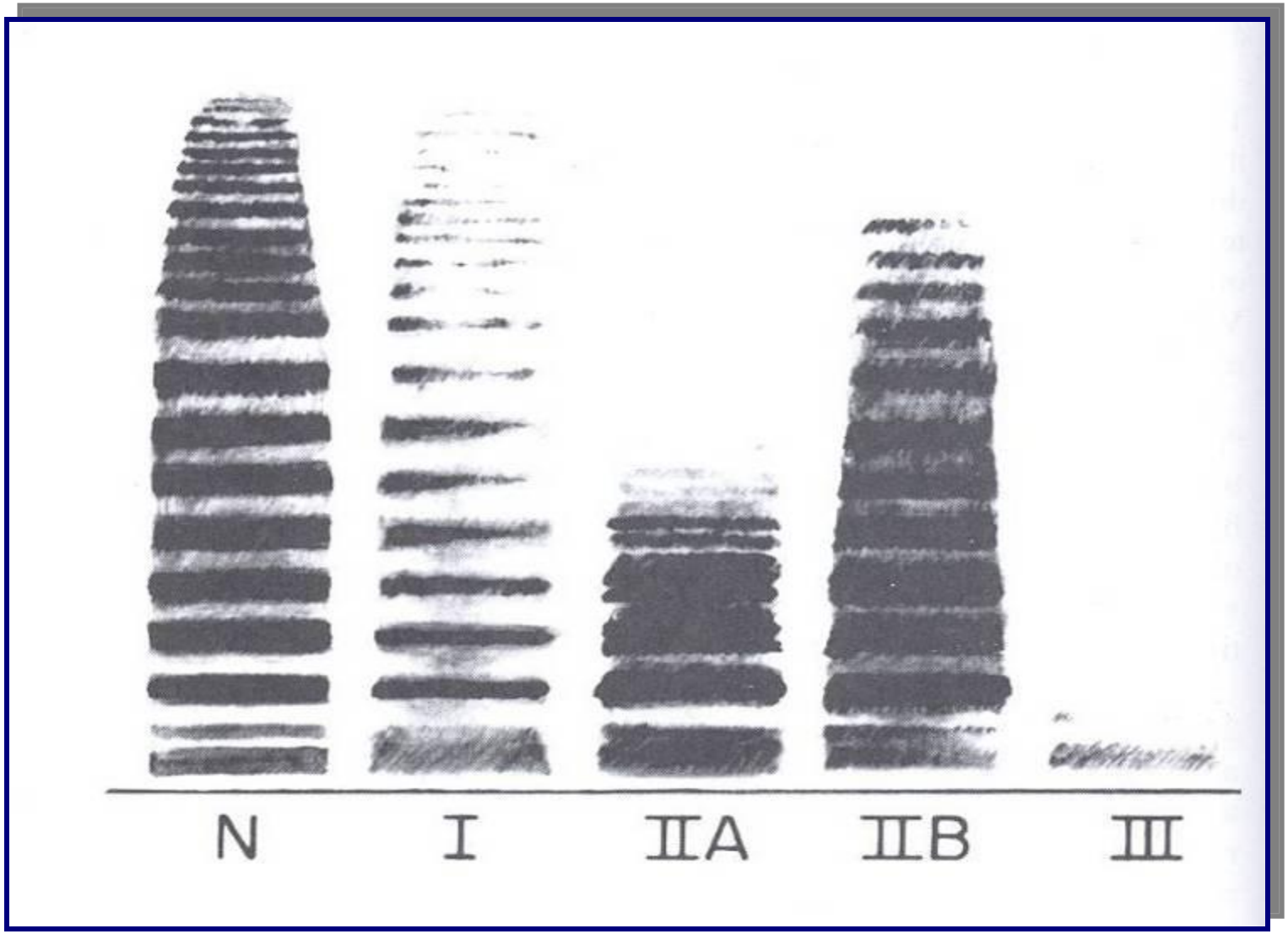
Normal tracing using platelet rich plasma (PRP)

vWF:RCo Standard Curve



vWF Multimer Analysis

Multimer analysis

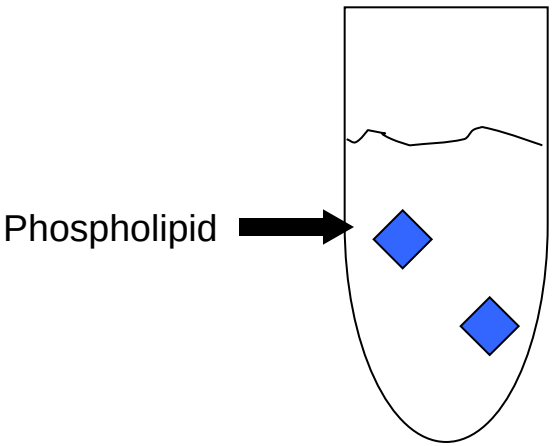


Lupus Anticoagulant

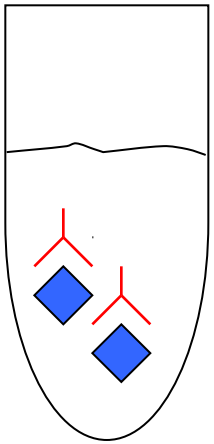
Evaluation of lupus anticoagulant

- Mixing studies
  - Mix equal parts patient and control plasma
  - aPTT will correct if prolongation due to factor deficiencies
  - If LA present will fail to correct aPTT
    - Usually immediate acting (before incubation)

Dilute Russell Viper Venom Time



Normal plasma
dRVVT 36-42 sec



Plasma with lupus anticoagulant
dRVVT > 43 sec

Evaluation of lupus anticoagulant

Neutralization study:

Addition of phospholipid will neutralize lupus anticoagulant

Two common neutralization tests:

- Platelet neutralization:

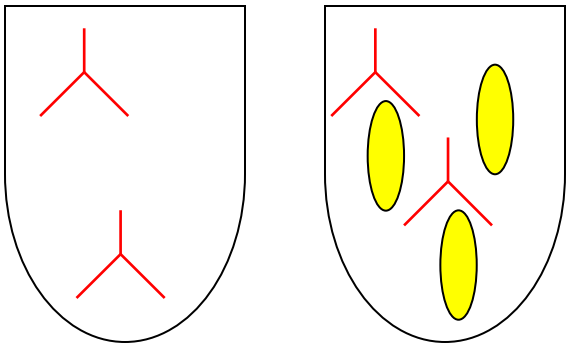
  lysates of frozen, thawed and washed platelets

- Hexagonal phase phospholipid neutralization:

  hexagonal phospholipid with high affinity for lupus anticoagulant

LA Confirmatory Tests

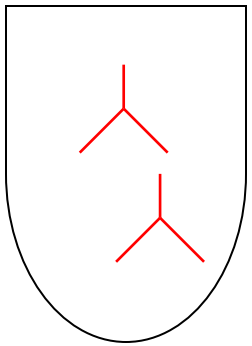
Platelet Neutralization



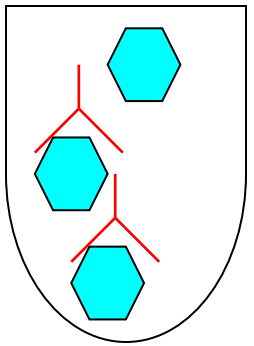
Prolonged aPTT

Shortened aPTT

Sta Clot-LA



Prolonged aPTT



Shortened aPTT

Clotting time >8 seconds shorter after addition of PL = + for LA

Anticardiolipid Antibodies

Anticardiolipin Antibodies

- ACA: IgG, IGM, IgA
- Laboratory: ACA by ELISA; high levels are associated with high risks of thrombosis

