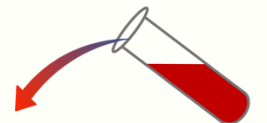


Laboratory Tests of Hemostasis (Part 1)



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Disclosures

- Research Support (Past 2 years):
 - Amgen
 - Janssen Scientific Affairs
 - Sobi/Dova Pharmaceuticals
 - Anthos Therapeutics
 - Alpine Immune Sciences (Data Safety Monitoring Committee)

- Advisory Boards (Past 2 years)
 - Janssen Scientific Affairs
 - Sobi/Dova Pharmaceuticals
 - Sanofi
 - Novartis
 - Agios Pharmaceuticals.



Learning Objectives:

Part 1: Laboratory Tests of Hemostasis

1. Describe analytical principles of routine coagulation screening tests and their clinical utilization.
2. Discuss methods and limitations of clot based, immunoassays, and chromogenic assays.
3. Interpret abnormal coagulation screening test results and what additional testing is recommended.



Material To Cover

1. *The Hemostatic Balance*
2. *Overview of The Coagulation Cascade and Testing*
3. *Functional and Immuno-assays*
4. *The Prothrombin and Activated Partial Thromboplastin Times*
5. *Other Tests:*
 - *Specific Factor Assays*
 - *Anti-Xa Heparin Assay*
 - *Thrombin Time*
 - *Fibrinogen Assay*
 - *D-Dimer*
 - *Thromboelastography (TEG) and Thromboelastometry (ROTEM)*
6. **Interpretation of Prolonged PT and/or aPTT Results**
7. *Tests Of Thrombotic Disease*
8. *Heparin Induced Thrombocytopenia/Thrombosis (HITT): Pathophysiology*
9. *Antiphospholipid Antibody Syndrome*
10. *Laboratory Testing for Thrombophilia (Hypercoagulable State)*
11. *APC-Resistance—Screening Assay For Factor V Leiden*
12. *Conditions That Impact Tests for Thrombotic Risk Factors.*
13. *If/When to Do Hypercoagulable Work-up*



The Hemostatic Balance

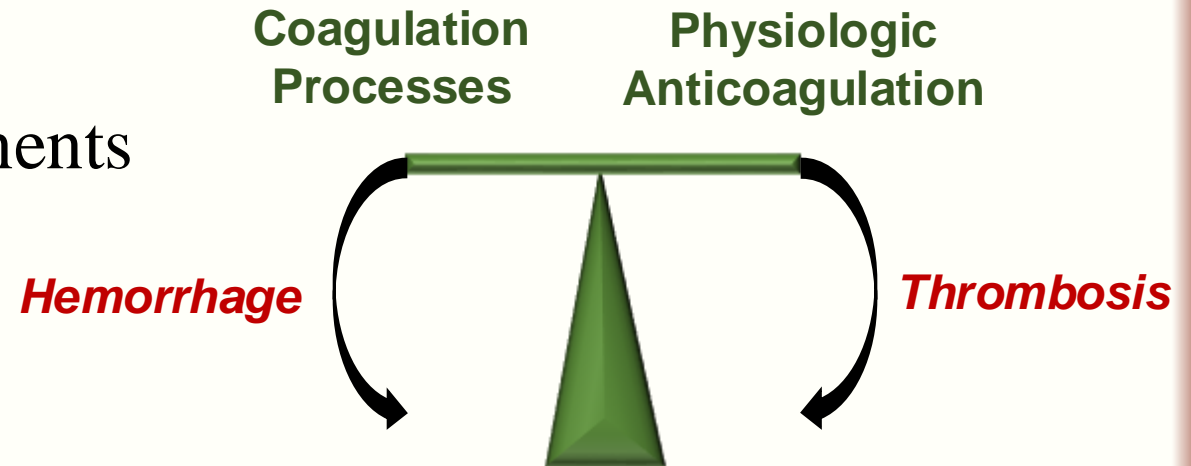
➤ Hemostasis is the balance between bleeding and clotting and involves both cellular and soluble enzymatic components of the blood and vasculature.

➤ Primary Hemostasis/Cellular Components

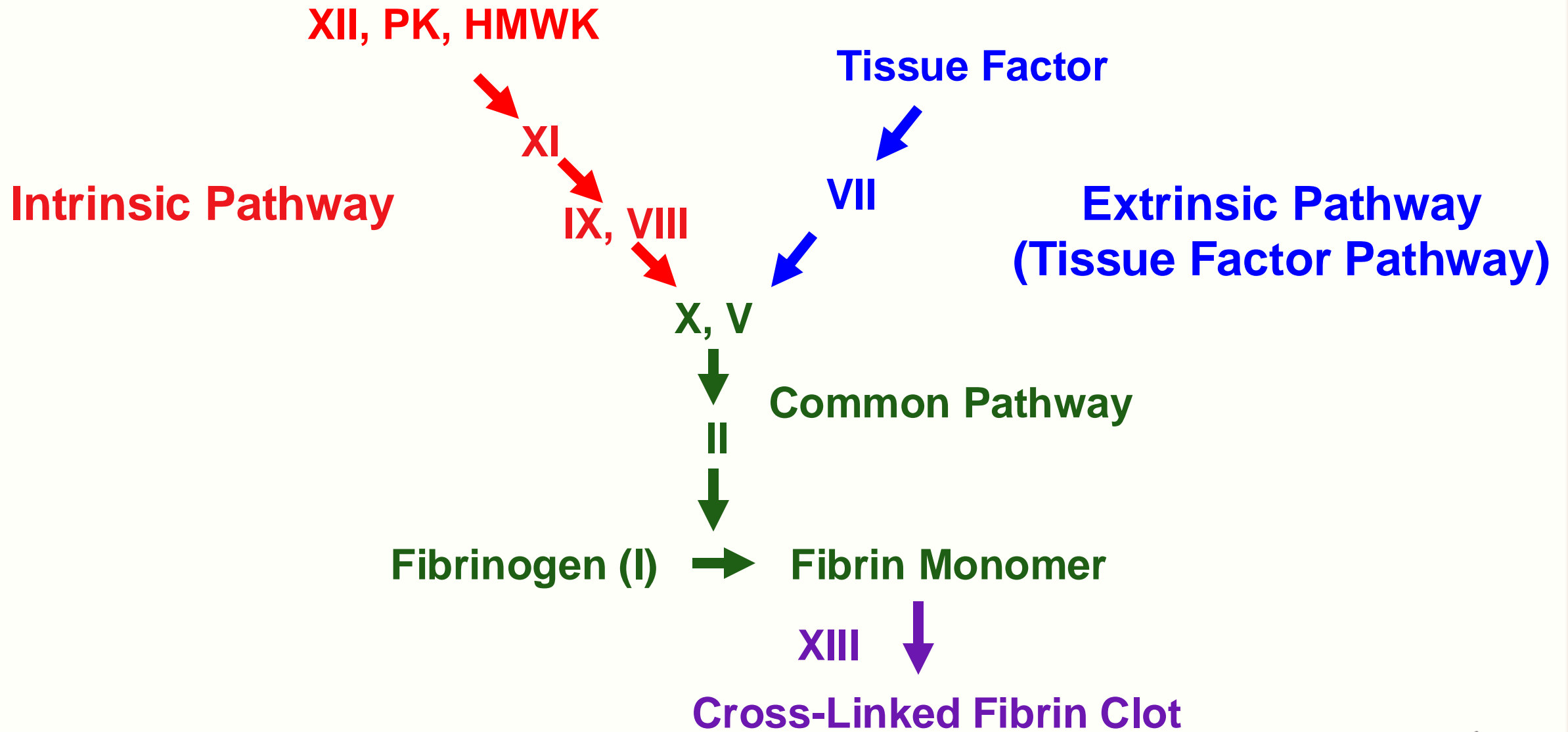
- Vascular endothelial cells
- Platelets

➤ Secondary Hemostasis/Fluid Phase Components

- Coagulation proteins:



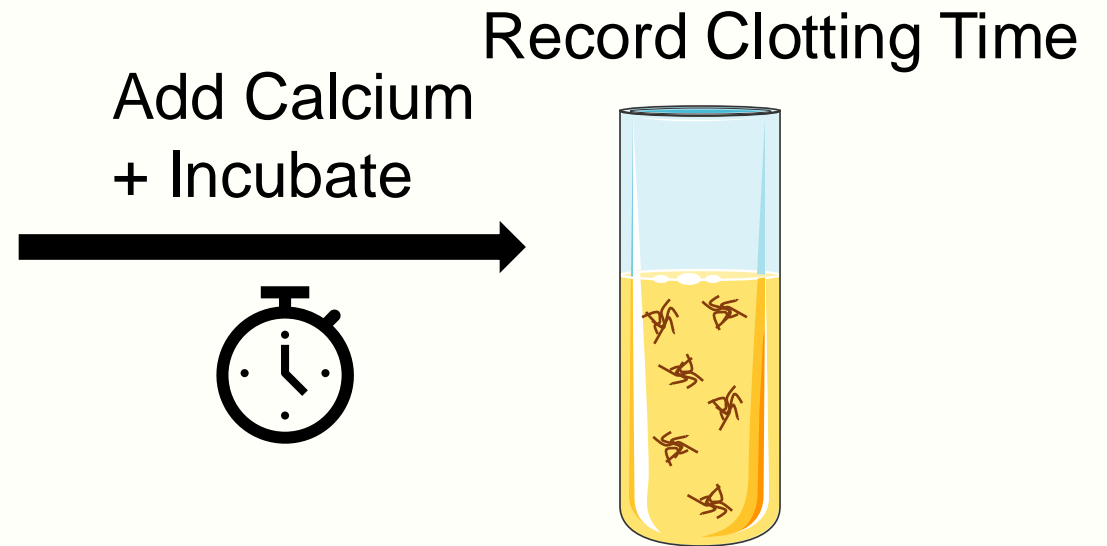
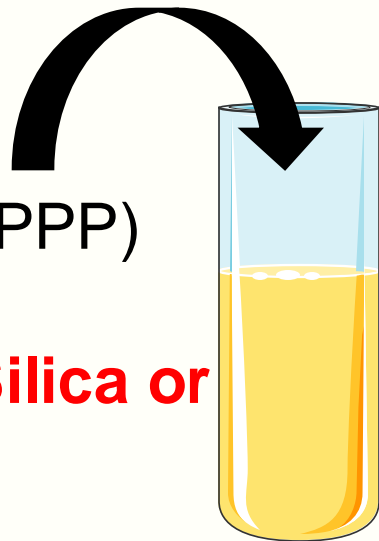
Contemporary Representation of the Coagulation Cascade



Prothrombin Time & activated Partial Thromboplastin Time

- Functional Assays: Clot-Based Assays.
- Good screening assays.
- Based on a functioning coagulation cascade.
- Results in fibrin clot.
- Subject to exogenous and intrinsic interferences.

