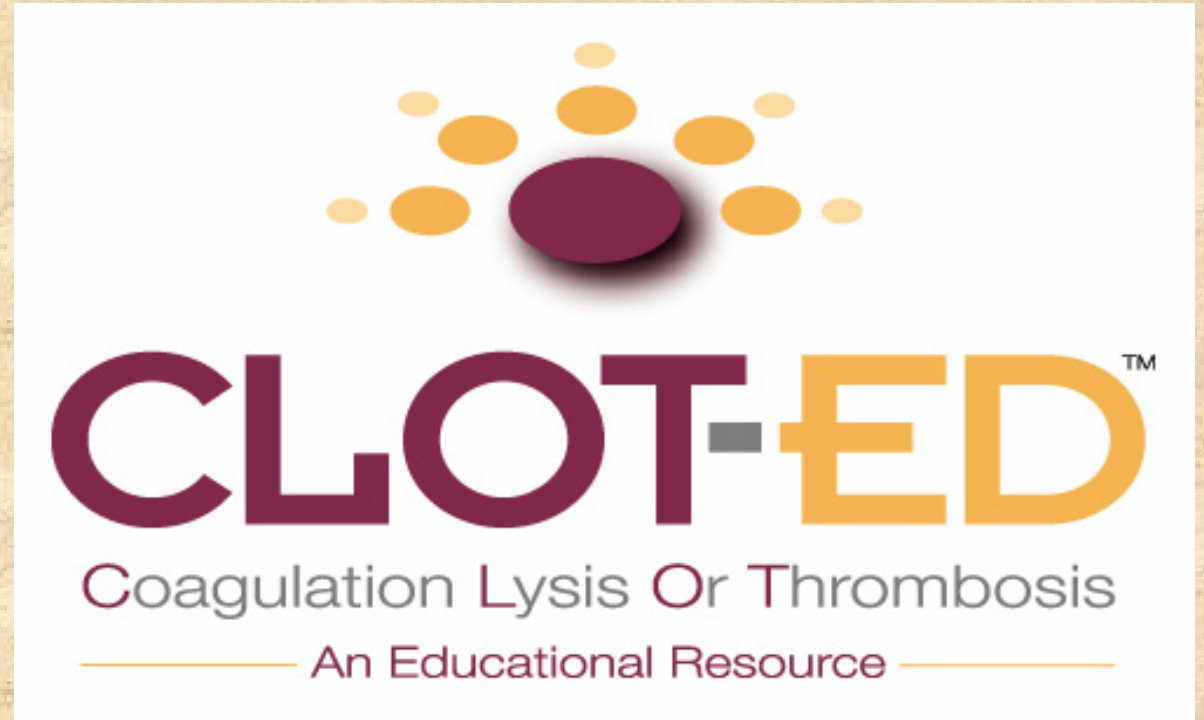
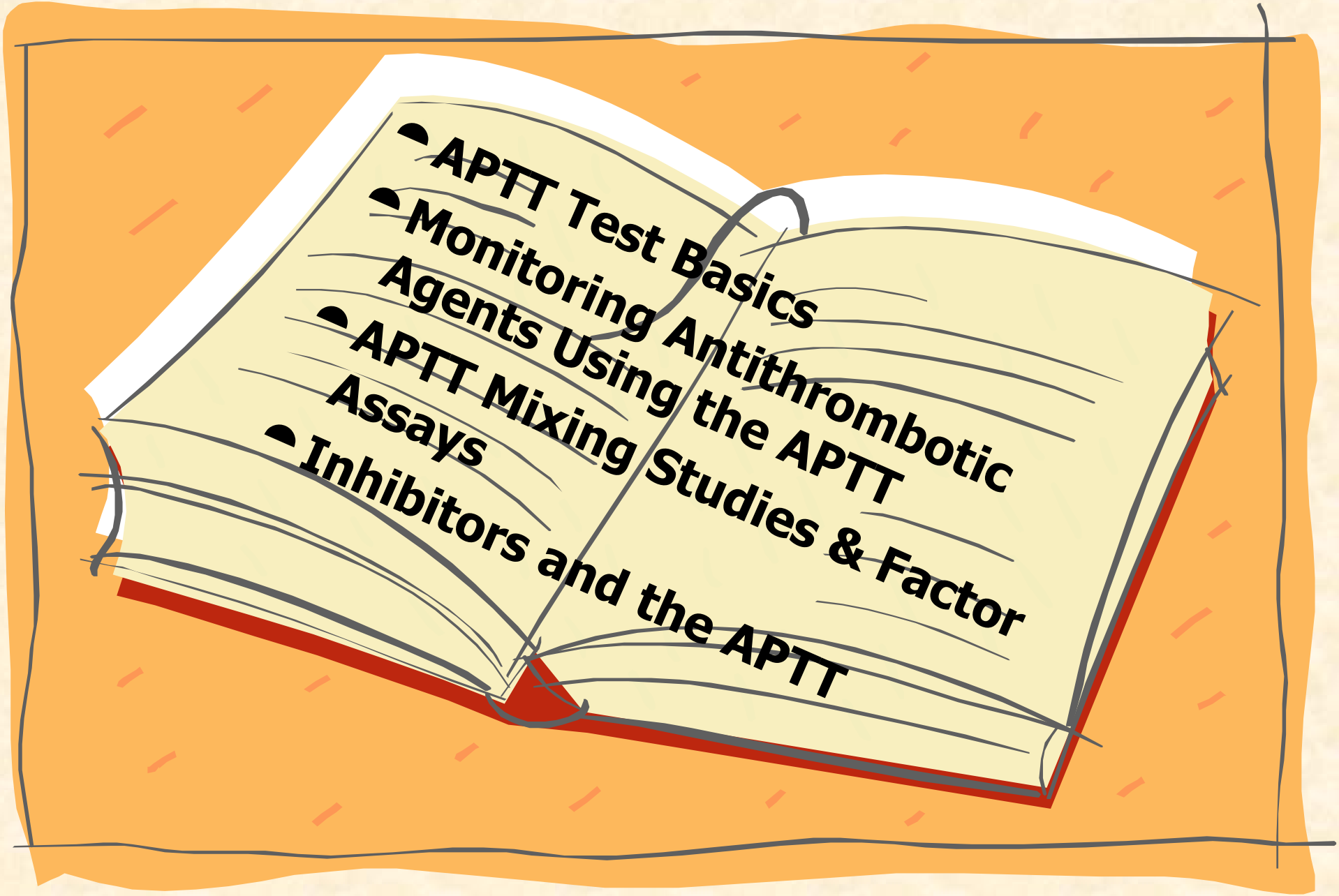
Activated Partial Thromboplastin Time (APTT) Testing