Anticardiolipin Antibodies (cont'd)

	Normal range	Clinically insignificant	Moderate risk	High risk
IgG	< 15 GPL	15-20	20-80	> 80
IgM	< 12.5 MPL	12.5-20	20-80	> 80
IgA	< 15 APL	15-20	20-80	> 80

Factor VIII Inhibitor Assay

Factor VIII Inhibitor Assay

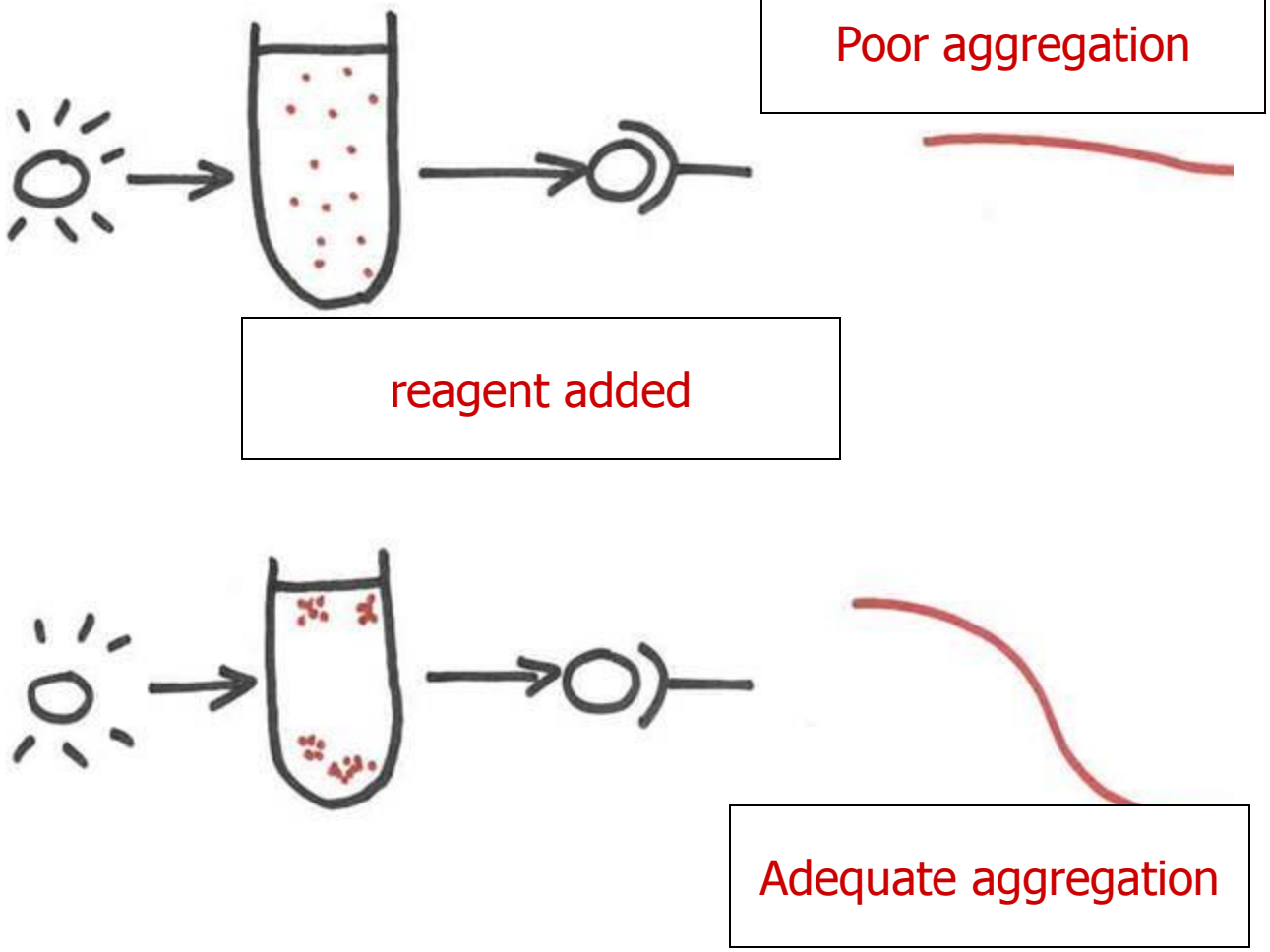
- Measured in Bethesda Unit (BU)
- 1 BU = quantity of inhibitor in patient's plasma that results in loss of 50% factor activity in normal plasma sample (1:1 mix) after incubation for 2 hours at 37°C
- Positive for inhibitor: > 0.5 BU
- High responding inhibitor: titer > 5 BU
- Low responding inhibitor. titer < 5 BU despite repeated exposure

Platelet Aggregation

Platelet aggregation study

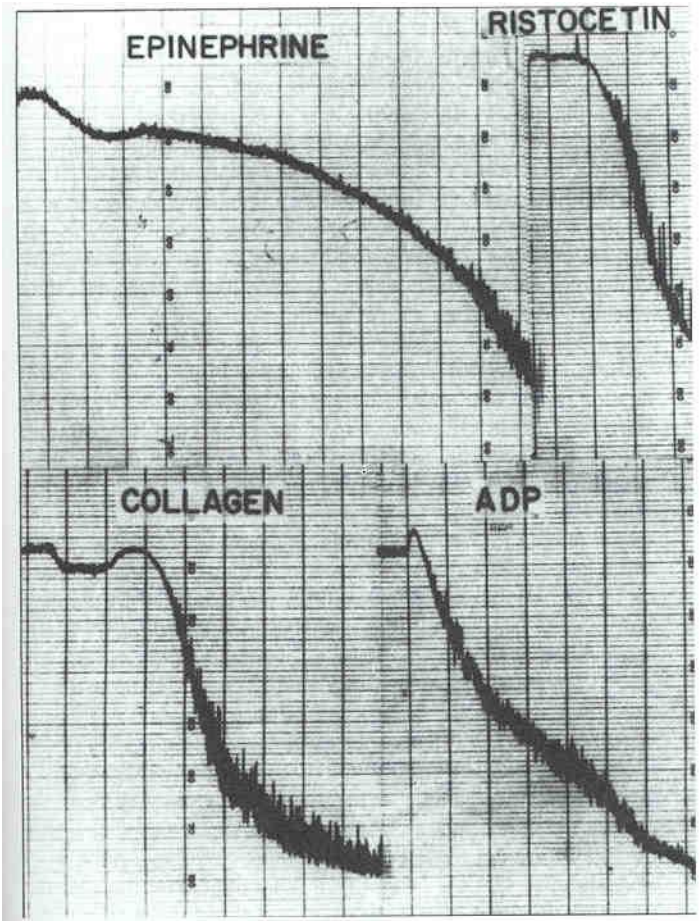
- Principle: aggregation in response to an added chemical stimulus can be monitored by change in transmittance
- Stimulating agent: arachidonic acid, ADP, collagen, epinephrine, and ristocetin
- Platelet functional disorders have typical aggregation patterns