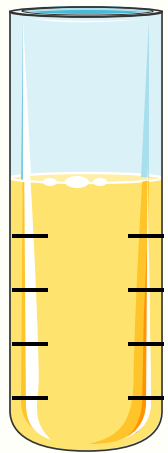
- Platelet Poor Plasma (PPP)
- Phospholipid
- **aPTT: Activator (i.e. Silica or Ellagic acid,)**
- **PT: Tissue Factor**



There Are Two Ways to Initiate Coagulation System *in Vitro*

Intrinsic Pathway:

Initiated by Negatively Charged Surface



XII, PK, HMWK



XIIa

(aPTT)

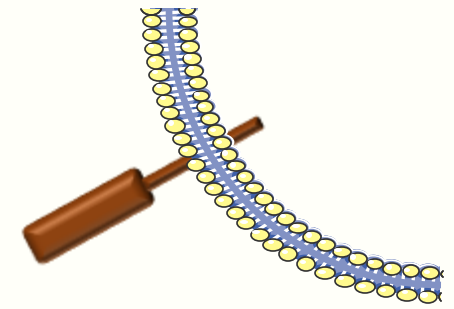
Extrinsic Pathway:

Initiated by addition of Tissue Thromboplastin (Tissue Factor and phospholipid)

TF:VII

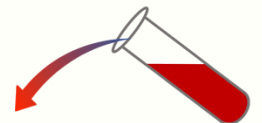


VIIa



TF bound to cell surface

(PT)



Prothrombin Time, Partial Thromboplastin Time, activated Partial Thromboplastin Time

- Prothrombin Time uses “complete” Thromboplastin.
 - Thromboplastin: Consists of phospholipid and Tissue Factor.
- “Partial Thromboplastin” uses phospholipid, without Tissue Factor.
 - Cephalin is a phospholipid substitute.
 - Activators: (ellagic acid, silica, kaolin, or celite).
 - “Contact” activates Factor XII.
- Both require recalcification for the reactions to go to completion/fibrin clot.

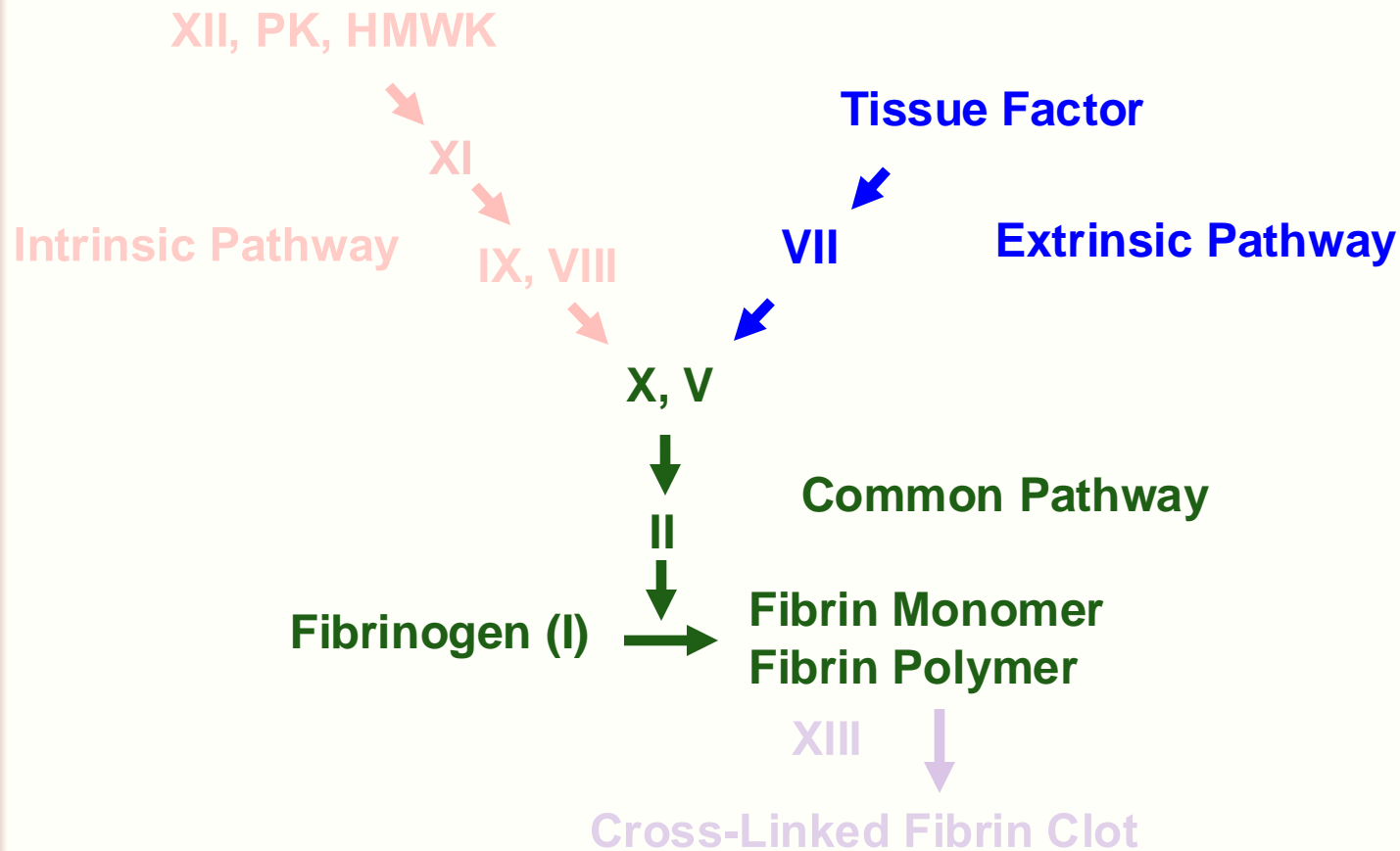


Uses of the Prothrombin Time, activated Partial Thromboplastin Time

- The PT and PTT/aPTT were developed to screen for and factor deficiencies.
 - Still widely used for this indication.
- Currently, much of the use is for monitoring and titrating the doses of anticoagulation.
 - The PT used for warfarin.
 - With increasing use of direct oral anticoagulants (DOAC) instead of warfarin, the PT is used much less frequently for this indication.
 - The aPTT has traditionally been the standard test to monitor and titrate heparin.
 - This is being replaced with the anti-Factor Xa assay for heparin and low molecular weight heparin.
 - To be discussed further below.



Prothrombin Time



➤ Measures:

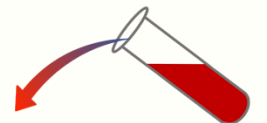
- Extrinsic and Common Pathways
- Endpoint is Fibrin Polymer

➤ Major Uses:

- Hemostasis Screening
- Monitoring Warfarin Anticoagulation

➤ Results:

- Reported in Seconds and INR.

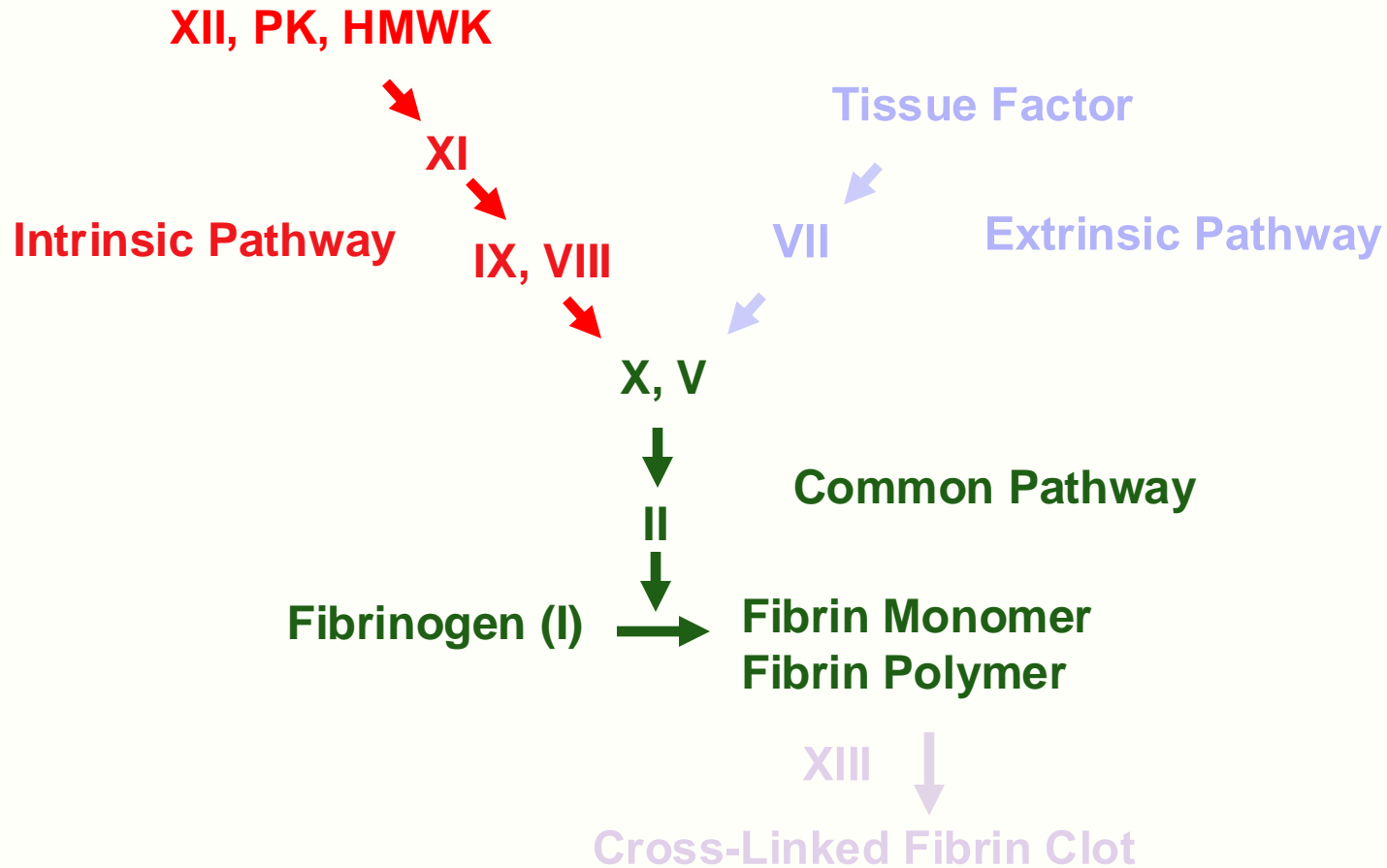


INR (International Normalized Ratio)