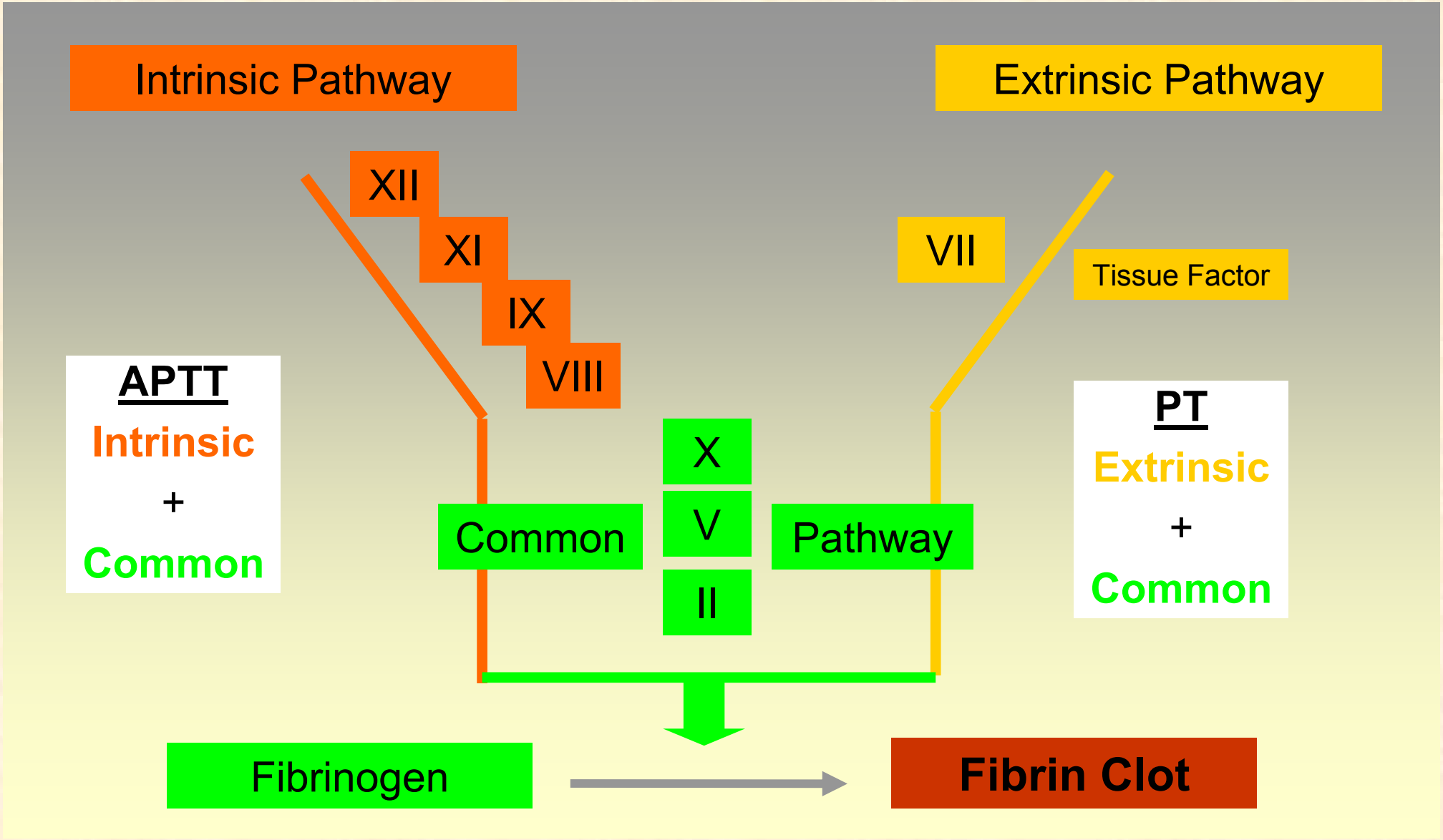


Marlies Ledford-Kraemer,
MBA, BS, MT(ASCP)SH

Topics for Discussion



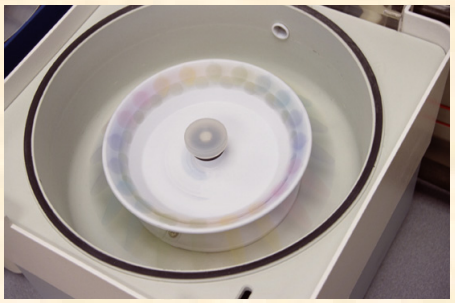
Coagulation in the Laboratory

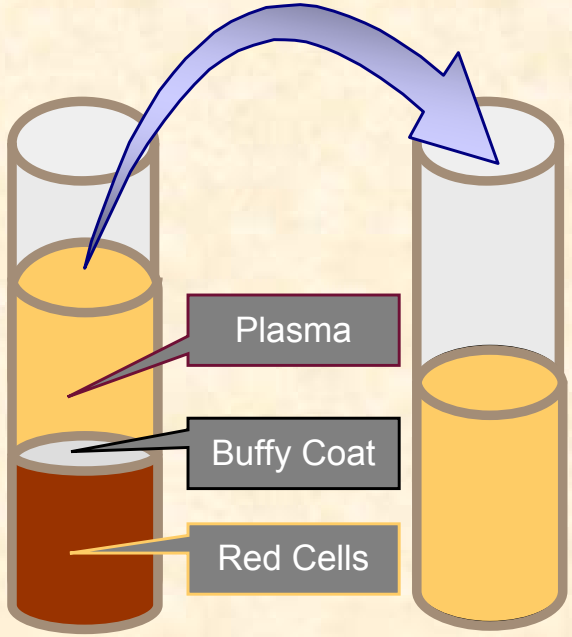


APTT Test Basics

The Specimen

- Blood is collected into a tube containing 3.2% sodium citrate as the anticoagulant
- Blood fluidity is maintained because sodium citrate binds calcium ions, which are critical to the coagulation process
- Tube is centrifuged in order to separate plasma from buffy coat and red blood cells
  - Plasma is "platelet poor" (PPP)

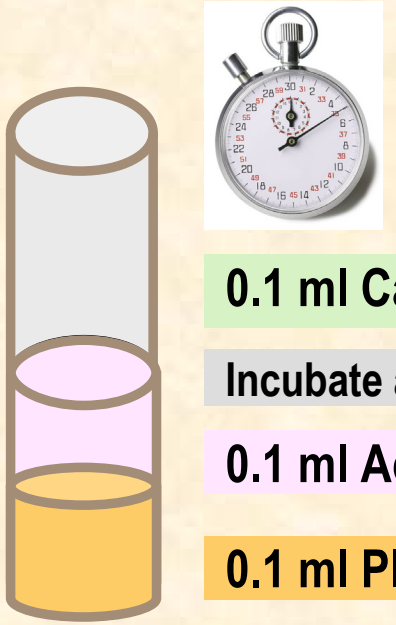




- Plasma is used for testing
  - PLASMA contains FIBRINOGEN
  - Serum does not contain Fibrinogen

APTT Test

- "Partial thromboplastin"
  - Tissue Factor not used to initiate (activate) coagulation
- Two-stage assay
  - Activation & re-calcification
- Reagent composition
  - Activator
    - Converts FXII to FXIIa
  - Phospholipid
    - Replaces *in vivo* platelet phospholipid surface on which coagulation reactions occur
  - Buffer (minimizes pH changes in plasma reaction mixture)
  - $CaCl_2$
    - Re-introduces calcium ions that were chelated by sodium citrate anticoagulant