Platelet aggregation



Aggregometer

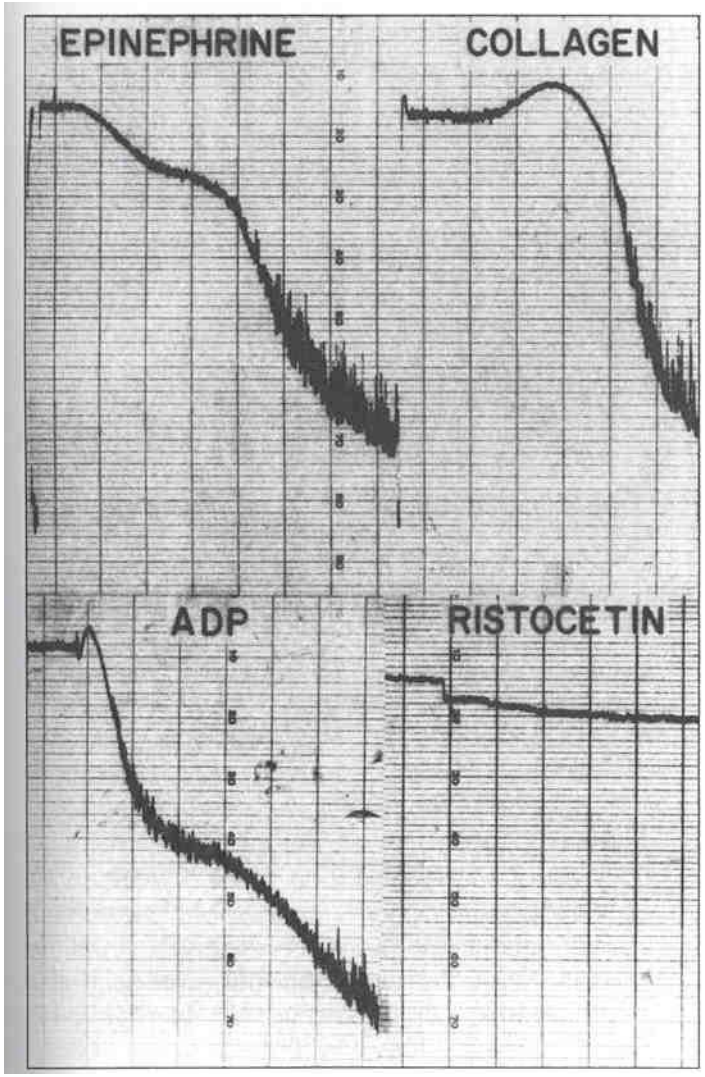
Normal pattern



ADP and epinephrine exhibit two waves of aggregation, primary and secondary

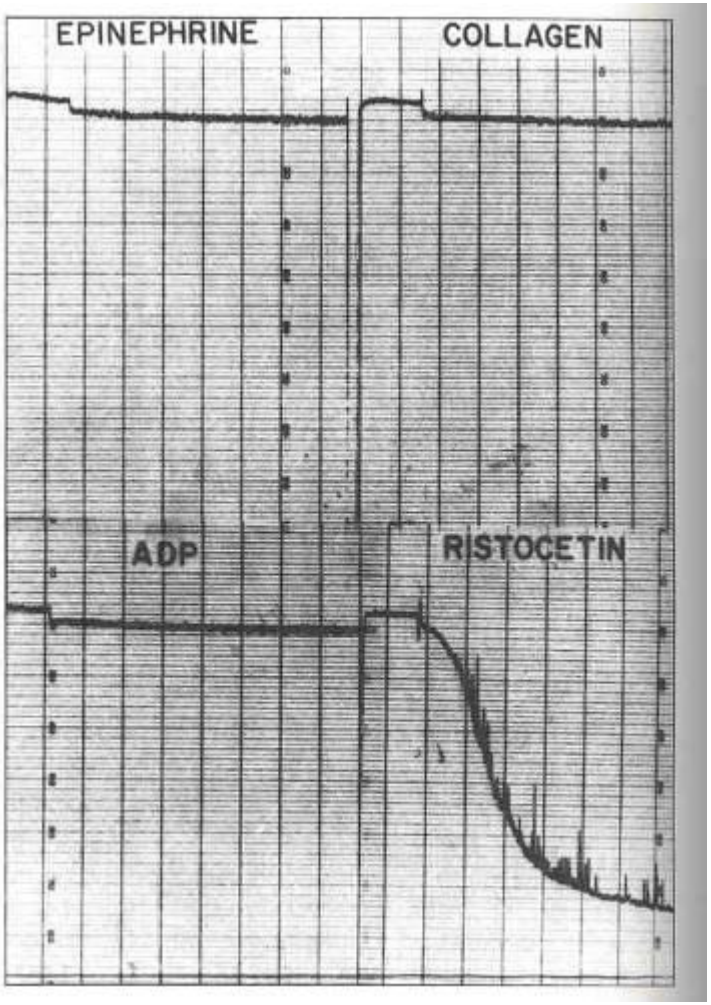
The secondary wave is due to release of endogenous ADP

Abnormal platelet aggregation studies



VWD and Bernard Soulier have similar aggregation pattern: Aggregation in response to ristocetin is abnormal

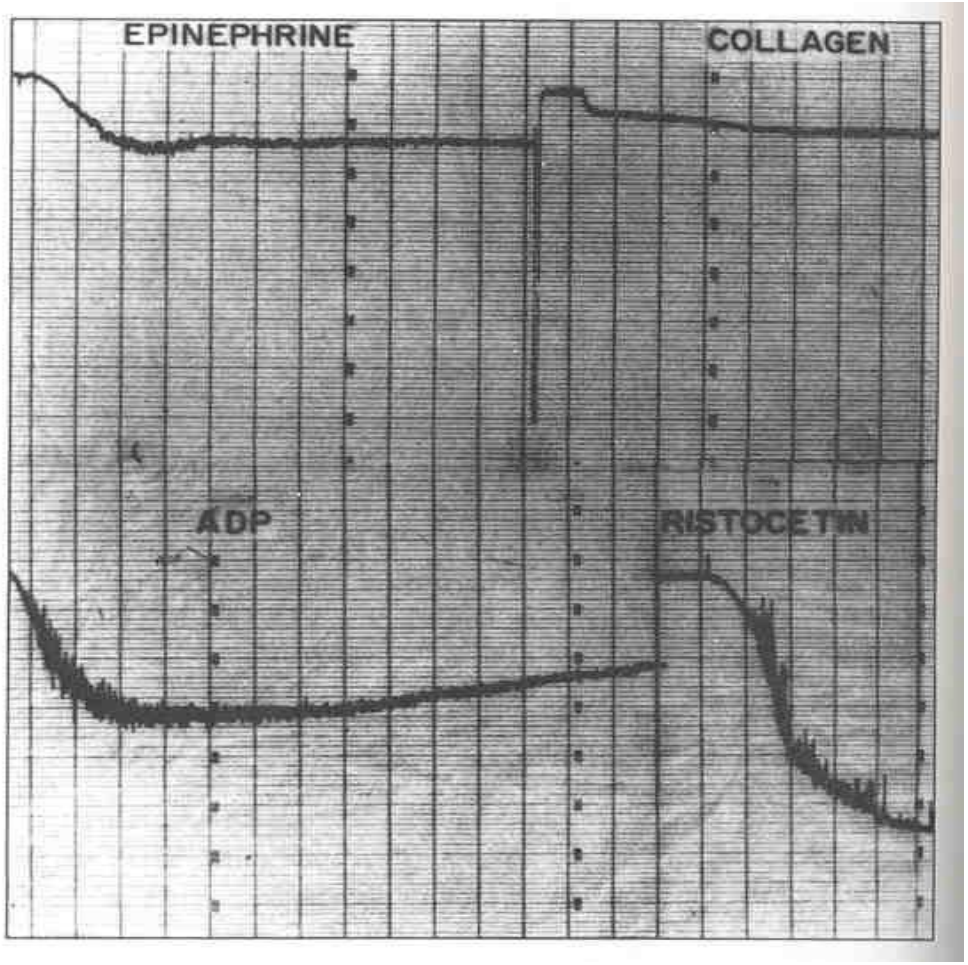
Abnormal platelet aggregation studies: Glanzmann's thrombasthenia



