Improving Acute Care Using Coagulation Mixing Studies

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The Fritsma Factor,
Your interactive Hemostasis Resource℠
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Surgeons and physicians order PT and PTT assays to predict bleeding risk, often generating isolated, unexplained prolonged PTs or PTTs. In follow-up, the laboratory practitioner mixes patient plasma with normal plasma and repeats the assay. Mixing studies may be provided at community hospitals and acute care facilities. The information gained from mixing studies is an essential first step in the diagnosis of many hemostatic abnormalities. During this presentation, we discuss the importance of mixing studies, describe how they are performed, and show how their results contribute to the diagnosis.
Bottom Line at the Start (BLAST)

The participant...

• Lists the clinical applications for a PTT mixing study.
• Lists the steps to perform a PTT mixing study.
• Explains why the mixing study is an acute care assay.
• Correlates mixing study results with lupus anticoagulant and specific inhibitor testing.
Mixing Study: An Acute Care Assay
Differentiates a coagulopathy from a specific inhibitor or from a lupus anticoagulant

Case: 32-yo Female
Pre-op Screen

- Six weeks post-partum
- Easy bruising, frequent nosebleeds, vaginal bleeding
Pre-op Screen
32-yo Female, 6 Weeks Post-partum

<table>
<thead>
<tr>
<th>Assay</th>
<th>Patient</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB</td>
<td>11.8 g/dL</td>
<td>12–15 g/dL</td>
</tr>
<tr>
<td>PT</td>
<td>12.4 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT (APTT)</td>
<td>42.5 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT count</td>
<td>310,000/µL</td>
<td>250–450,000/µL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>320 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>

Isolated, prolonged PTT—response? Go to 1:1 PTT mix
### Rule Out Heparin, DOACs

<table>
<thead>
<tr>
<th>Assay</th>
<th>Patient</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin time (TT)</td>
<td>14 s</td>
<td>&lt;21 s</td>
</tr>
<tr>
<td>Anti-Xa</td>
<td>0.0 U/mL</td>
<td>0.4–0.7 U/mL</td>
</tr>
</tbody>
</table>

- **Inpatient**—unrecorded UFH flush of vascular access device
  - neutralize w/ Hepsorb (polybrene) or Hepzyme, proceed if TT normal
- **Outpatient**: direct oral anticoagulant (DOAC): discontinue
  - Dabigatran, direct thrombin inhibitor, TT markedly prolonged
  - Direct anti-Xa like rivaroxaban elevates anti-Xa, may prolong PTT
Direct Oral Anticoagulants (DOACs)

• Oral direct anti-Xa anticoagulants
  – Rivaroxaban; Xarelto
  – Apixaban: Eliquis
  – Edoxaban; Savaysa
  – Betrixaban; now at the FDA

• Oral direct thrombin inhibitor (DTI)
  – Dabigatran; Pradaxa

• If no A/C, perform 1:1 PTT mix to differentiate factor deficiency from factor-specific inhibitor or the “non-specific inhibitor,” lupus anticoagulant (LA, LAC)
PTT Mixing Study: Cheap and Simple

- Start within 2 hours of collection to avoid specimen degradation
  - Factors V (FV) and VIII (FVIII) deteriorate
- Ensure patient plasma is platelet-poor, < 10,000/uL
  - If not, platelets release platelet factor 4, coagulation factor V
- Mix patient plasma 1:1 with pooled normal plasma (NP) and perform immediate PTT on mixture
- Correction: If PTT of 1:1 mix is ≤10% longer than NP PTT
  - Factor deficiency? (But first you must incubate and repeat)
- No correction: 1:1 mix is >10% longer than NP PTT
  - Non-specific inhibitor, usually LA
  - Specific inhibitor (anti-FVIII), usually requires 37°C incubation
PTT Mixing Study

Patient plasma

PTT 42.5 s

Equal volumes

Normal plasma

PTT 30 s

1:1 mix

Manufacturer’s value confirmed by laboratory QA supervisor
PTT Mixing Study
Using 10% Limit