Variables Affecting APTT

- Pre-analytical variables
  - Phlebotomy, specimen transport, specimen examination, centrifugation & aliquoting, time to testing & storage
    - Please refer to Focus Article entitled *Garbage In—Garbage Out: Talking Trash About Pre-Analytical Variables (Part 1)* for more details
- Analytical variables
  - Reagent sensitivity to coagulation factors, heparin, Direct Thrombin Inhibitors (DTI), and Lupus Anticoagulant (LA)
    - Types of activators
    - Source, concentration, and types of phospholipid
  - Instrument clot detection method
    - Mechanical
    - Photo-optic
    - Nephelometric

APTT Reagent Activators

- Types of activators
  - Particulate activators
    - Kaolin (diatomatacious earth)
    - Micronized or colloidal silica
    - Celite
  - Non-particulate activator
    - Ellagic acid (liquid activator)
- Rationale for using activators
  - Provide large surface areas for reactions to occur
  - Influence time required for test incubation
    - Replace glass surfaces originally used to perform Partial Thromboplastin Time (PTT)
    - Shorten clotting times that historically were seen with PTT thereby reducing test imprecision

Phospholipids in APTT Reagent

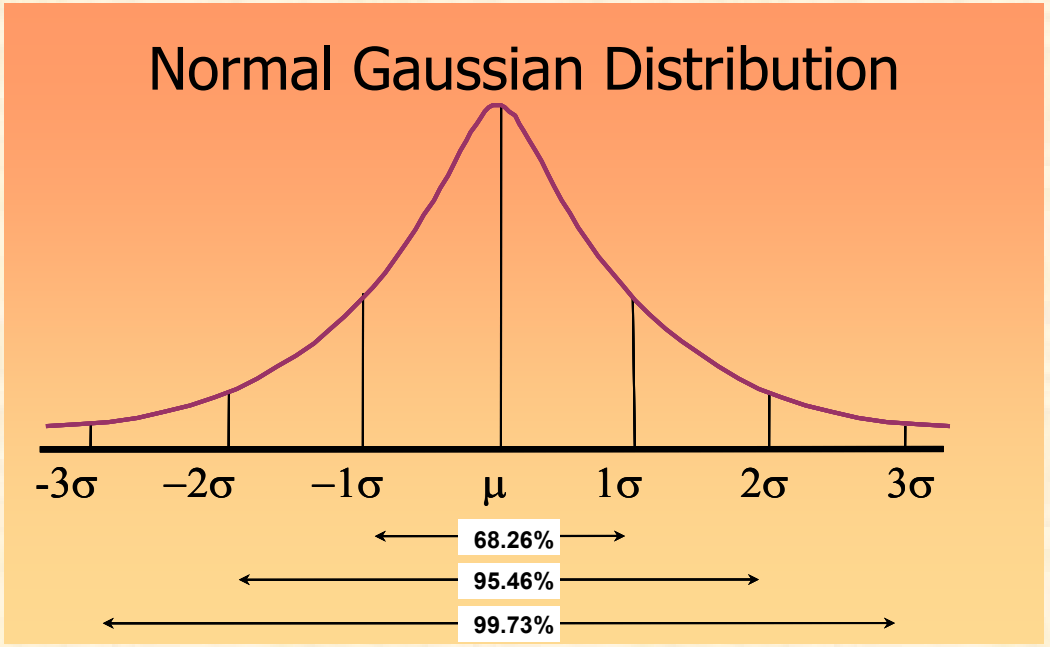
- Source
  - Human placenta, rabbit brain, bovine brain, soybean (purified), or synthetic lipidation in liposomes
- Concentration
  - High levels
    - Insensitive to the Lupus Anticoagulant (overwhelm or mask the inhibitor)
  - Low levels
    - Sensitive to the Lupus Anticoagulant (accentuate inhibitor effect)
- Types of phospholipids
  - Reagents with lower levels of phosphatidylserine are more sensitive to the Lupus Anticoagulant

Intended Use for APTT

- Screening test
  - APTT reagent should be sensitive to a reduction in coagulation factors that are associated with bleeding
    - Intrinsic (and severe common pathway) factor deficiencies
- Laboratory monitoring
  - Unfractionated heparin
  - Direct Thrombin Inhibitors
- Laboratory detection of inhibitors
  - Lupus Anticoagulant is most common

What is Normal?

- Reference intervals provide a range of normalcy
- Establish using minimum of 20-30 normal healthy individuals



- Desired test (APTT, Factor VIII) is performed with instrumentation and reagents used for patient testing
- Determine mean and ± 2 SD (standard deviation or sigma [σ])
- A 2 SD range reflects normalcy for 95% of population whereas a 3 SD range includes 99.7%

Causes for Prolonged APTT

- Most common causes
  - Heparin (contamination from lines or therapeutic)
  - Lupus Anticoagulant
  - Normal after retesting (pre-analytical issues)
- Other causes
  - Reduction in or deficiencies of coagulation factors
    - FVIII, FIX, FXI, FXII, Contact Factors, FV, FVIII Inhibitor (↓ FVIII)
  - Liver disease (site for production of most coagulation factors)
  - Consumption of coagulation factors as seen in Disseminated Intravascular Coagulation (DIC)
  - Vitamin K deficiency (warfarin: affects FII, FIX, FX)
  - Hypo and Dys-Fibrinogenemias

APTT Monitoring of Antithrombotic Agents

Unfractionated Heparin

- Tests to detect unfractionated heparin
  - APTT
  - Thrombin Time (TT)
  - Reptilase Time
  - Hepzyme® (Dade Behring®)
    - Heparinase I enzyme
    - Neutralizes up to 2.0 Units of unfractionated heparin in 1.0 ml of citrated plasma
- If heparin is present, is it a contaminant or a therapeutic?