Primary wave defect for all reagents except Ristocetin

Autosomal recessive

Abnormal platelet aggregation studies:
Storage pool disease or defective release of storage pool contents (aspirin-like defect)



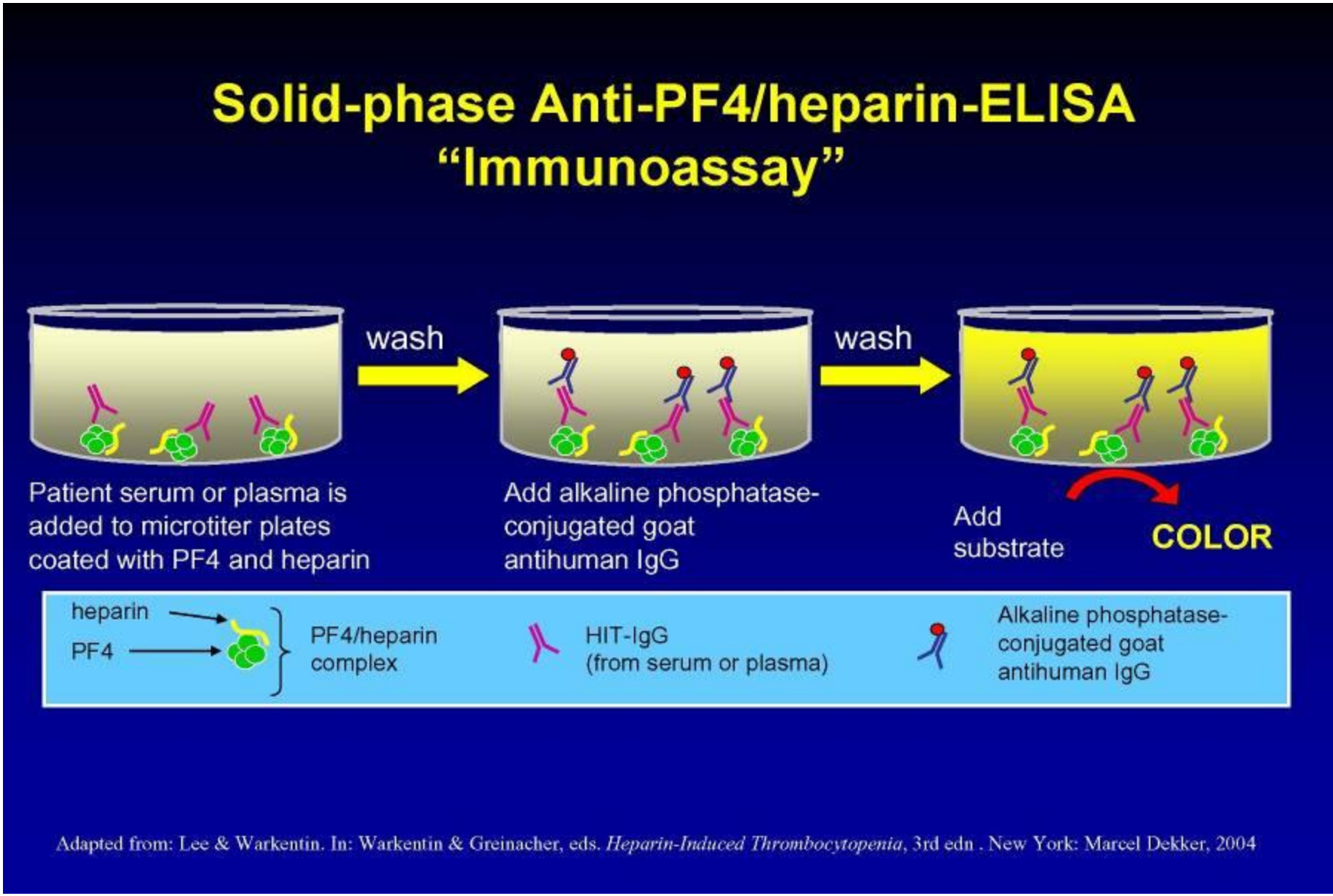
Secondary waves to ADP and epinephrine absent

Heparin-associated Antibody

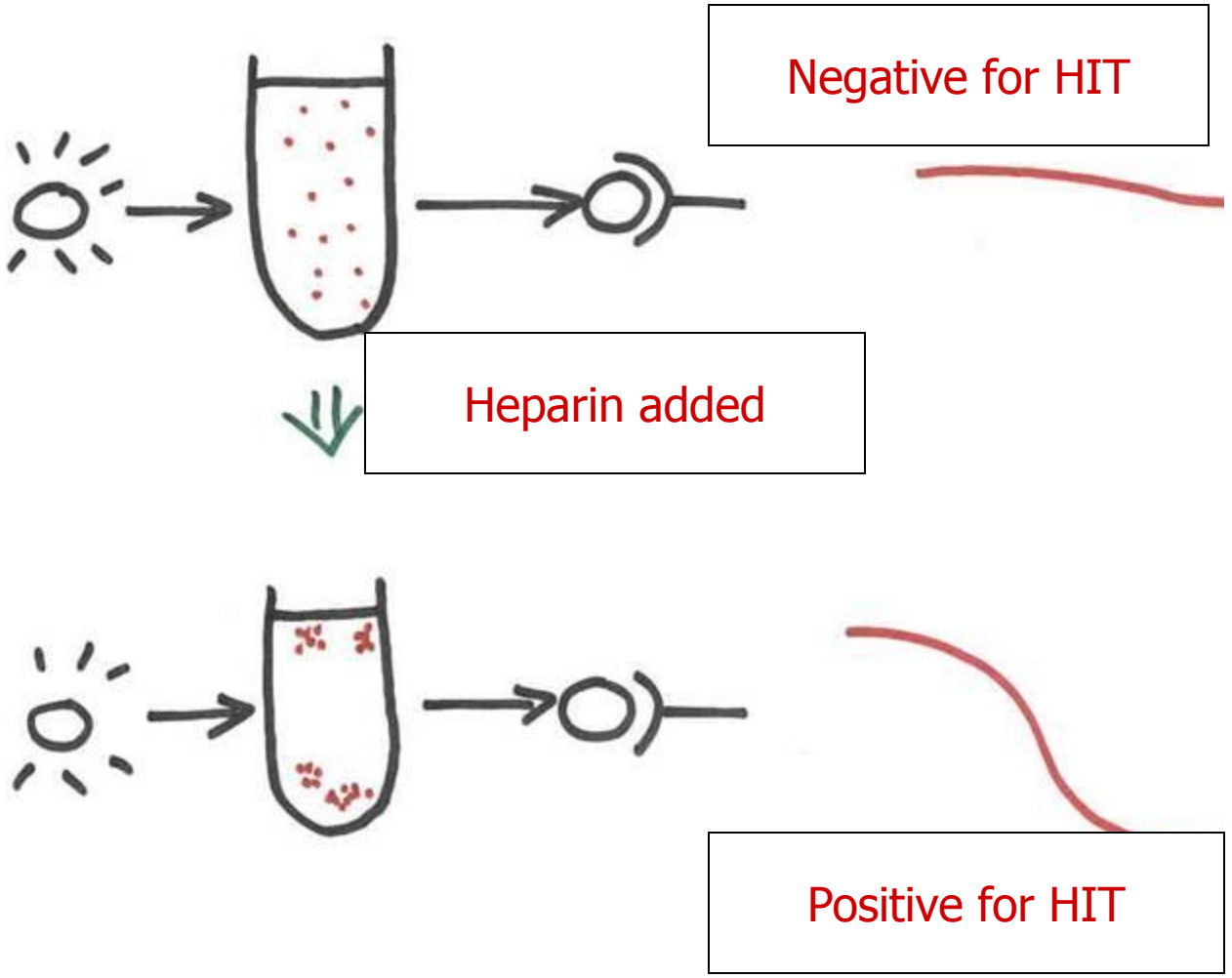
Testing for Heparin Antibody

- Heparin-induced platelet aggregation: sensitivity 70%, specificity 85%
- Serotonin release assay: sensitivity 80%, specificity 85%, limited use due to radioisotope (^{14}C)
- Heparin-PF4 antibody (ELISA): sensitivity 82%, specificity 70%