100 uL PTT reagent + 100 uL CaCl₂ =

1:1 mix + PTT rgt + CaCl₂

PTT

≤33 s: Correction
>33 s: No correction
1:1 PTT Mix with Incubation

- PTT of immediate mix ≤10% longer than NP
  - Correction: factor deficiency? But first…
  - Incubate 1:1 mix 1–2 hours and repeat
- Correction after incubated mix = factor deficiency
- No correction: PTT remains >10% above NP
  - Specific inhibitor such as anti-FVIII
    - IgG₄: Temp dependent, usually requires incubation
    - However, some inhibitors neutralize FVIII within 10 min
    - May detect in immediate mix
1:1 PTT Mix with Incubation

- Reflex to incubation if unincubated mix corrects
- Must also incubate NP
- Compare mix PTT to incubated NP PTT
- May also detect temperature-dependent LA
  - ~15% of LAs are temperature-dependent

37°C Incubated 1:1 PTT Mix

Patient plasma (not incubated)

PTT 42.5 s

+ NP (not incubated)

1:1 mix: incubate 1–2 h, repeat PTT

PTT of Mix
≤38.5 s: Correction
>38.5 s: No correction

Mean while:

NP: incubate 1–2 h, repeat PTT

Compare to incubated PTT: 35 s
## Mixing Study Result

**32-yo Female, 6 Weeks Post-partum**

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<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTT</td>
<td>42.5 s</td>
<td>25–35 s</td>
<td>Confirms previous PTT</td>
</tr>
<tr>
<td>PTT/control 1:1 mix immediate</td>
<td>32.1 s</td>
<td></td>
<td>NP: 30s</td>
</tr>
<tr>
<td>PTT/control 1:1 mix 1–2 h at 37°C</td>
<td>37.3 s</td>
<td></td>
<td>Incubate NP: 35s</td>
</tr>
</tbody>
</table>

**Conclusion:** both immediate *and* incubated mix PTTs correct, suspect factor deficiency, arrange for factor assays and von Willebrand disease profile.
### Factor Assay Results

**32-yo Female, 6 Weeks Post-partum**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Result</th>
<th>RI</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII</td>
<td>32%</td>
<td>50–150%</td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>92%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>131%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XII</td>
<td>113%</td>
<td></td>
<td>XII, HMWK &amp; PK deficiency not associated with bleeding</td>
</tr>
<tr>
<td>HMWK</td>
<td>ND</td>
<td>65–135%</td>
<td></td>
</tr>
<tr>
<td>PK</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Extrinsic**

PT reagent: tissue factor, Ca$^{++}$, phosphatidyl serine; prolonged by VII, X, V, prothrombin, Fg deficiency; coumadin Rx, direct anti-Xa Rx

**Intrinsic**

PTT reagent: Ca$^{++}$, particulate neg activator, phosphatidyl serine; test prolonged by XII, PK, HMWK, XI, IX, VIII, X, V, prothrombin, Fg deficiency; heparin Rx, LAC

**Common**

Figure courtesy of Margaret G. Fritsma, Rodak’s Hematology, 5th Edition, 2015
PT and PTT Results in Inherited Single-factor Coagulopathies

<table>
<thead>
<tr>
<th>PT</th>
<th>PTT</th>
<th>Single Factor Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long</td>
<td>Normal</td>
<td>VII</td>
</tr>
<tr>
<td>Long</td>
<td>Long</td>
<td>X, V, II, and fibrinogen(^1)</td>
</tr>
<tr>
<td>Normal</td>
<td>Long</td>
<td>VIII, IX, XI(^2)</td>
</tr>
</tbody>
</table>

\(^1\)PT & PTT prolonged only when fibrinogen is <100 mg/dL, perform fibrinogen assay (fibrinogen assay often added to initial screen)

\(^2\)Contact factor deficiencies XII (1–3% prevalence), prekallikrein (PK, Fletcher), or high molecular weight kininogen (HMWK, Fitzgerald) also prolong PTT results, but not associated with bleeding
PTT Mix: Why Does This Work?