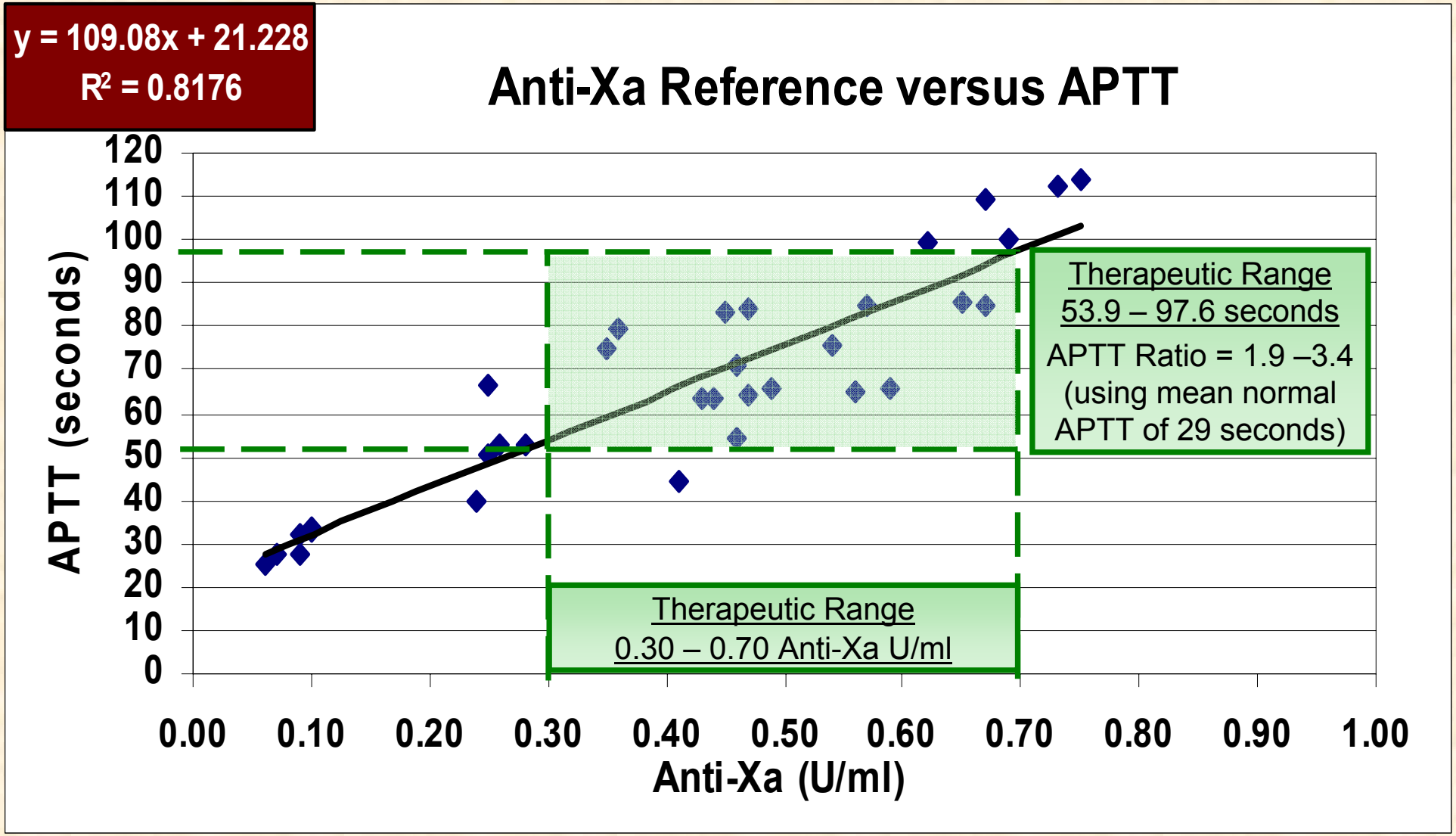
APTT Monitoring of Heparin

- Assumes antithrombotic (anti-IIa) effect parallels anticoagulant effect
- Limitations
  - Pre-treatment APTT of patient
    - Baseline APTT of patient prolonged due to Lupus Anticoagulant
    - Baseline APTT of patient sample below or at low end of reference interval (-2 to –3 SD) due to high levels of FVIII (apparent "heparin resistance")
  - APTT reagents vary in sensitivity to heparin
    - Laboratories must determine responsiveness of their APTT reagent to unfractionated heparin
    - Determine APTT therapeutic interval (seconds) for reagent used to monitor heparin therapy

Assessing Heparin Sensitivity

- Response curve: comparing APTT to Anti-Xa levels
  - Perform APTT and Anti-Xa assays simultaneously on plasma samples from patients receiving heparin (*ex vivo* samples)
  - Note APTT values that correspond to low (0.3 U/ml) and high (0.7 U/ml) ends of therapeutic interval when using Anti-Xa assay (see next slide for example)
- Comparing APTT reagents (current lot number to new)
  - Perform APTT on minimum of 30 *ex vivo* samples using both reagents
  - Sum data for each reagent, determine means, and compare difference between means
  - Repeat process for each new reagent change (yearly) and determine cumulative change
  - Difference of more than 7 seconds between 1) reagent means or 2) cumulative means, indicates an unacceptable level of variation that can adversely affect therapeutic interval currently in use

Heparin Response Curve



Direct Thrombin Inhibitors

- Hirudin [Lepirudin (rDNA)–trade name: Refludan®]
  - Approved in USA, Canada, and EU for Heparin Induced Thrombocytopenia (HIT) complicated by thrombosis
  - Target APTT is 1.5-2.5 x patient baseline APTT
  - In absence of severe thrombosis, some experts recommend a target APTT of 1.5-2.0 x patient baseline APTT and monitor every 4 hours
- Argatroban [non-US trade name: Novastan]
  - Approved for HIT with or without thrombosis and also for anticoagulation during percutaneous coronary intervention (PCI) in patients with, or at risk for, HIT
  - Target APTT 1.5-3.0 x patient baseline APTT (maximum 100 seconds)
- Hirulog [Bivalirudin-trade name: Angiomax™]
  - Undergoing evaluation for use as an anticoagulant for "on-pump" and "off-pump" cardiac surgery in patients with HIT
  - Target APTT is 1.5-2.5 x patient baseline APTT

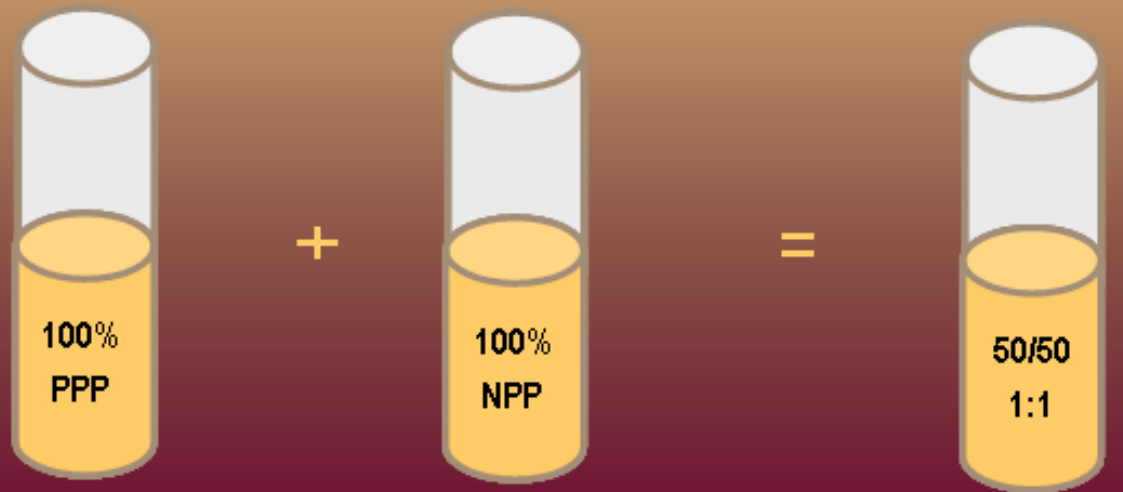
APTT Mixing Studies and APTT Factor Assays