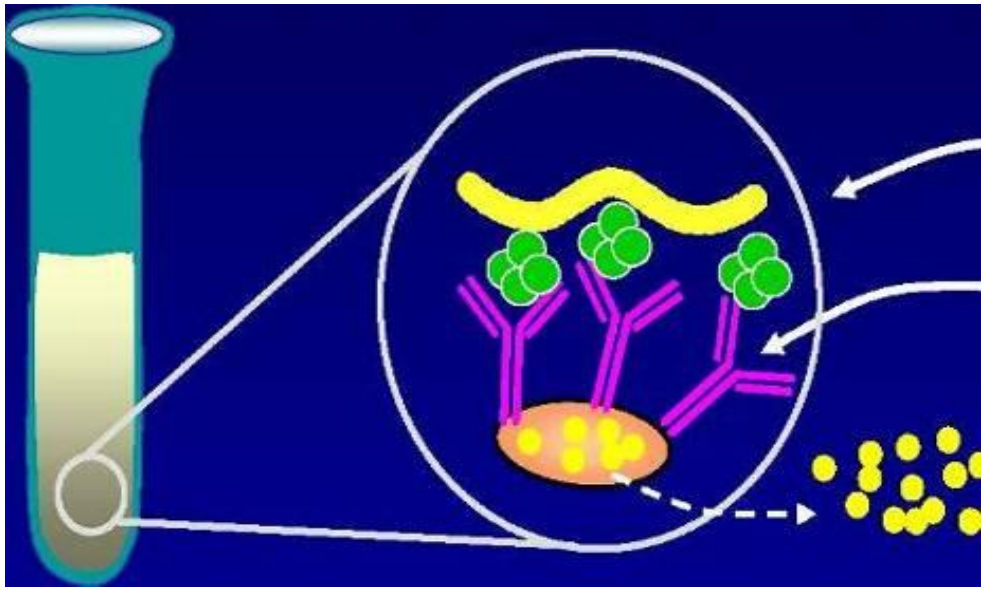
ELISA


Solid-phase Anti-PF4/heparin-ELISA
"Immunoassay"
wash
wash
Patient serum or plasma is added to microtiter plates coated with PF4 and heparin
Add alkaline phosphatase-conjugated goat antihuman IgG
Add substrate
COLOR
heparin
PF4
PF4/heparin complex
HIT-IgG (from serum or plasma)
Alkaline phosphatase-conjugated goat antihuman IgG
Adapted from: Lee & Warkentin. In: Warkentin & Greinacher, eds. Heparin-Induced Thrombocytopenia, 3rd edn . New York: Marcel Dekker, 2004

Heparin Antibody Testing by Heparin-induced platelet aggregation



Serotonin release assay



Heparin/PF4 complex

HIT IgG

Radiolabeled serotonin released from platelets

ADAMTS-13 Testing

Assay Methods for ADAMTS-13

- Used to assess ADAMTS-13 activity levels
- Substrate – VWF (purified or recombinant)
- VWF unfolding – urea or guanidine
- Activation – $BaCl_2$
- Detection –decrease in related function
- ADAMTS-13 activity inhibited by EDTA
  - Must use citrate instead

Collagen-Binding Assay

- Gerritsen, et. al.
- Small vWF fragments do not bind collagen; large forms do
- Dilutions of patient's plasma mixed with purified vWF
- Incubation for 2 hours
- ELISA – Microtiter plates coated with collagen type III
- Collagen-bound VWF quantified using labeled antibodies: detection of (large) vWF bound to collagen by ELISA indicates poor ADAMTS-13 activity

Bethesda Inhibitor Assay

- Mixing studies
  - Normal human plasma mixed with patient's plasma
- Residual activity measured via ADAMTS-13 assay
- One Bethesda Unit = quantity of inhibitor that neutralizes 50% of the ADAMTS-13 activity in normal plasma
  - Increase in Bethesda units is exponential
  - Normal is ≤ 0.3 Bethesda Units

When ADAMTS-13 assay is ordered here at MHH…

- Sent to the Blood Center of Southeastern Wisconsin Reference Laboratory
- Gerritson method and Bethesda Inhibitor Assay
- Sample collected in citrate and sent frozen
- Assay run 2x per week
- Turnaround time 7-10 days