- Hypothetical 20% F VIII level prolongs PTT
  - PTT rghts are calibrated to prolong at 30–40% FVIII, IX, XI
- Add NP with established 100% factor level
  - 1:1 mix, average of 100% and 20% = 60%, PTT corrects
- Hypothetical anti-FVIII or lupus anticoagulant
  - With typical avidity, retains its ability to prolong the mix

\[
\text{Patient} \quad \text{(20\% FVIII)} \quad + \quad \text{Normal} \quad \text{(100\% FVIII)} \quad = \quad \text{1:1 Mix: 60\% FVIII}
\]

PTT corrects to normal
52-yo Athletic Female

Pre-op screen for total hip replacement
# 52-yo Athletic Female
Screen Prior to Hip Replacement Surgery

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB</td>
<td>14.1 g/dL</td>
<td>12–15 g/dL</td>
</tr>
<tr>
<td>PT</td>
<td>11.2 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT</td>
<td>58 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT</td>
<td>170,000/µL</td>
<td>150–400,000/µL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>410 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>

Patient reports no bleeding or bruising, no thrombosis
Isolated Prolonged PTT: Differential

- Could be nothing: 5% of normals exceed limit
- Preanalytical variable: green or lavender-closure tube, hemolysis, lipemia, clotted specimen, short draw
- Outpatient: dabigatran
- Inpatient: unreported UFH
- Congenital single factor deficiency: VIII, IX, or XI, hemophilia A, B, or C with bleeding; VWD
- Congenital FXII, PK, or HMWK without bleeding
- Acquired FVIII inhibitor with severe bleeding
  - Anti-factor VIII, “Acquired hemophilia”
- Lupus anticoagulant (LA)
# 52-yo Female PTT Mixing Study

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>17 s</td>
<td>RI: &lt; 21 s, rules out dabigatran</td>
</tr>
<tr>
<td>PTT</td>
<td>58 s</td>
<td>RI: 25–35 s</td>
</tr>
<tr>
<td>PTT NP</td>
<td>28 s</td>
<td>Correction if &lt; 30.8 s (10%)</td>
</tr>
<tr>
<td>1:1 mix</td>
<td>35 s</td>
<td>25% longer than NP = no correction</td>
</tr>
</tbody>
</table>

What is the next step?
Acute Care PTT Mixing Study Algorithm

TT long

Heparinase or polybrene, recheck TT

If TT long, dabigatran, stop here

TT

TT normal

Patient & NP 1:1 mix

Correction

Incubated patient & NP 1:1 mix

Correction

Factor assay

TT normal

Isolated prolonged PTT

No correction

LA profile

FVIII inhibitor: Bethesda assay
Mixing Study Considerations

- Preanalytical variables
  - Anti-Xa rivaroxaban, apixaban, edoxaban prolong PT, PTT
  - Dabigatran and UFH prolong PT, PTT
  - Clotted, hemolyzed, lipemic specimen
  - Underfilled tube, wrong anticoagulant
  - Must be platelet-poor, <10,000/uL patient and NP
- Heparinase neutralizes ≤1 unit/mL UFH
- 15% of anti-FVIII inhibitors are detected in immediate mix
- 15% of LAs require incubation
- Weak LAs may be missed in 1:1 mix
  - Select a more LA-sensitive PTT reagent or prepare a 4:1 mix
The “LA Cofactor” Effect

- Initial PTT = 48 s, RI 25–35; 1:1 mix *prolongs* to 54 s
- LA binds IIa, slows clot formation, NP in mix adds IIa?
  - Or placental annexin V?

Normal Plasma Source?