- For many years, the prothrombin time was essential to titrate warfarin dosing.
 - Target was 2-3 X normal for optimal balance of efficacy and safety.
 - The reagents for the assay were derived from animal (mostly rabbit) brains, and there was inconsistency of results.
- The **INR= International Normalized Ratio** was developed to standardize results, accounting for variation in thromboplastin reagents.
- The **INR= International Normalized Ratio**
 - **(patient PT/mean normal PT)^{ISI}**
 - **ISI= International Sensitivity Index**
- INR validated for warfarin titration, but practically used in other settings.
 - Dorgalaleh, A, Favaloro, EJ, Bahraini, M, Rad, F. Standardization of Prothrombin Time/International Normalized Ratio (PT/INR). Int. J. Lab Hematol. 2021; 43: 21– 28. <https://doi.org/10.1111/ijlh.13349>



Activated Partial Thromboplastin Time (aPTT)



➤ Measures:

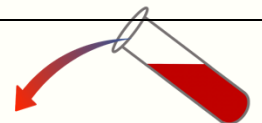
- Intrinsic and Common Pathways
- Endpoint is Fibrin Polymer

➤ Major Uses:

- Hemostasis Screening
- Monitoring unfractionated heparin therapy.
- (Anti-Factor Xa assay is replacing the aPTT for heparin monitoring).

➤ Results:

- Reported in Seconds



APTT: Monitoring UFH Therapy

- **APTT reagents vary in their sensitivity to UFH**
 - Laboratories establish reagent-specific therapeutic range.
 - Reagent standardization has not been successful.
- **APTT response to heparin may be exaggerated by**
 - **Conditions that elevate the baseline APTT:**
 - Concomitant warfarin therapy
 - Lupus anticoagulant
 - Liver disease
- **APTT response to heparin may be blunted by**
 - **Conditions that shorten the baseline APTT:**
 - Cause of *in vitro* drug “resistance”
 - Elevated Factor VIII (inflammation, cancer, etc.)
 - Antithrombin deficiency
- **Alternative: Chromogenic anti Xa assay**



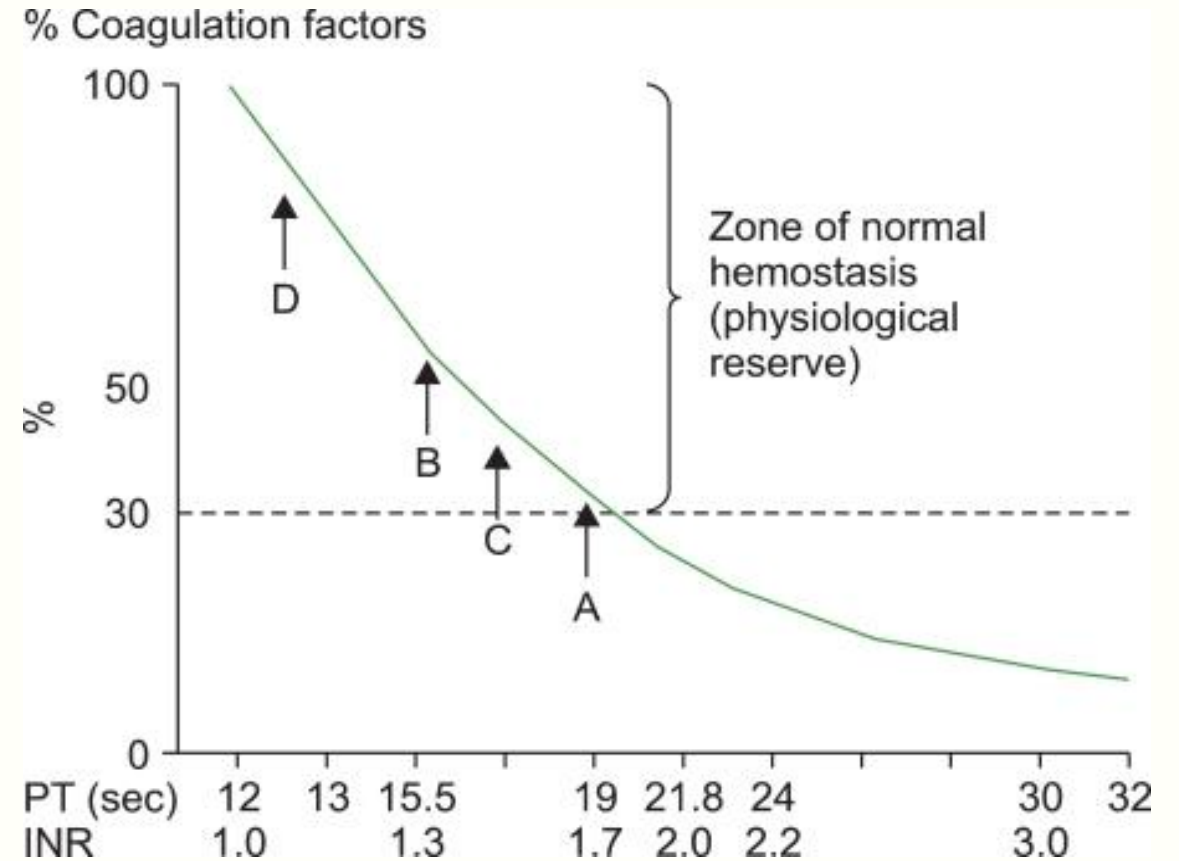
The PT/aPTT Reflect *In Vitro* Coagulation, Not *In Vivo* Coagulation.

- **An elevated aPTT does not necessarily mean an increased risk of bleeding.**
- Deficiencies of factors not associated with bleeding (i.e. Contact Factors: Factor XII, Prekallikrein, High Molecular Weight Kininogen), prolong aPTT to same extent, or more, as clinically relevant factors.
- Lupus Anticoagulant may prolong aPTT, and is associated with thrombosis.
- But still widely used use for coagulation screening and anticoagulant monitoring, due to near universal access and low cost.
- PT does not reflect the activation of Factor IX by the TF:VIIa complex. i.e. no TFPI present in the test.



Use of PT/aPTT To Screen For Factor Deficiencies

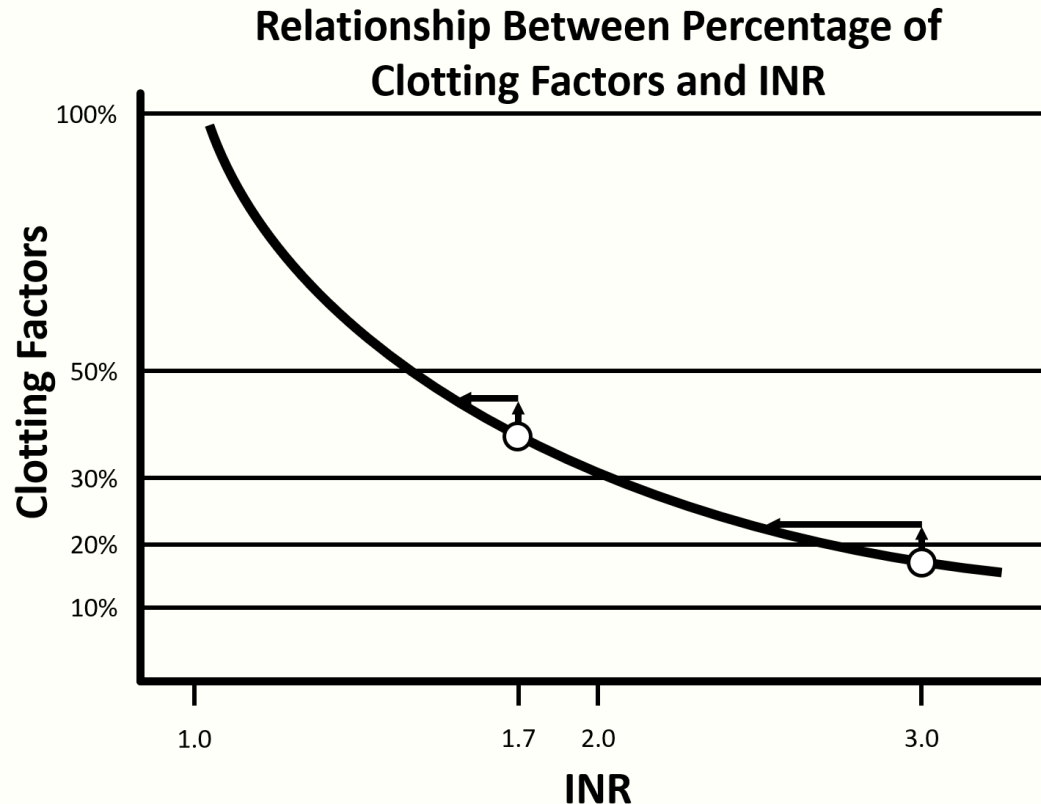
- Assays are valuable as screening tests for factor deficiencies.
- In general, only when factor levels are under 50% is the PT/aPTT prolonged.
 - Falay et al. J Clin Lab Anal. 2018;32:e22415.



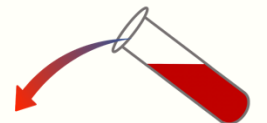
Dzik WH. Component therapy before bedside procedures.
2nd ed. Baltimore, MD: AABB Press, 2005.



Use of PT/INR for Warfarin Titration or Factor Deficiencies



- As an approximate rule of thumb:
 - INR 2.0: Vit K dependent factors ~30%
 - INR 3.0: Vit K dependent factors ~20%
-
- <https://www.aliem.com/inr-reduction-ffp/>
 - Adapted from Dzik W “Sunny.” Reversal of drug-induced anticoagulation: old solutions and new problems. *Transfusion*. 2012;52(s1):45S-55S. doi: 10.1111/j.1537-2995.2012.03690.x. PMID: 22578371.