Thromboelastograph

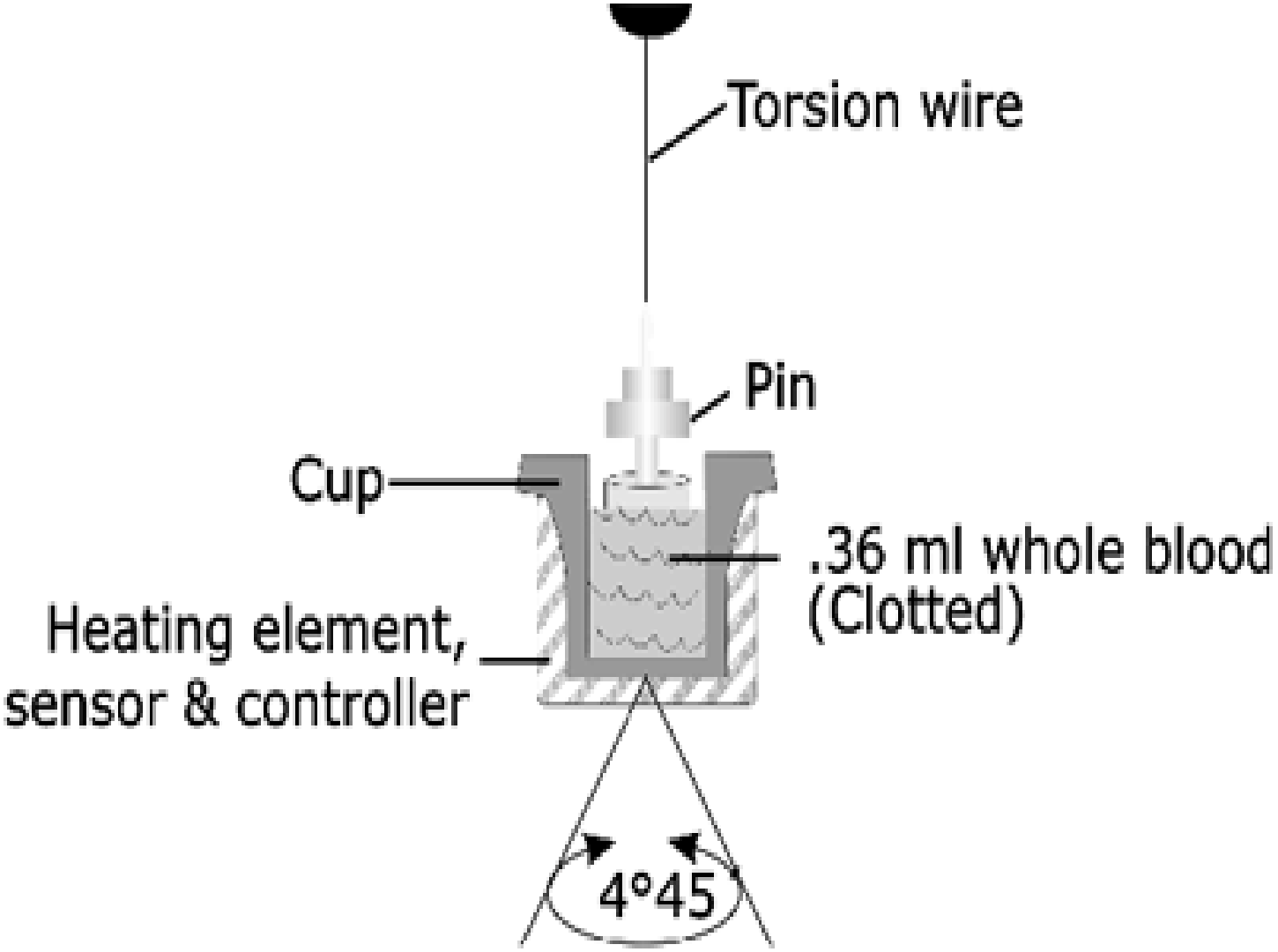
Thromboelastograph (TEG): principles

Measuring the mechanical properties of the developing clot:

- The time it takes until initial fibrin formation.
- The kinetics of the initial fibrin clot to reach maximum strength.
- The ultimate strength and stability of the fibrin clot, ie. its ability to mechanically impede hemorrhage without permitting inappropriate thrombosis.

TEG® 5000
Thrombelastograph®
Hemostasis Analyzer

- The TEG analyzer has a sample cup that oscillates back and forth constantly at a set speed through an arc of 4°45'. Each rotation lasts ten seconds. A whole blood sample of 360 ul is placed into the cup, and a stationary pin attached to a torsion wire is immersed into the blood.
- When the first fibrin forms, it begins to bind the cup and pin, causing the pin to oscillate in phase with the clot. The acceleration of the movement of the pin is a function of the kinetics of clot development.

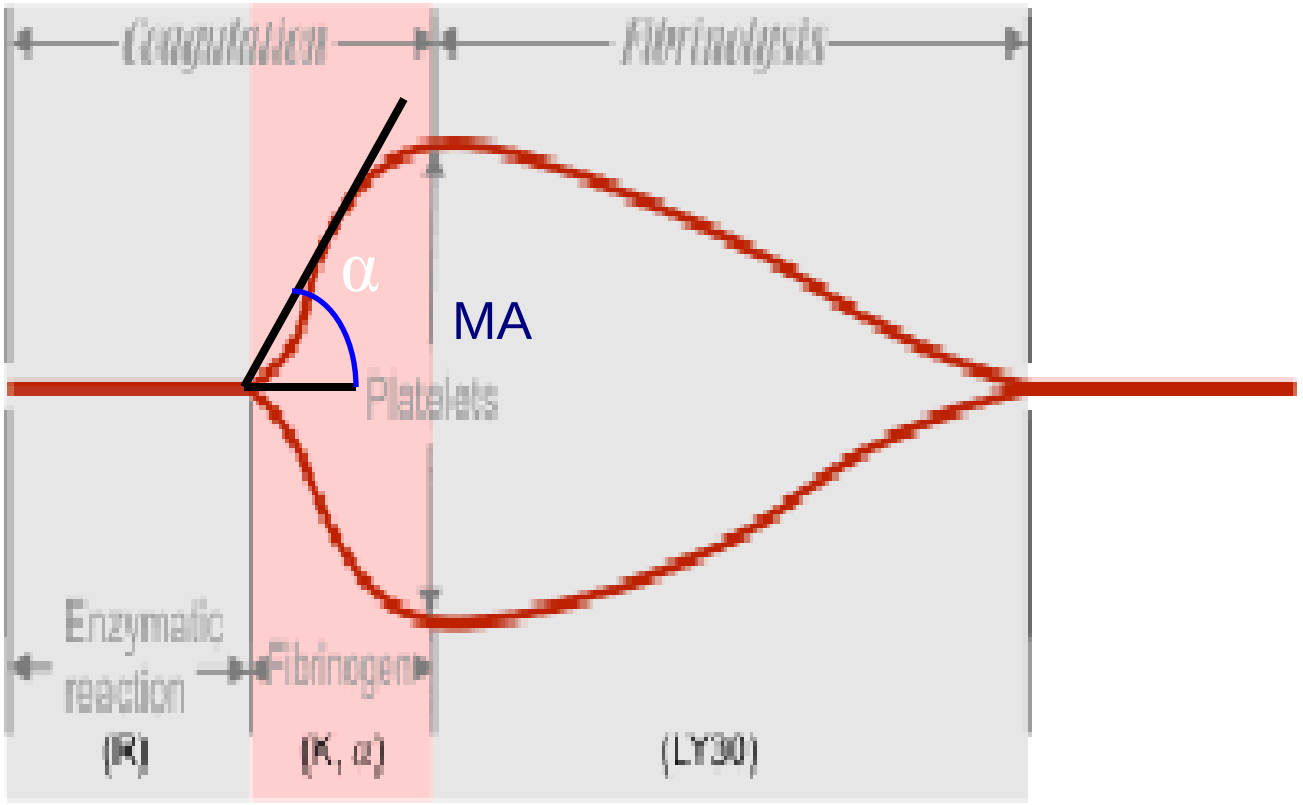

Torsion wire
Pin
Cup
.36 ml whole blood
(Clotted)
Heating element,
sensor & controller
4°45

- The torque of the rotating cup is transmitted to the immersed pin only after fibrin-platelet bonding has linked the cup and pin together. The strength of these fibrin-platelet bonds moves the pin directly in phase with the cup motion. Thus, the magnitude of the output is directly related to the strength of the formed clot.
- As the clot retracts or lyses, these bonds are broken and the transfer of cup motion is diminished. The rotation movement of the pin is converted by a mechanical-electrical transducer to an electrical signal which can be monitored by a computer.

- The resulting hemostasis profile is a measure of:

  -The time it takes for the first fibrin strand to be formed,
  -The kinetics of clot formation,
  -The strength of the clot (in shear elasticity units of dyn/cm2), and
  -Dissolution of clot.

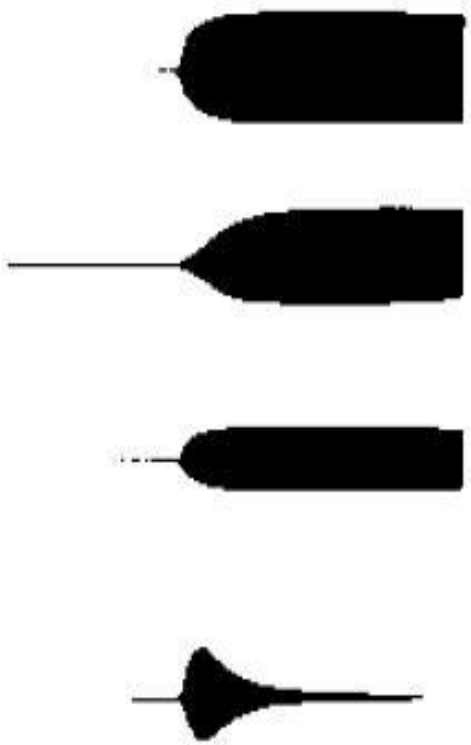
Parameters of clot dynamics



Parameters of clot dynamics

Clotting time	**R**	**The period of time of latency from the time that the blood was placed in the TEG analyzer until the initial fibrin formation (MA= 2 mm).**
Clot kinetics	**K**	**A measure of the speed to reach a specific level of clot strength (MA= 20 mm).**
	alpha	**Measures the rapidity of fibrin build-up and cross-linking (clot strengthening)**
Clot strength	**MA,G**	**A direct function of the maximum dynamic properties of fibrin and platelet bonding via GPIIb/IIIa and represents the ultimate strength of the fibrin clot.**
Hemostasis profile	**CI**	**Coagulation Index, which is a linear combination of the above parameters.**
Clot stability	**LY30**	**Measures the rate of amplitude reduction 30 minutes after MA.**