• Home brew: ~pool 20 normal plasmas, male ≈ female
  – Ensure plasma is platelet-poor; < 10,000/uL; PTT ≅ mean of RI
  – Ensure NP has ~100% of all factors, especially VIII, IX, and XI
  – Elevated FVIII causes false negative results
  – Screen each for LA, specific factor inhibitors. HBV, HCV, HIV
  – Aliquot and freeze

• Or purchase commercial plasma
  – GMP & frozen meets all criteria
  – Lyophilized plasma acceptable when validated
    • Processed with stabilizers

What Limit Defines Correction?

No Consensus; Fritsma Factor 2015 Quick Question Answers

- Limits based on a fixed PTT value such as reference interval
  - 1:1 mix within RI upper limit (95% or 99% confidence interval, 39%)
  - 1:1 mix within RI upper limit + 5 seconds (8%)
- Limits based on the pooled normal plasma PTT value
  - 1:1 mix within NP PTT value + 5 seconds (14%)
  - 1:1 mix within NP PTT + 10% (32%)
- Rosner or Chang limit formula using patient, NP, and 1:1 mix results
  - Rosner formula produces a ratio
  - Chang’s formula produces % deviation, requires incubation of patient plasma
- Other (7%): combination of RI and Rosner
  - Dedicated RI for mix
Chang Index: Limit Based on % Correction

% Correction = \frac{\text{Patient PTT} - \text{1:1 mix PTT}}{\text{Patient PTT} - \text{NP PTT}} \times 100

% Correction = \frac{42.5 - 32.1}{42.5 - 30} = \frac{10.4}{12.5} = 0.83 = 83\%

Factor Deficiency = \geq 75\%
Inhibitor = \less than 75\%

% Correction verified by local laboratory

Rosner Index

\[
\text{Rosner Index} = \frac{1:1 \text{ mix PTT} - \text{NP PTT}}{\text{Patient PTT}} \times 100
\]

\[
\text{Rosner Index} = \frac{32.1 - 30}{42.5} \times 100 = 4.9
\]

Inhibitor

Correction

\(\geq 11\)

\(< 11\)

Rosner index validated by local laboratory

59-yo Male
Former Hockey Player
Total knee replacement preop labs
### 59-yo Male Former Hockey Player
**Screen Prior to Knee Replacement Surgery**

<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>HGB</td>
<td>14.8 g/dL</td>
<td>12–15 g/dL</td>
</tr>
<tr>
<td>PT</td>
<td>11.2 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT</td>
<td>38 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT</td>
<td>310,000/µL</td>
<td>150–400,000/µL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>390 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>

Patient reports no bleeding or bruising, no thrombosis
When to Perform Mixing Study

• Any PTT > RI upper limit
• Any PTT > RI upper limit + 5 seconds
• Any PTT > RI upper limit with consult
  – Is patient bleeding or clotting?
  – Possible “weak” LA: use 4:1 mix
  – Lupus sensitive PTT reagent
  – Factor sensitive PTT reagent

# 59-yo Male Former Hockey Player

<table>
<thead>
<tr>
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<th>Result</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>17 s</td>
<td>RI: &lt; 21 s, rules out dabigatran</td>
</tr>
<tr>
<td>Anti-Xa</td>
<td>0.0</td>
<td>0.4–0.7 U/mL, r/o direct anti-Xa</td>
</tr>
<tr>
<td>PTT</td>
<td>38 s</td>
<td>RI: 25–35 s</td>
</tr>
<tr>
<td>PTT NP</td>
<td>31 s</td>
<td>Correction if &lt; 34.1 s (10%)</td>
</tr>
<tr>
<td>1:1 mix</td>
<td>35 s</td>
<td>Correction? No correction?</td>
</tr>
</tbody>
</table>

What is the next step?
59-yo Male Former Hockey Player
Clinical Consult

• Consult: if no medical conditions go on to TKR
• Prior thrombotic events (VTE)
  – Perform mix using 4:1 patient plasma to NP
  – Or choose PTT reagent that is LA-sensitive
• If anatomic bleeding, test for FVIII, FIX, FXI
  – Vitamin K deficiency: factor VII
  – Renal insufficiency
  – Liver disease (factor V), malignancy, VWD
2 YO Hemophilic Boy