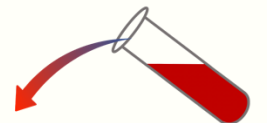
Preanalytical Considerations (I)

➤ Contamination:

- Blood drawn through a heparinized central venous catheter.
- Blood drawn through a central venous catheter, with "stale" blood.
- **Only use blood from venipuncture.**

➤ Specimen Collection

- 3.2% sodium citrate
- 9:1 volume of blood to anticoagulant
- Hct <25% or >50% may affect results. (Citrate:plasma ratio)
 - Severe anemia may prolong PT/aPTT.
- Nair M, et al. BMJ Open 2021;11:e050815. doi: 10.1136/bmjopen-2021-05081



Preanalytical Considerations (II)

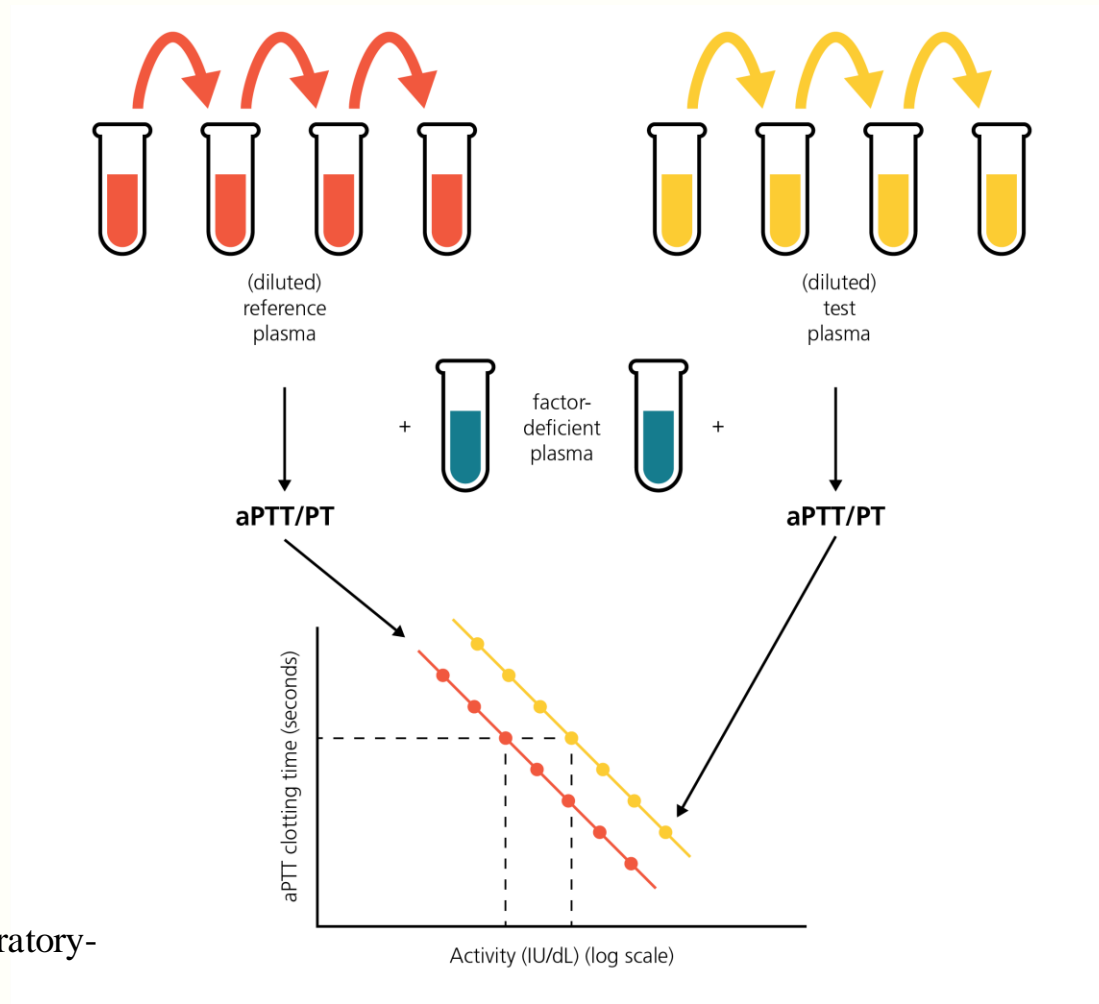
➤ Specimen Stability

- Assays become unreliable in non-refrigerated whole blood.
- Assays also become unreliable with time in refrigerated whole blood, although longer time.
- Platelet Poor Plasma more stable.
- If assays not to be done “promptly,” best to freeze platelet poor plasma.
 - Separated from cells and frozen at -80°C

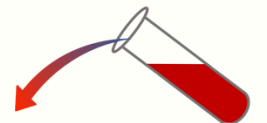


Factor Assays:

- PT or aPTT are adopted by mixing diluted patient's plasma (or reference plasma for standard curve) with known specific factor deficient plasma and performing the PT or aPTT.

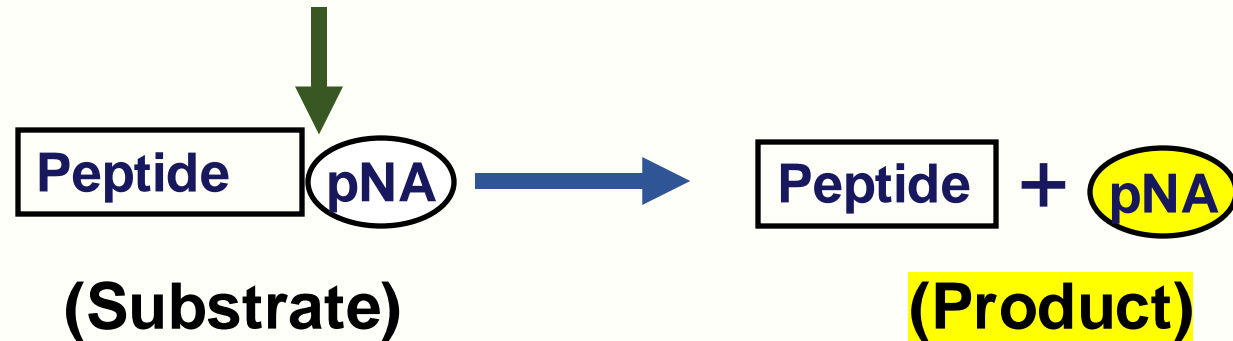


https://www.coagulationassays.com/en_gb/home/laboratory-assays/Clot-based-activity-assays.html



Functional Assay: Chromogenic Assay

Enzyme of interest cleaves substrate



- p-nitroanilide (chromogenic substrate)
- After enzymatic cleavage is released as p-nitroaniline.

- Color develops.
- Change in absorbance over time, correlates with enzyme activity.
- Quantify spectrophotometrically.
- Discreet measure of a specific enzyme activity.
- Can be adapted to measure inhibition of the enzyme (i.e. anti-Xa)
- Affected by *fewer* preanalytical variables.



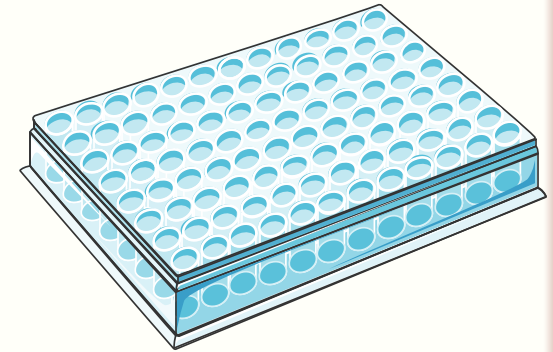
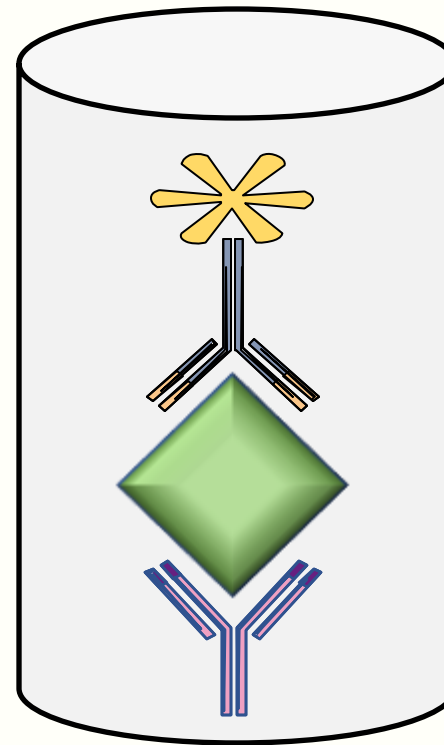
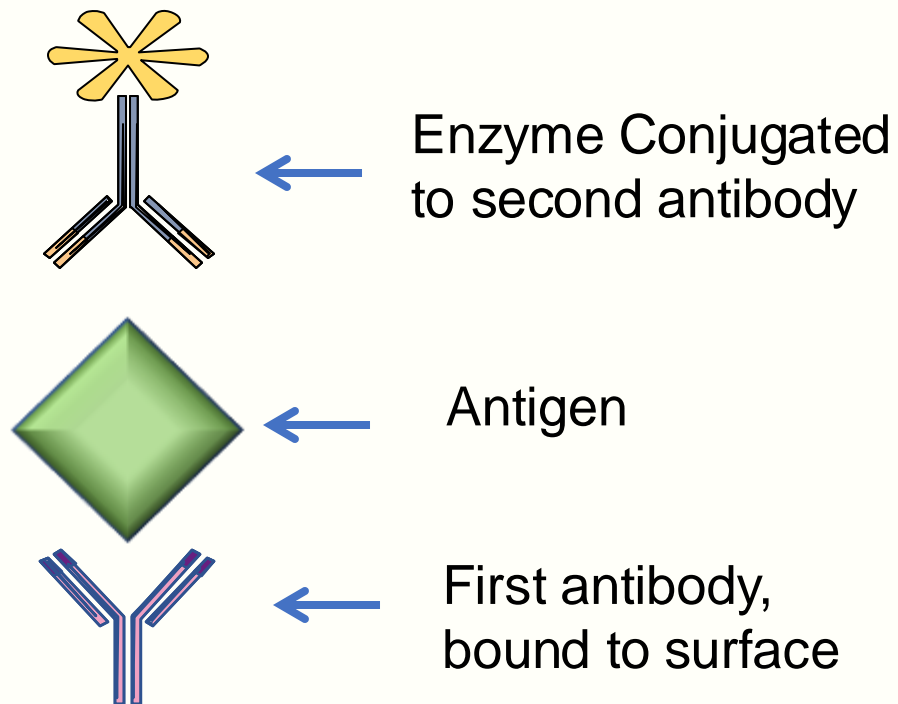
Antigen Assays

- Measures the amount of protein antigen present rather than function.
- Measured Immunologically
 - Latex Induced Agglutination Assays (LIA)
 - Enzyme-Linked Immunosorbent Assay (ELISA)