Patterns of TEG Tracings



- **Normal**
  R;K;MA;Angle = Normal
- **Anticoagulants/hemophilia**
  Factor Deficiency
  R;K = Prolonged
  MA;Angle = Decreased
- **Platelet Blockers**
  Thrombocytopenia/Thrombocytopathy
  R ~ Normal; K = Prolonged
  MA = Decreased
- **Fibrinolysis**
  R ~ Normal;
  MA = Continuous Decrease

Patterns of TEG Tracings

- **Hypercoagulation**
  R;K = Decreased
  MA;Angle = Increased

Thromboelastograph

- **D.I.C.**
  Stage 1- Hypercoagulable state with secondary fibrinolysis

- Stage 2 - Hypocoagulable state

Factor V Leiden

Testing for Factor V Leiden

- Clot-based testing (blue top tube)
- Polymerase chain reaction (PCR) testing (purple top tube)

Clot-based Testing

- Determines the resistance to APC, using platelet-poor plasma
- Principle of test: in patient with APC resistance, Factor V is not inactivated by APC, hence (PTT with APC) is not prolonged. This will shorten the APC Ratio (APCR)

Clot-based Testing (cont'd)

- APCR = (PTT with APC)/(PTT without APC)

  APCR > 2 -> negative for APC resistance
  APCR < 2 -> positive for APC resistance

- Considerable overlap between FV Leiden heterozygous and normal

  Note: cut-off value is dependent on particular test kits

Clot-based Testing (cont'd)

- Inaccurate result with: intrinsic factor deficiency, lupus anticoagulant, anticoagulant (need to get pre-treatment sample)
- New generation test (COATEST by Chromogenix)
  1. Predilution of patient sample with FV deficient plasma before testing: alleviates coumadin interference
  2. Polybrene: alleviates heparin intereference

PCR Testing

- Amplifies the mutated gene fragment. Results: negative, heterozygous, homozygous.
- Results not effected by factor deficiency, lupus anticoagulant, anticoagulant
- PCR testing cannot detect APC resistance that is not due to FV Leiden

PCR Testing (cont'd)

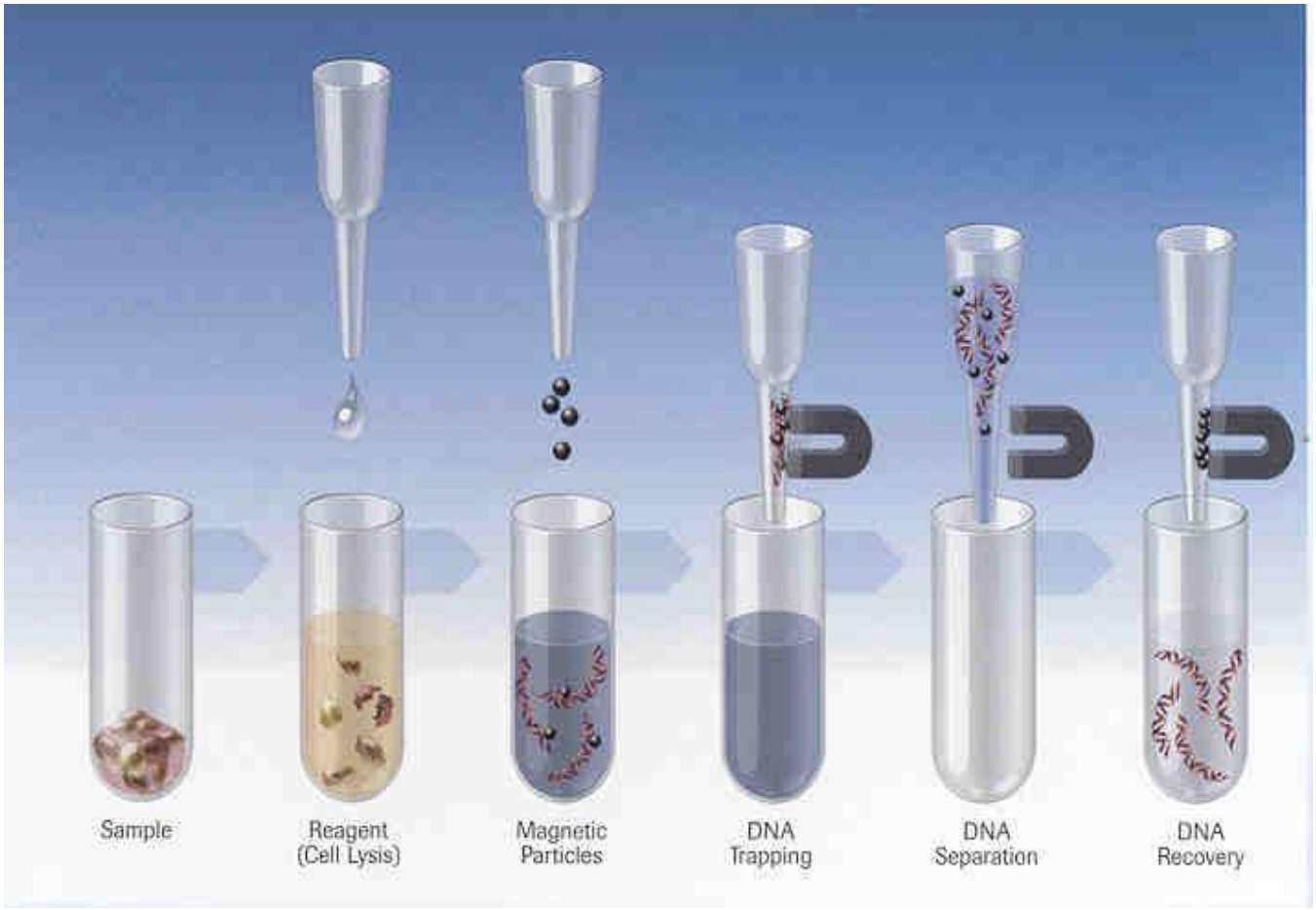
- Genomic DNA from lymphocytes
- DNA sequence flanking the mutation site is amplified by PCR, resultant product is analyzed by restriction enzyme digestion
- Normal (wild type): two normal FV alleles
  Heterozygous: one abnormal allelle
  Homozygous: two abnormal allelles

New PCR Testing: LightCycler (Roche)

- Melting curve analysis method
- “Real time” analysis
- 35 thermal cycles in 25 min, followed by melting curve analysis in 5 min -> results in 30 min
- Batch of 32 samples
- Designed for clinical lab setting
- Optional module for automated DNA extraction (60 min for 32 sample extraction)