Classical Mixing Study

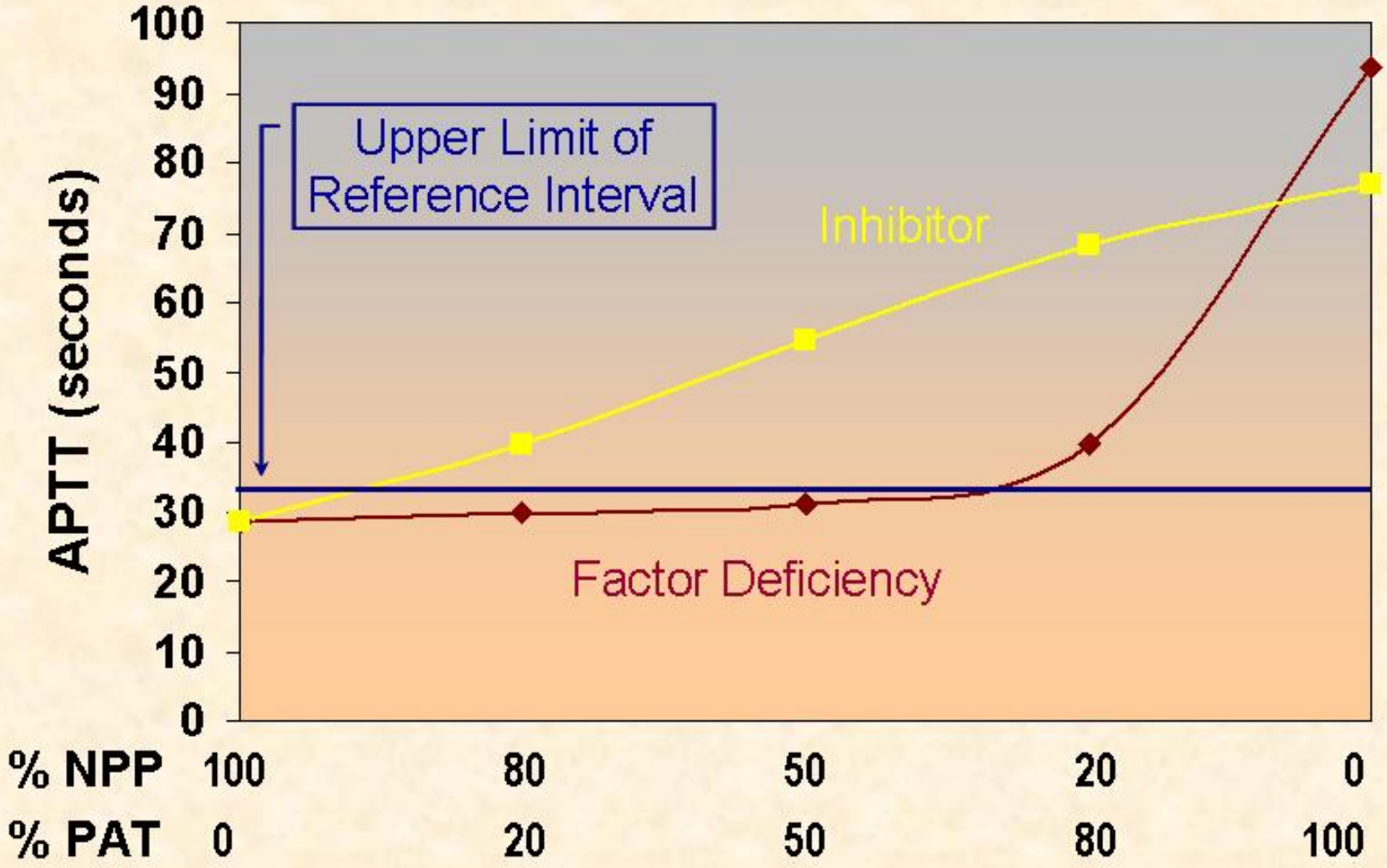
- Use normal pooled plasma (NPP)
- Mix with patient plasma
- Perform APTT
- Controversy as to what constitutes a "correction"
  - Value within APTT reference interval?
  - Value within 5 seconds of upper limit (+2SD) of reference interval?

IF baseline APTT is prolonged:

- Mix patient platelet poor plasma (PPP) with normal pooled plasma (NPP) and repeat APTT
  - Most common mix is "1:1" Mix (50/50 Mix)
- Failure to "correct" (upper limit of reference interval) indicates presence of an inhibitor
- "Correction" indicates a factor deficiency



Mixing Study Patterns



% NPP = Percent Normal Pooled Plasma: % PAT = Percent Patient Plasma

APTT Factor Disorders

- Reagent must prolong APTT above upper limit (+2SD) of reference interval if factor level is below ~40% activity

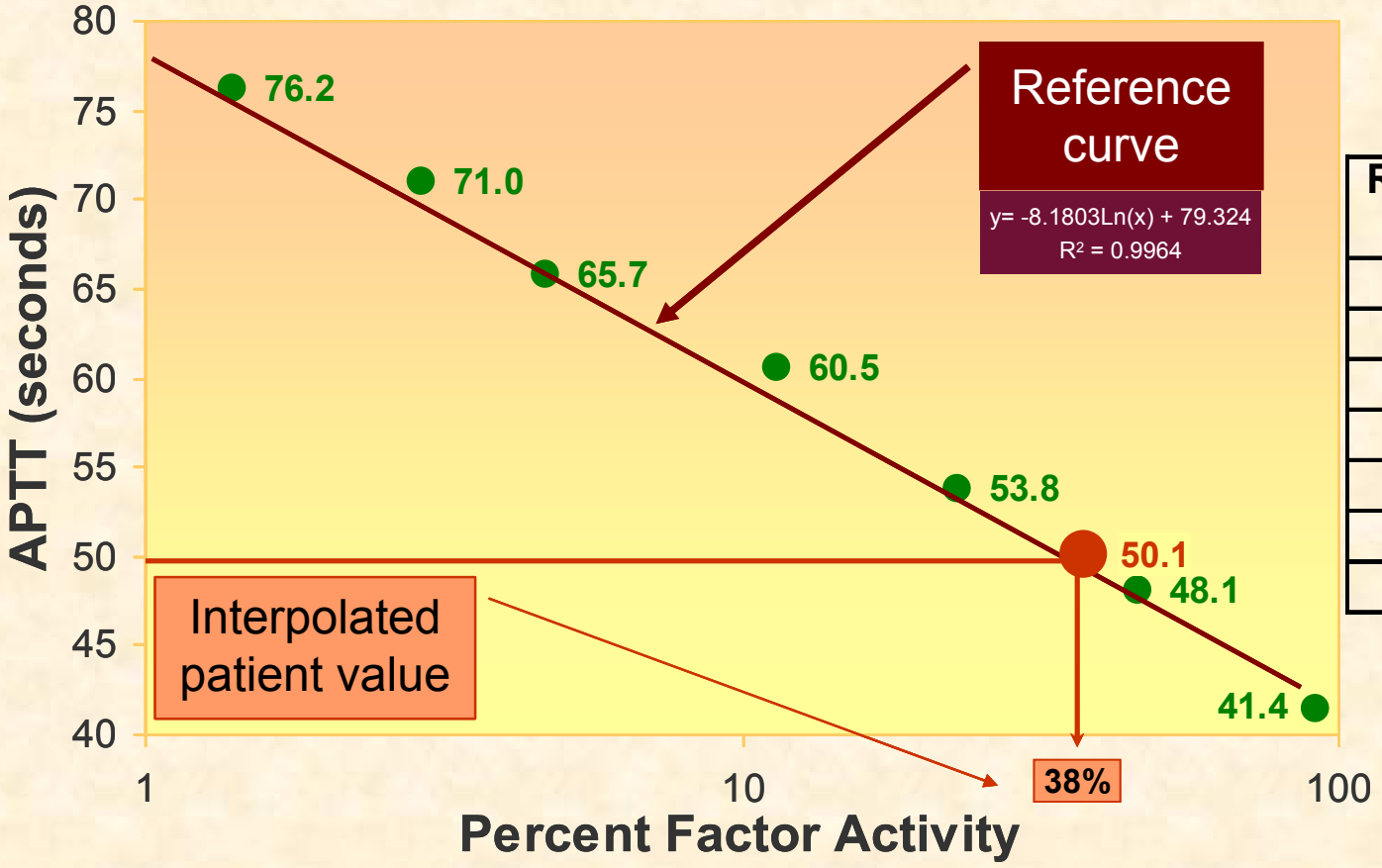
- Deficiency of Factor VIII
  - Associated with Hemophilia A and Von Willebrand Disease
- Deficiency of Factor IX (Hemophilia B)
- Deficiency of Factor XI (Hemophilia C)
  - Found predominantly in individuals of Ashkenazi Jewish descent