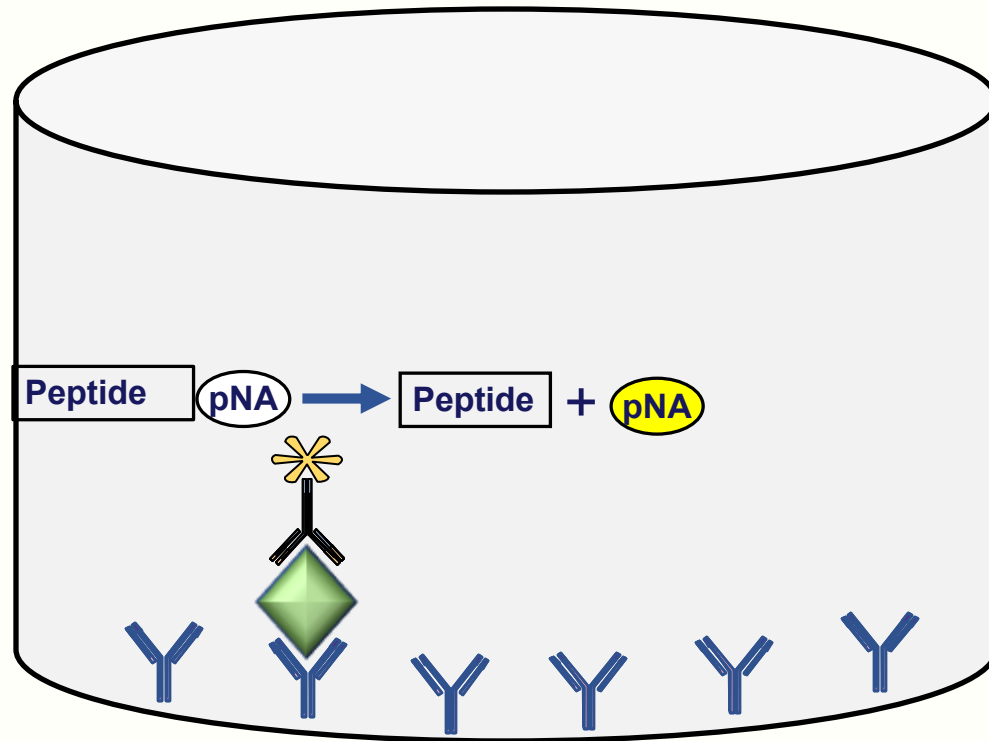
Sandwich ELISA

- First antibody captures antigen to surface.
- Second antibody, labelled with enzyme, binds to immobilized antigen.
- Substrate cleaved by conjugated enzyme
- Color development a function of enzyme capture
- Spectrophotometric quantification

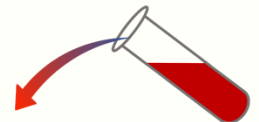
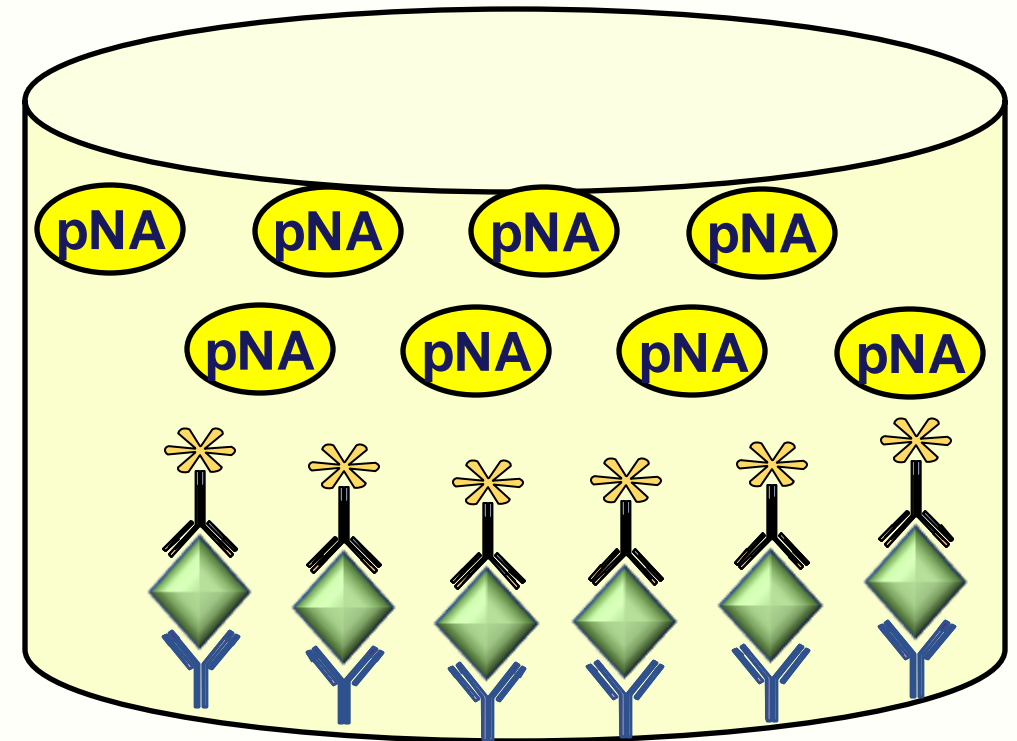


Sandwich ELISA

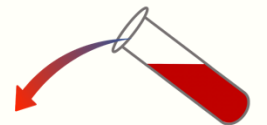
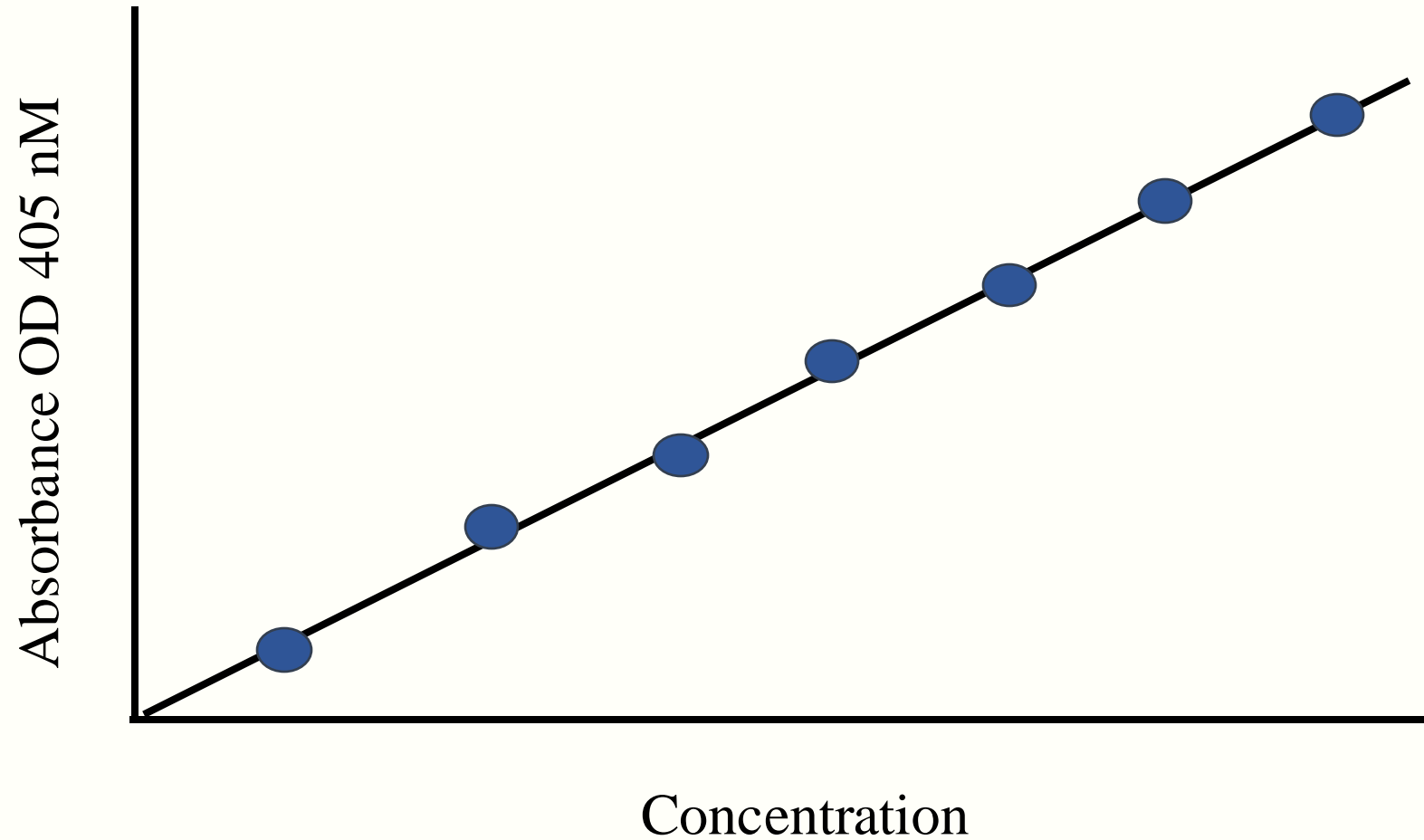
Low Level of Antigen