MagNA Pure LC and LightCycler

Magnetic Bead Technology for DNA Extraction



LightCycler Schematics

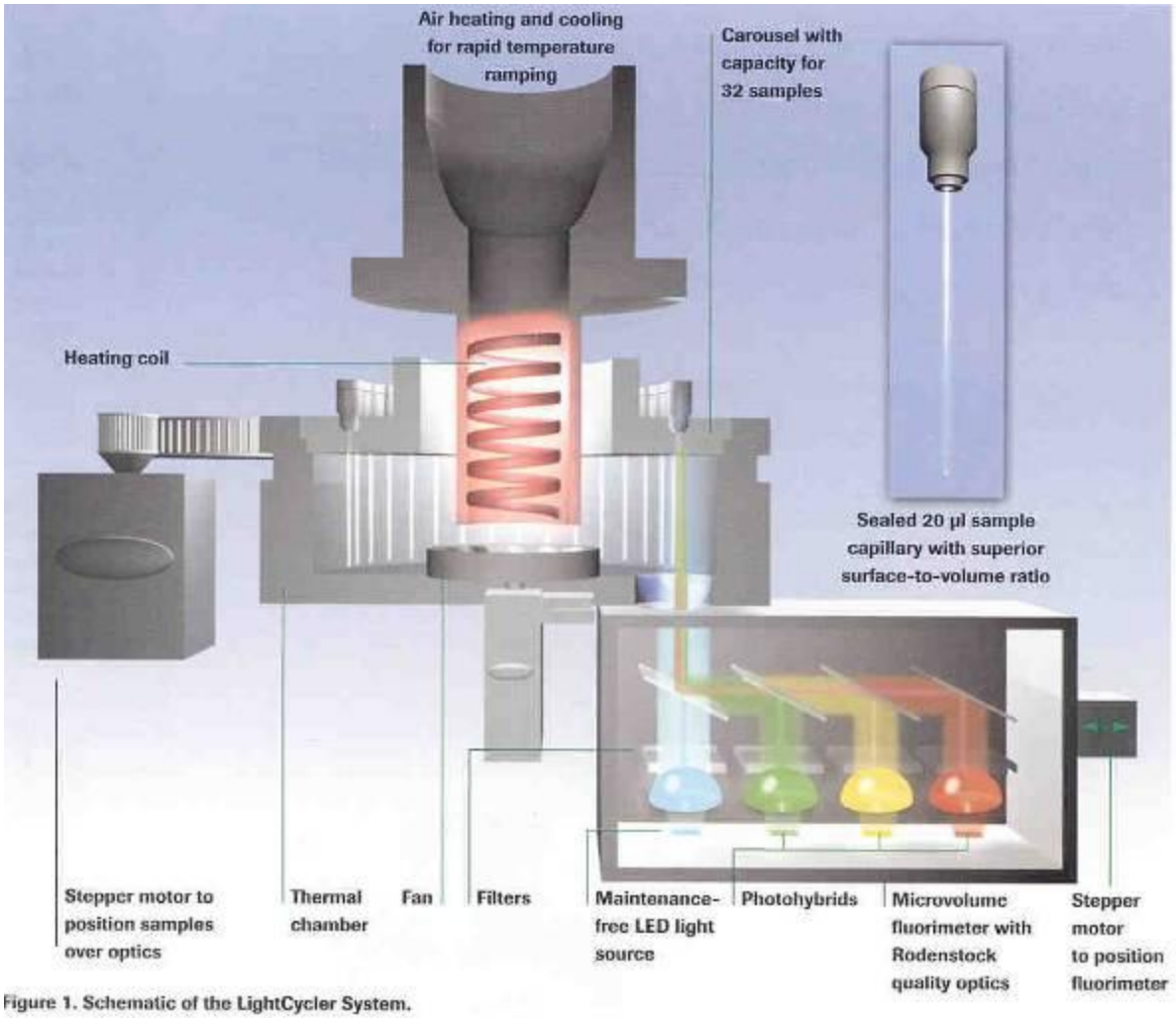
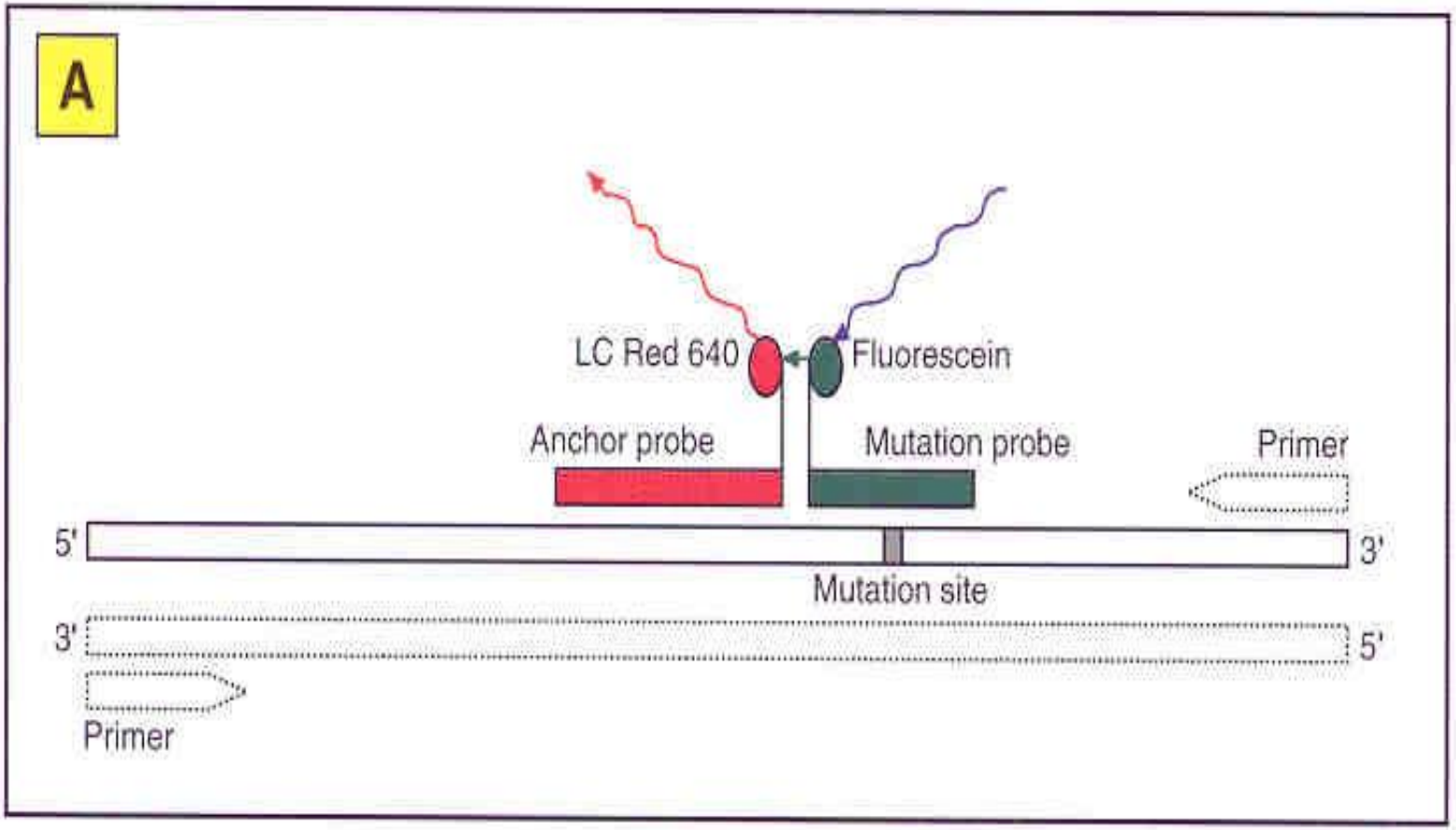


Figure 1. Schematic of the LightCycler System.

FV Leiden Mutation: Hybridization Probe with Fluoresence Resonance Energy Transfer (FRET)



FV Leiden Mutation: Melting Curve Analysis