- Contact factors
  - FXII, Prekallikrein, HMW Kininogen
  - Reagents are generally relatively insensitive to these factors
  - Patients do not have a bleeding history

Factor Assays

- Based on the APTT
- Test performed in a dilute system (1:10, ie 1 part plasma to 9 parts buffer)
- Assays are "mixing studies" that use diluted patient plasma to correct specific deficiency in a "deficient substrate plasma"
  - For example: diluted patient plasma is used to "correct" the deficiency of FVIII (complete absence) in FVIII deficient substrate plasma
- Quantify by interpolating "mixing study" clotting time from a standard curve

Factor Assay Curve



Reference Dilution	% FVIII X (log)	Seconds Y (lin)
1:10	92	41.4
1:20	46	48.1
1:40	23	53.8
1:80	11.5	60.5
1:160	5.7	65.7
1:320	2.9	71.0
1:640	1.4	76.2

Example

Patient value is determined by interpolating time, 50.1 seconds using a 1:10 dilution, from the reference curve to yield a FVIII activity of 38%. This indicates that the patient's FVIII activity level is only 38% that of normal activity.

Inhibitors and the APTT

"Circulating Anticoagulants"

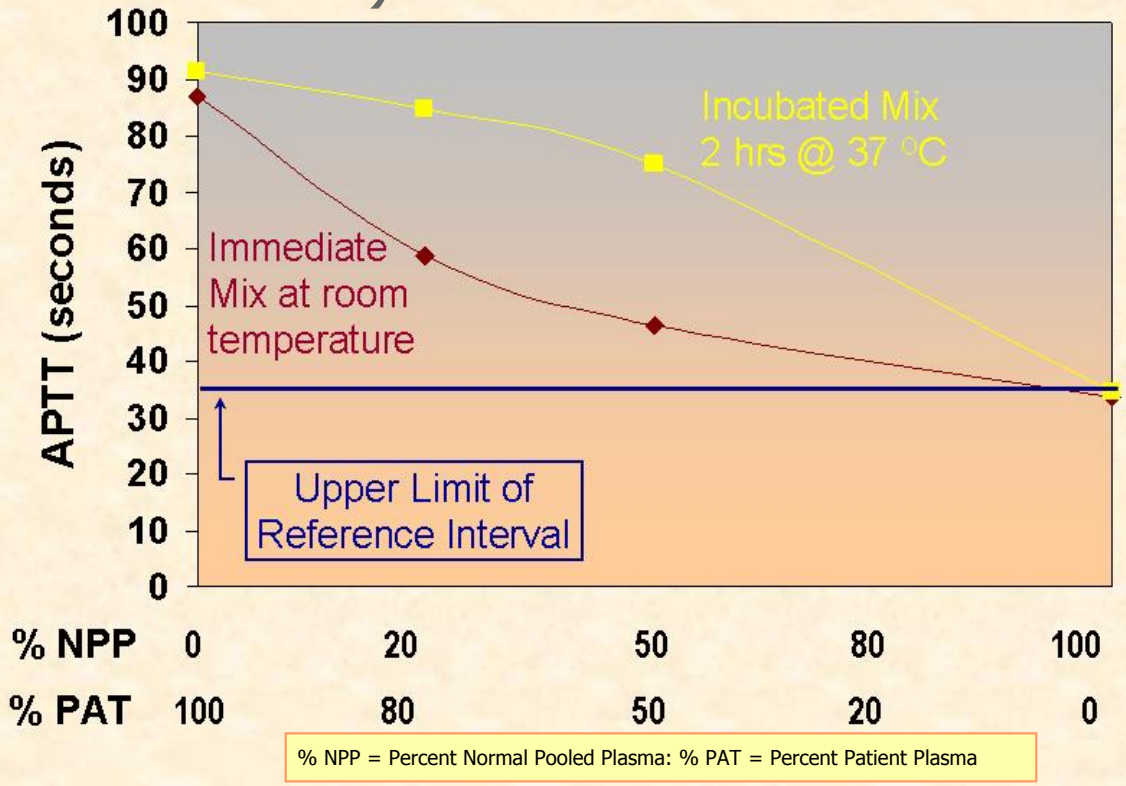
- Inhibitors to specific factors
  - Neutralizing antibodies
  - Non-neutralizing antibodies
- Non-specific inhibitors
  - Lupus Anticoagulant, Fibrin Degradation Products, paraproteins (Dysproteinemias)
- Global inhibitors
  - Heparin-like activity (glycosaminoglycans)
  - Heparin (unfractionated) and Direct Thrombin Inhibitors (hirudin, argatroban, hirulog)

Specific Inhibitors

- Immunoglobulins (generally IgG4) with epitope specificity for a single protein/site that interfere with reaction(s) involved in generating Thrombin and formation of a stable fibrin clot
  - Neutralizing (completely neutralize factor)
    - FVIII
    - FV, FIX, FXI, FXII, VWF, Thrombin, Fibrinogen
  - Non-neutralizing
    - FVIII (acquired)
    - FIX (cleared in nephrotic syndrome)
    - FX (amyloidosis)
    - FII
    - VWF

FVIII Inhibitors

- Alloantibodies develop in ~15-35% of Hemophilia A (HA) patients who have received exogenous (non-self) FVIII through replacement therapy
- Autoantibodies are found in "acquired hemophilia" (antibodies develop against self FVIII)
- Antibodies lead to neutralization of exogenous (transfused) or endogenous FVIII
- Time (and temperature) dependent
- Quantify by Bethesda assay