High Level of Antigen



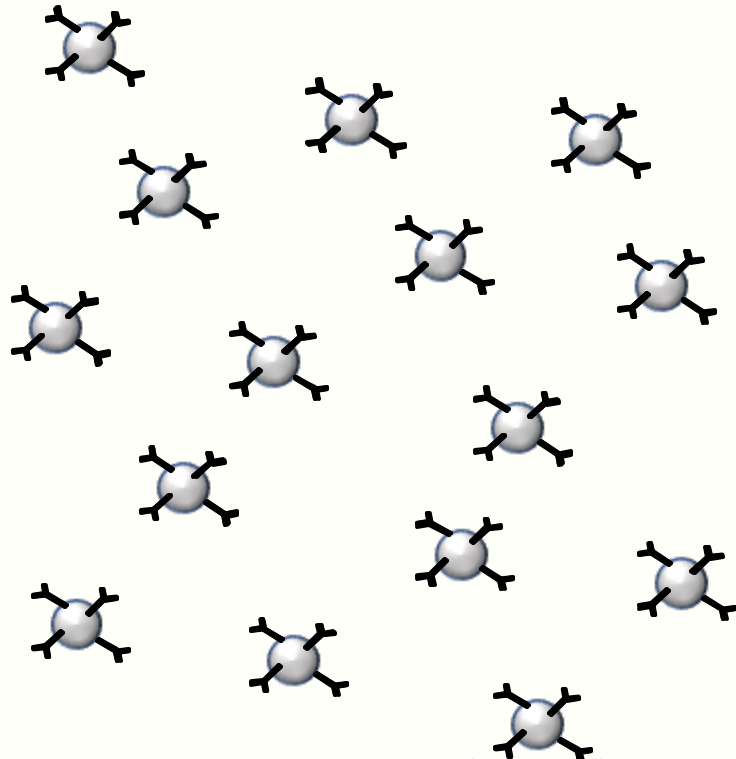
Sandwich ELISA: Results



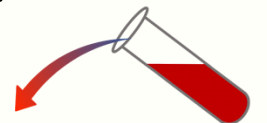
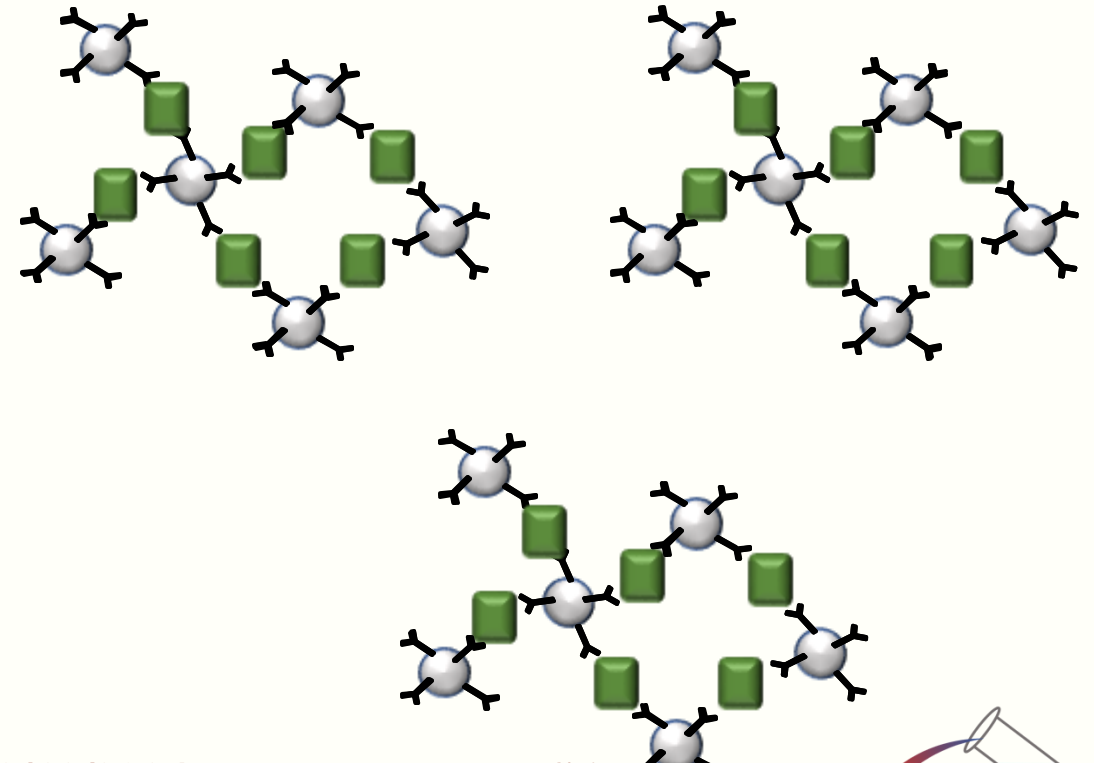
Latex Agglutination

- Antibody coated latex beads
- Agglutination in presence of antigen
- Agglutination is measured optically

Absence/Low Level of Antigen



High Level of Antigen



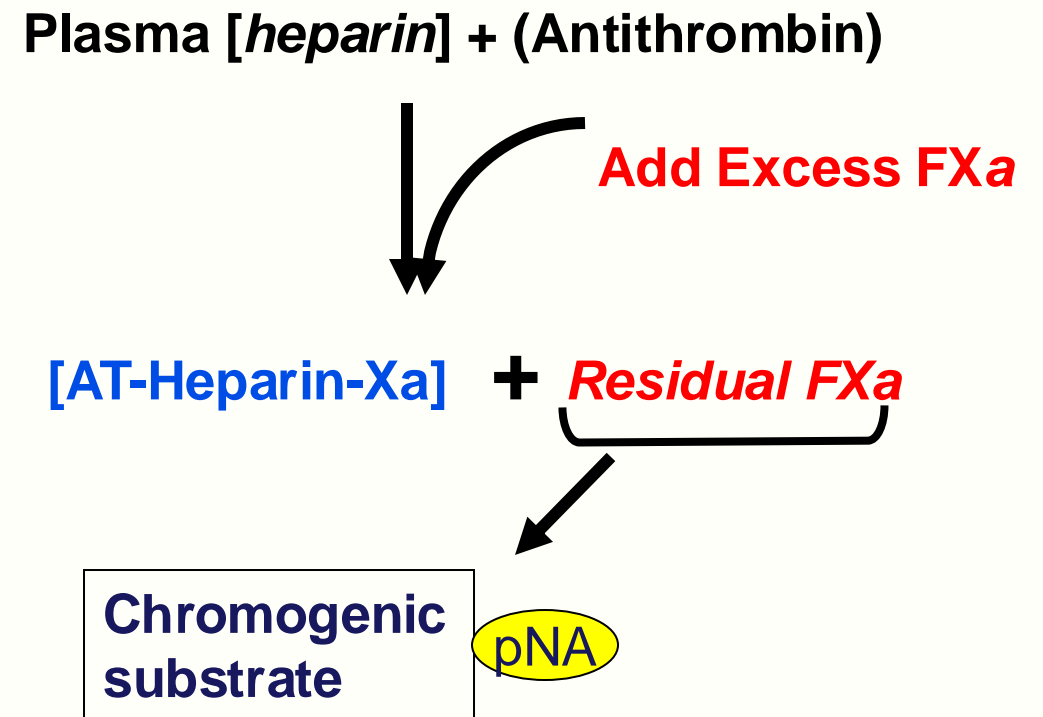
Other Tests

- Anti-Factor Xa Chromogenic Assay
- Thrombin Time
- Fibrinogen Assay
- D-Dimer
- Thromboelastography (TEG) and Thromboelastometry (ROTEM)

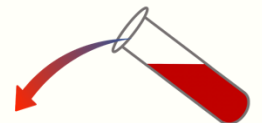


Anti-Factor Xa Chromogenic Assay: Monitoring Unfractionated Heparin and Low Molecular Weight Heparin

- Specifically determines *anticoagulant activity* of LMWH and UFH by measuring ability of heparin-bound antithrombin to inhibit F Xa.
- More specific than aPTT since it measures inhibition of a **single enzyme**.
- Major advantage is **lack** of biologic interference.
 - Eikelboom JW. Thromb Haemost 2006;96:547-52.
 - Francis JL. Pharmacotherapy 2004;24:108S-19S.



Color development is Inversely proportional to the anticoagulant concentration in the plasma sample



Anti-Factor Xa Chromogenic Assay

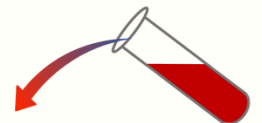
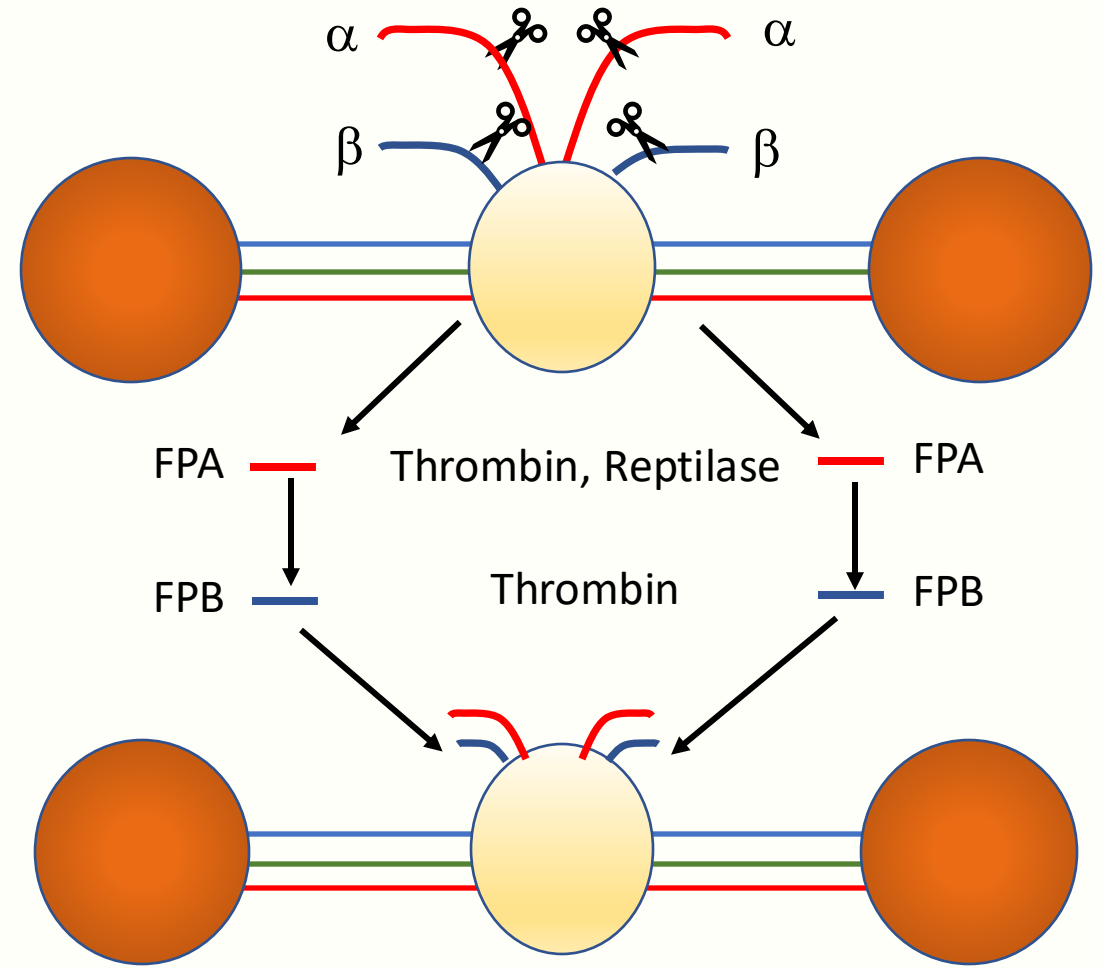
- There is an inverse relationship between the amount of anticoagulant present in the sample and color development.
- Assay can be utilized for unfractionated heparin, Low Molecular Weight Heparin, and Anti-Factor Xa DOACs.



Thrombin Time:

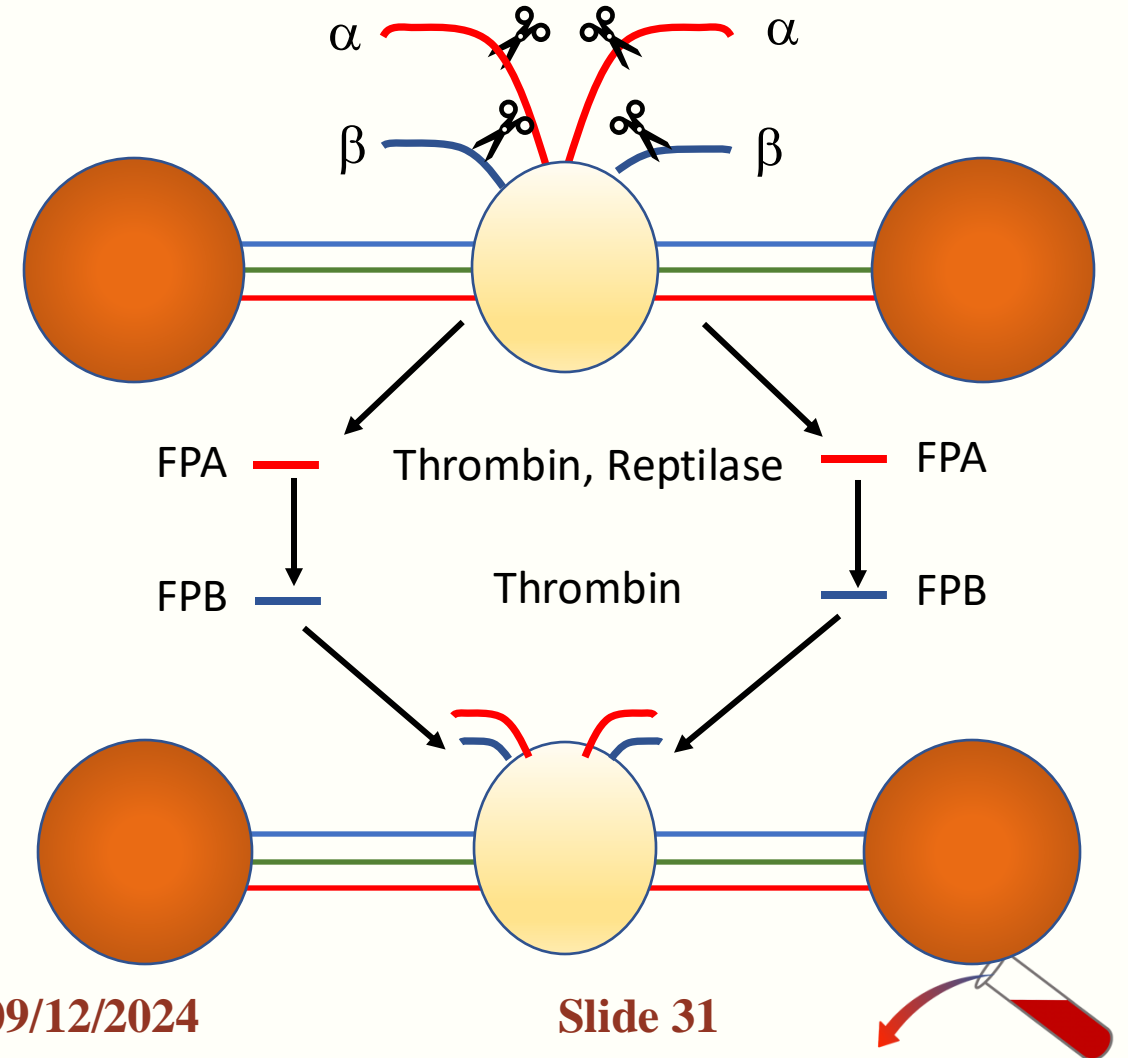
Evaluates the Conversion of Fibrinogen to Fibrin

- Screening test for fibrinogen quantity and quality.
- Thrombin cleavage of Fibrinopeptides A and B, resulting in fibrin monomer.
- Fibrin monomers spontaneously polymerize into fibrin clot as endpoint.
- Thrombin Time is prolonged by hypofibrinogenemia or dysfibrinogenemia.
 - Need to assess fibrinogen antigen levels.
- Thrombin Time is prolonged by some anticoagulants (i.e. heparin, dabigatran).

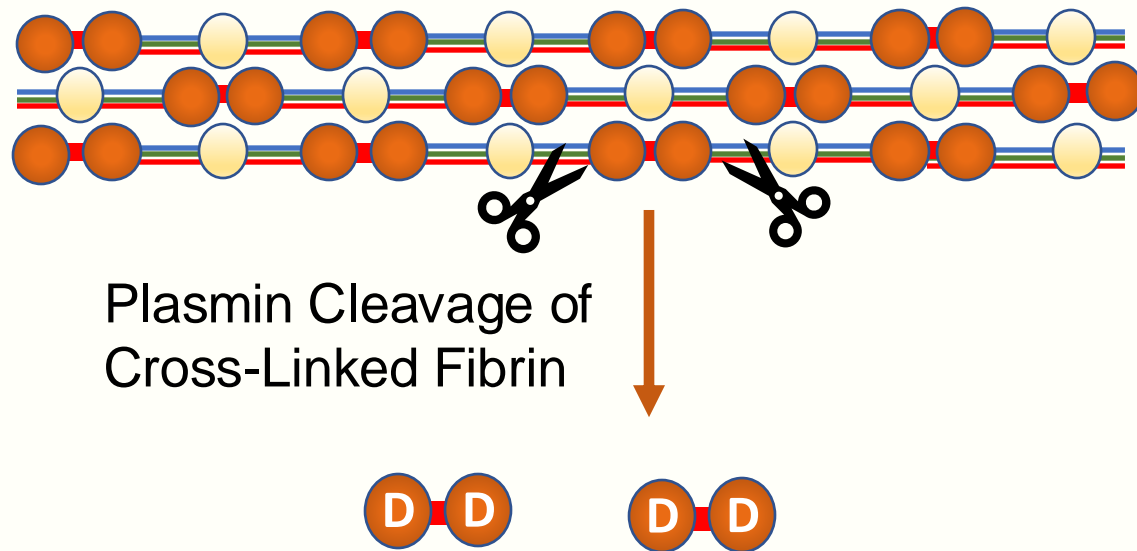


Thrombin and Reptilase Times:

- Reptilase Time often run in parallel.
- Effect of Heparin:
 - Thrombin Time is prolonged.
 - Reptilase Time not prolonged.
 - Thrombin & Reptilase Times may be used as quick screening test for possible heparin contamination.
- Dysfibrinogenemia
 - Thrombin: FPA & FPB
 - Reptilase: FPA (Not FPB)
 - Need fibrinogen result for interpretation.



D-Dimer: Degradation Product of Crosslinked Fibrin



Plasmin Cleavage of
Cross-Linked Fibrin

Quantitation

LIA TEST

- Immunoassay.
- MoAb to D-dimers linked to microbeads.
- Agglutination of beads occurs in the presence of D-dimers.
- Agglutination is measured optically.

Presence of D-Dimer indicates activation of both coagulation (thrombin) and fibrinolysis (plasmin).



Utilization of D-Dimer Testing

- Evaluate for DVT/PE
 - Rule out thrombosis in the outpatient setting in individuals with low suspicion for thrombosis.
 - Good negative predictive value.
 - Cut off: <230 ng/ml (Reference Range: <243 ng/ml)
 - Negative Predictive Value 100%
 - The lower the cutoff, the better the NPV.
 - Specificity 49%
- DIC
- Elevated by any process that activates the coagulation system:
 - Cancer
 - Inflammatory conditions
- A positive D-Dimer does not indicate a thrombosis or DIC is occurring.