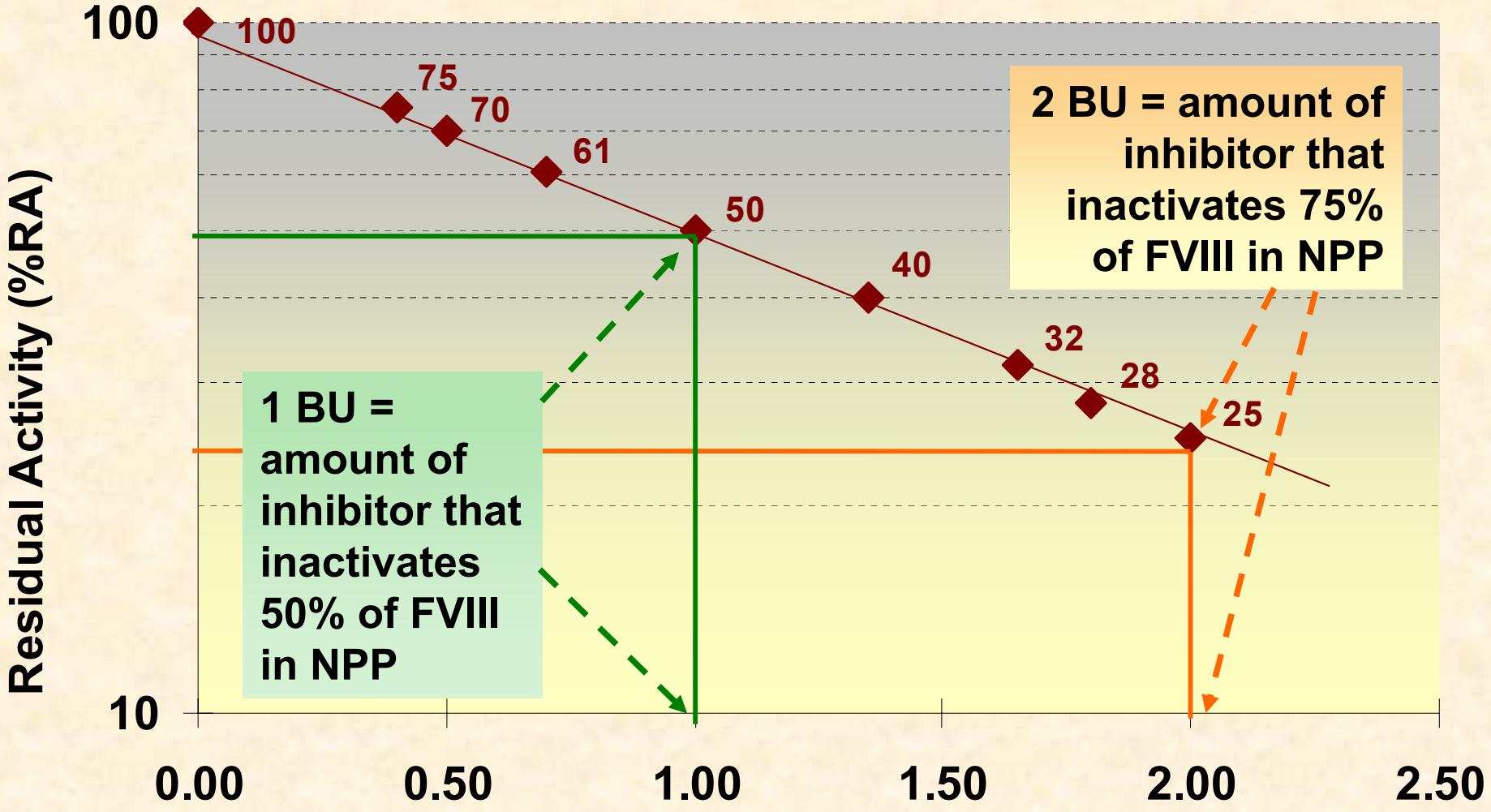


Bethesda Assay

- Prepare serial dilutions of patient plasma in buffer
- Mix patient plasma dilutions with equal part normal pooled plasma (NPP)
  - NPP is source for FVIII
  - NPP + buffer (NPP Mix) is control for FVIII lability
- Incubate for 2 hours at 37 ^{o}C
- Prepare 1:10 dilution of each mixture and perform FVIII assay
- Patient mix / NPP mix = corrected residual FVIII activity (RA)
  - (20% "FVIII" patient/40% "FVIII" NPP) x 100 = 50% RA
- RA utilized to quantify inhibitor activity from curve
  - 50% RA = 1.0 x 2 (1:2 mix) = 2 BU

Bethesda Assay Curve

Patient FVIII / Control FVIII = Corrected Residual FVIII Activity (RA); Read RA from Graph; (RA x Dilution = BU)



(20% "FVIII" patient / 40% "FVIII" NPP) x 100 = 50% RA
From Curve: 50% RA = 1.0 x 2 (1:2 mix) = 2 BU

Non-specific Inhibitors

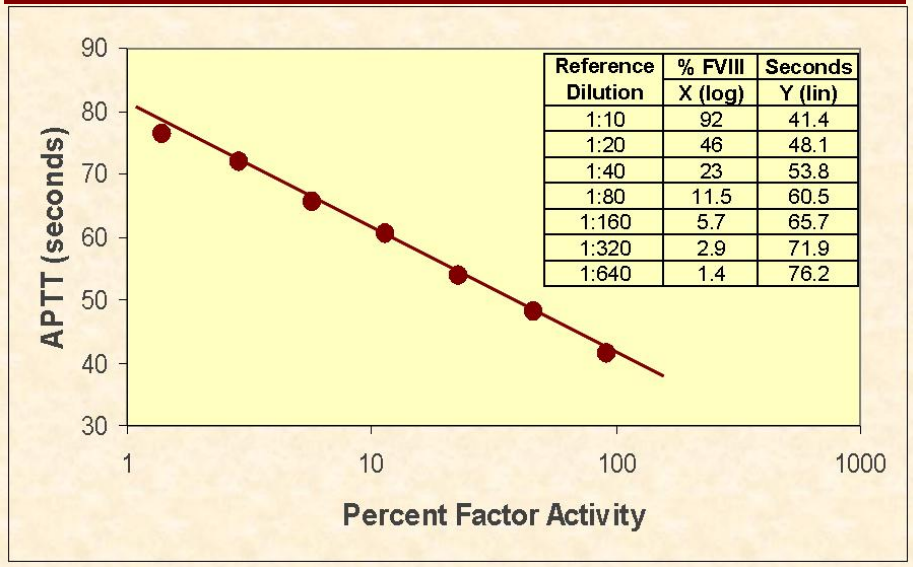
- Lupus Anticoagulant (LA)
  - Please refer to Slide Presentation entitled *Laboratory Diagnosis of the Lupus Anticoagulant* for more details
- Paraproteins
  - Bind to coagulation proteins
  - Complexes readily cleared
- Fibrin(ogen) Degradation Products (FDP or FSP)
  - "Natural anticoagulants"
  - FDPs truncate Fibrinogen protofibril formation

LA Effect: APTT Based Assays

- Factor assays
  - Non-parallelism of patient curve to reference (calibrator) curve
  - Factor activity increases with increasing dilution of plasma
- Protein C & Protein S assays based on APTT
  - False prolongation of clotting times leads to over-estimation of Protein C and Protein S activities

Non-Parallelism Due to LA Effect

Reference Curve



Reference Dilution	% FVIII X (log)	Seconds Y (lin)
1:10	92	41.4
1:20	46	48.1
1:40	23	53.8
1:80	11.5	60.5
1:160	5.7	65.7
1:320	2.9	71.9
1:640	1.4	76.2