Interpretation of Prolonged PT and/or aPTT Results

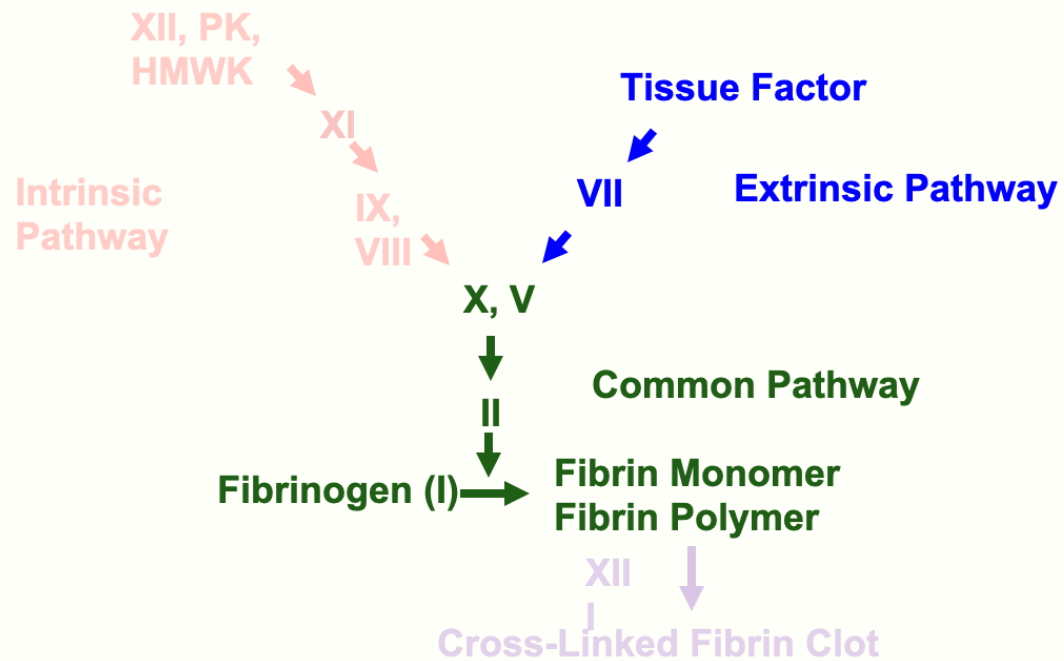


Interpretation of Prolonged PT and/or aPTT Results

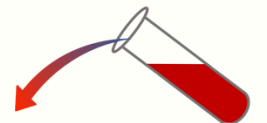
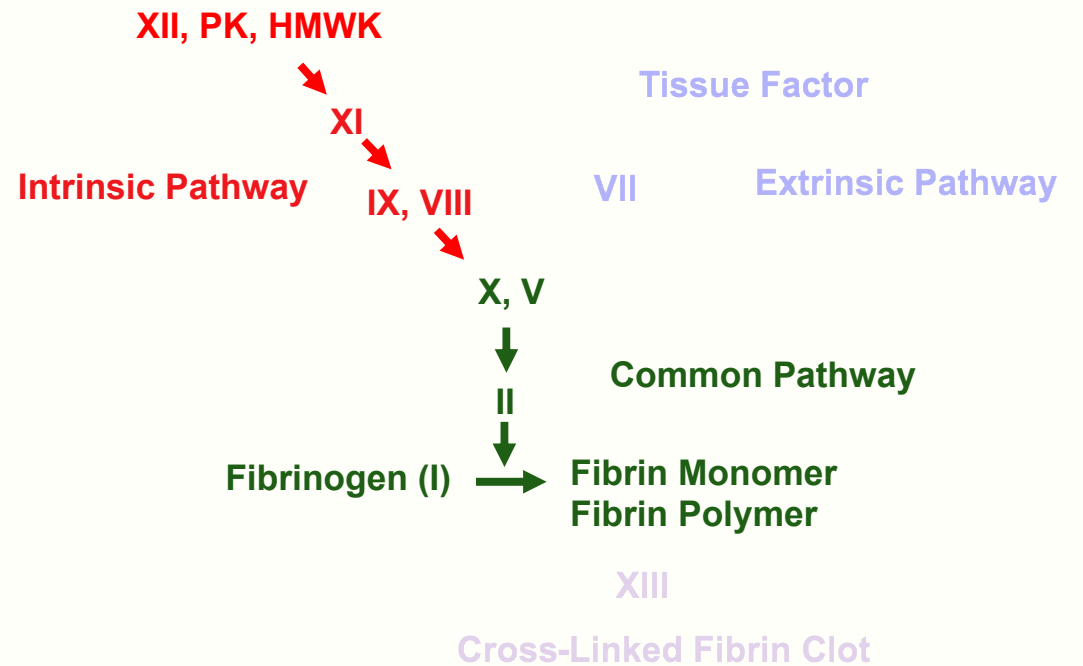
- Factor Deficiency
 - Single vs multiple deficiencies.
 - In general, factor levels must be under 40-50% of normal to prolong the test.
 - Factor XIII deficiency does not prolong PT or aPTT.
- Acquired Inhibitors
 - Specific factor inhibitor (i.e. F VIII)
- “Global” Anticoagulants
 - Lupus Anticoagulant
 - Paraproteins
- Therapeutic Anticoagulants: UFH, LMWH, Direct Oral Anticoagulants.



Prothrombin Time

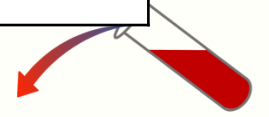


aPTT



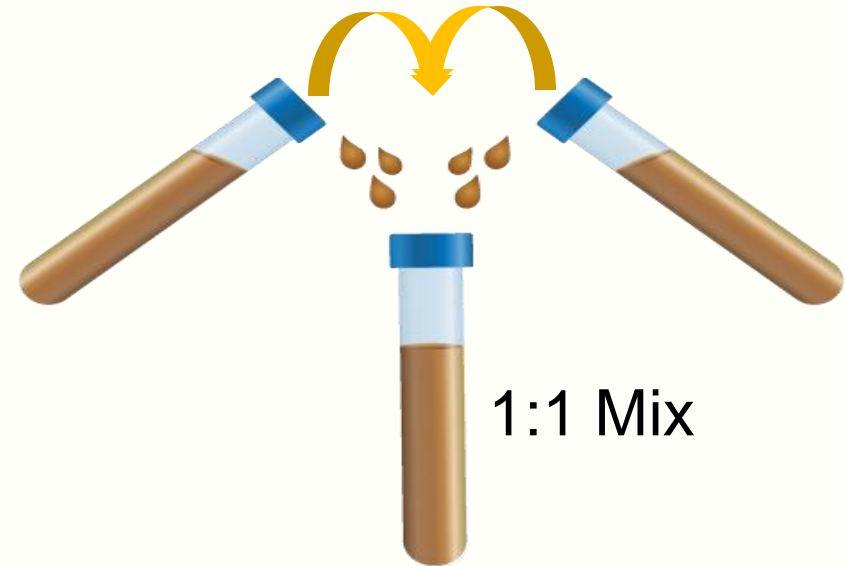
Sensitivity of PT/aPTT to Factor Deficiencies

Factor	PT	aPTT
I (Fibrinogen)	Yes	Yes
II (Prothrombin)	Yes	Yes
V	Yes	Yes
VII	Yes	No
VIII	No	Yes
IX	No	Yes
X	Yes	Yes
XI	No	Yes
XII	No	Yes
XIII	No	No
Prekallikrein (PK)	No	Yes
High Molecular Weight Kininogen HMWK)	No	Yes



Prolonged PT/APTT Work-Up: Mixing Studies

- Mix patient and normal plasma 1:1
- Perform aPTT and/or PT immediately and after 1-hour incubation at 37°C
- In presence of an inhibitor, the 1:1 mix “fails to correct”.
- Specific antibodies require time to bind to the antigen target.
- Common inhibitors: heparin, DOACs, Lupus Anticoagulant, dysproteins, paraproteins, Fibrin Split Products (DIC), factor-specific antibodies.



Mixing Studies

➤ **Factor Deficiency**

aPTT	Patient	Normal	1:1
Immediate	51"	29"	33"
1 Hour Incubation @ 37°C	52"	29"	32"

➤ **Lupus Anticoagulant**
➤ **Anticoagulants**

aPTT	Patient	Normal	1:1
Immediate	51"	29"	48"
1 Hour Incubation @ 37°C	52"	29"	50"

➤ **Anti-Factor VIII Antibody**

aPTT	Patient	Normal	1:1
Immediate	51"	29"	33"
1 Hour Incubation @ 37°C	52"	29"	50"



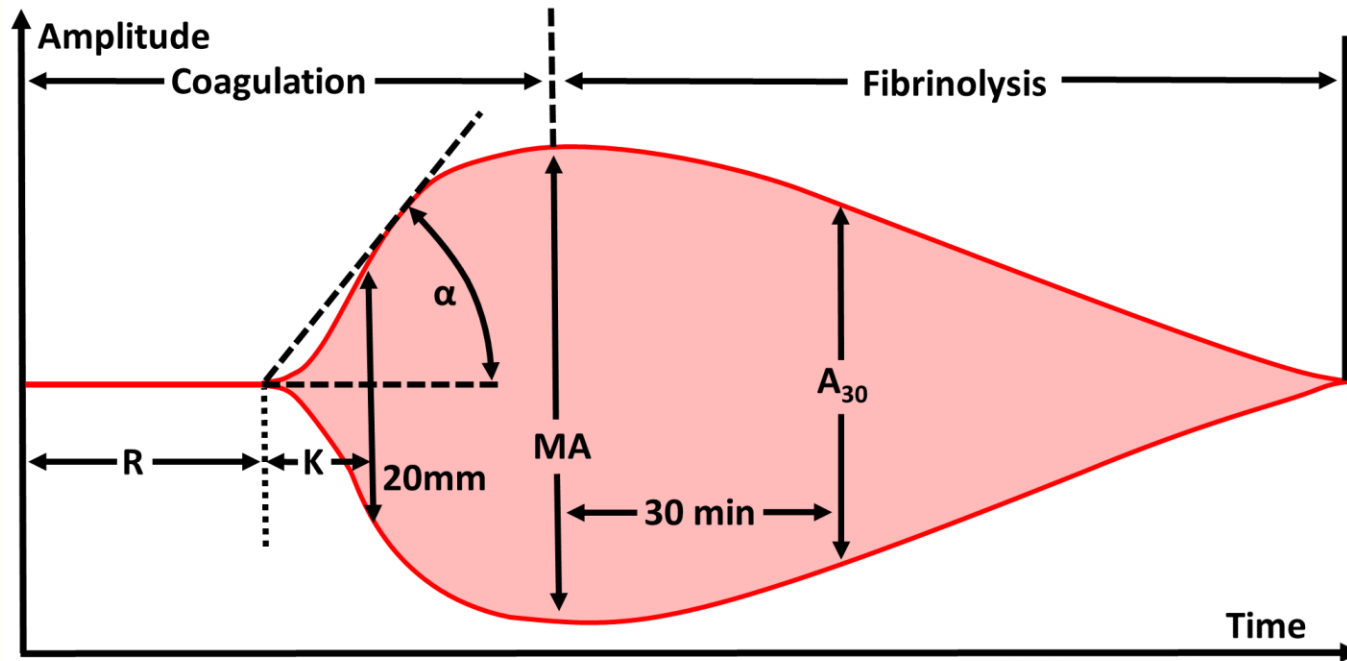
Thromboelastography (TEG) and Thromboelastometry (ROTEM)

- Global assessment of hemostatic function.
 - Mostly used for Point Of Care testing, in Operating Room, Critical Care.



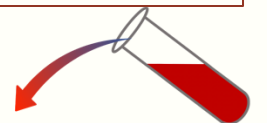
Thromboelastography (TEG) and Thromboelastometry (ROTEM)

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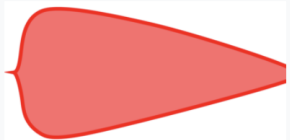


R time: Time to initial clot formation
K time: Time from initial clot formation until reaching 20 mm in amplitude
Alpha angle (α): Angle between the baseline at initial clot formation, and a tangent line that intersects the tracing curve.
Maximum amplitude (MA): Maximum deviation of tracing to baseline.
A₃₀: Amplitude 30 minutes after reaching maximum amplitude.

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Use of TEG/ROTEM

Condition	Appearance	Main treatment
Normal		
Hemodilution or clotting factor deficiency		Fresh frozen plasma
Fibrinogen deficiency		Cryoprecipitate
Low or dysfunctional platelets		Platelets
Thrombosis		Anticoagulant
Primary fibrinolysis		Antifibrinolytics or tranexamic acid
Secondary fibrinolysis		Treating disseminated intravascular coagulation

Kreitzer et al. Review of Thromboelastography in Neurocritical Care, Neurocritical Care volume 23, pages 427–433 (2015)



Any Questions?