LA Sample showing inhibitor effect

Factor activity 1:40 > 1:20 > 1:10 dilution

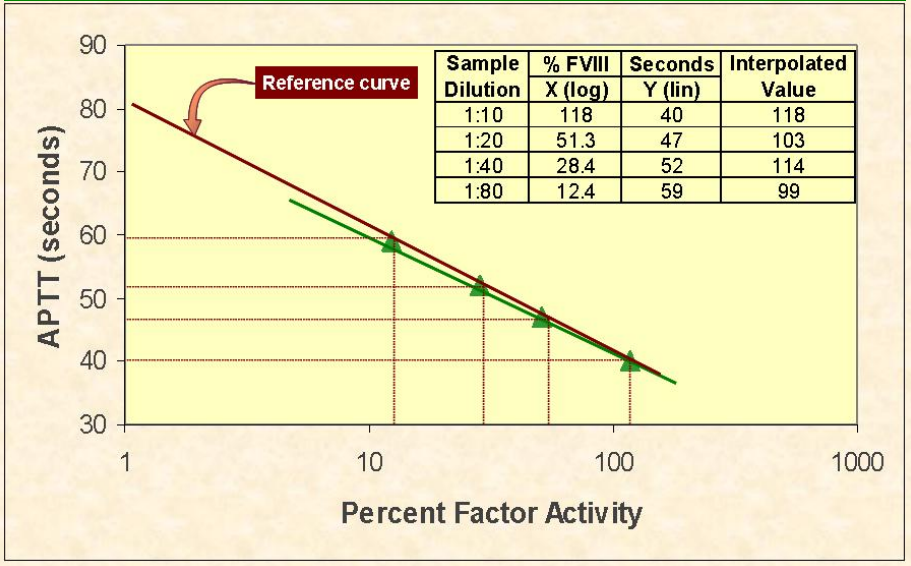
- 11% x 1 (1:10) = 11%
- 18% x 2 (1:20) = 36%

Inhibitor effect

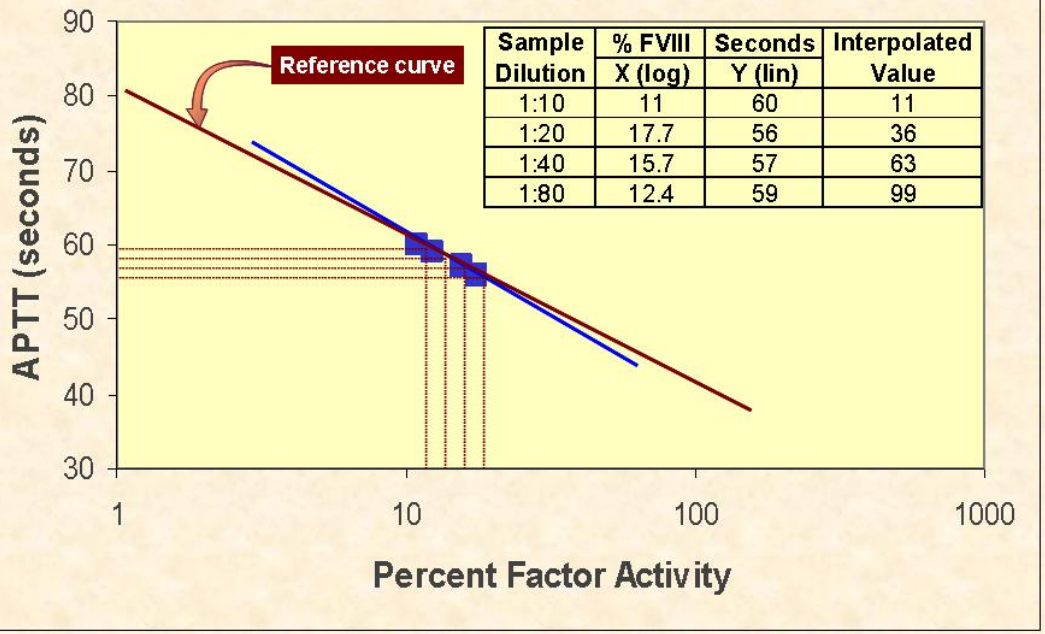
- 15.8 x 4 (1:40) = 63%
- 12.4% x 8 (1:80) = 99%

Inhibitor diluted

Normal Sample (parallel to reference curve)



Sample Dilution	% FVIII X (log)	Seconds Y (lin)	Interpolated Value
1:10	118	40	118
1:20	51.3	47	103
1:40	28.4	52	114
1:80	12.4	59	99



Sample Dilution	% FVIII X (log)	Seconds Y (lin)	Interpolated Value
1:10	11	60	11
1:20	17.7	56	36
1:40	15.7	57	63
1:80	12.4	59	99

Summary

- APTT test is a global assay that must be sufficiently sensitive and robust to fulfill three requirements:
  - Screen for intrinsic coagulation factor defects that may lead to bleeding and to monitor replacement therapy used to correct these deficiencies
  - Monitor unfractionated heparin and Direct Thrombin Inhibitors
  - Detect the Lupus Anticoagulant
- Types and concentrations of activators and phospholipids define an APTT reagent and its ability to meet the above stated objectives
- Assays for quantification of intrinsic coagulation factors and specific inhibitors are based on the APTT and are subject to its limitations

References

- Brandt JT, et al on behalf of The Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the ISTH. Criteria for the diagnosis of lupus anticoagulants: an update. Thromb Haemost 1995;74(4):1185-90.
- Clinical and Laboratory Standards Institute (CLSI). One-stage Prothrombin Time (PT) test and Activated Partial Thromboplastin Time (APTT) test; Approved Guideline H47-A, 1996.
- Greaves M, Preston FE. Approach to the bleeding patient (Chapter 48). In: Colman RW, ed. *Hemostasis and Thrombosis, 4th ed*. Philadelphia: Lippincott Williams & Wilkins, 2001.
- Hirsh J, Raschke R. Heparin and low-molecular-weight heparin: The Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest 2004;126:188S-203S.
- Kasper CK. Laboratory diagnosis of Factor VIII inhibitors. In: Kessler C, ed. *Acquired Hemophilia, 2nd ed*. Princeton: Excerpta Medica, 1995.
- Konkle BA. Clinical approach to the bleeding patient (Chapter 77). In: Colman RW, ed. *Hemostasis and Thrombosis, 5th ed*. Philadelphia: Lippincott Williams & Wilkins, 2006.
- Olson JD, et al. College of American Pathologists Conference XXXI on Laboratory Monitoring of Anticoagulant Therapy: Laboratory monitoring of unfractionated heparin therapy. Arch Pathol Lab Med 1998;122(9):782-98.
- Physicians' Desk Reference, 60th ed. Montvale: Thomson PDR, 2006.
- Poller L. Activated Partial Thromboplastin Time (Chapter 5). In: Jespersen J, ed. *Laboratory Techniques in Thrombosis-A Manual, 2nd ed*. Dordrecht: Kluwer Academic Publishers, 2000.
- Warkentin TE. Heparin-induced thrombocytopenia (Chapter 114). In: Colman RW, ed. *Hemostasis and Thrombosis, 5th ed*. Philadelphia: Lippincott Williams & Wilkins, 2006.