Bleed into knee and ankle
# 2-yo Hemophilic Boy

<table>
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<tr>
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<th>RI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB</td>
<td>11.8 g/dL</td>
<td>9.6–15.6 g/dL</td>
</tr>
<tr>
<td>PT</td>
<td>11.2 s</td>
<td>9.8–12.6 s</td>
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<tr>
<td>PTT</td>
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<td>Fibrinogen</td>
<td>390 mg/dL</td>
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</table>

Inflamed, swollen knee and ankle
Mixing Study Result
2-yo Hemophilic Boy

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<tr>
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<th>Result</th>
<th>RI</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTT</td>
<td>65 s</td>
<td>25–35 s</td>
<td>Confirms previous PTT</td>
</tr>
<tr>
<td>PTT/control 1:1 mix immediate</td>
<td>33.5 s</td>
<td>NP 30 s</td>
<td>Correction (ambiguous)</td>
</tr>
<tr>
<td>PTT/control 1:1 mix 1 h at 37°C</td>
<td>47.9 s</td>
<td>NP 35 s</td>
<td>Control is incubated alone and with mix</td>
</tr>
</tbody>
</table>

Conclusion: Anti-FVIII inhibitor
Factor VIII Assay

- Dilute plasma 1:10
- Add factor VIII-depleted reagent plasma 1:1
- Add PTT reagent, incubate 3 minutes
- Add CaCl$_2$, record interval to clot formation
- Compare result in seconds to calibration curve
## Factor VIII Assay Dilutions
Parallelism Indicates No Inhibitor

<table>
<thead>
<tr>
<th>Plasma Dilution</th>
<th>Seconds</th>
<th>Raw Factor VIII Activity</th>
<th>Computed Factor VIII Activity (× dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 “undiluted”</td>
<td>90 s</td>
<td>20%</td>
<td>20%</td>
</tr>
<tr>
<td>1:20</td>
<td>104 s</td>
<td>10%</td>
<td>20% (parallel)*</td>
</tr>
<tr>
<td>1:40</td>
<td>107 s</td>
<td>5%</td>
<td>20% (parallel)</td>
</tr>
<tr>
<td>1:80</td>
<td>110 s</td>
<td>2.5%</td>
<td>20% (parallel)</td>
</tr>
</tbody>
</table>

* <10% difference from undiluted indicates parallelism, no inhibitor
## FVIII Assay Dilutions

**non-Parallelism Indicates Inhibitor**

<table>
<thead>
<tr>
<th>Plasma Dilution</th>
<th>Seconds</th>
<th>Raw Factor VIII Activity</th>
<th>Computed Factor VIII Activity (× dilution)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 “undiluted”</td>
<td>80 s</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>1:20</td>
<td>93 s</td>
<td>8%</td>
<td>16%</td>
</tr>
<tr>
<td>1:40</td>
<td>107 s</td>
<td>5%</td>
<td>20%</td>
</tr>
<tr>
<td>1:80</td>
<td>108 s</td>
<td>4%</td>
<td>32%</td>
</tr>
</tbody>
</table>

* >10% difference from undiluted, rising = non-parallel, implies inhibitor

55-yr Male with Atrial Fibrillation

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<tr>
<th>Test</th>
<th>Result</th>
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<tbody>
<tr>
<td>HGB</td>
<td>13.8 g/dL</td>
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</tr>
<tr>
<td>PT</td>
<td>17.2 s</td>
<td>9.8–12.6 s</td>
</tr>
<tr>
<td>PTT</td>
<td>159 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>PLT</td>
<td>310,000/µL</td>
<td>150–400,000/µL</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>20 mg/dL</td>
<td>220–498 mg/dL</td>
</tr>
</tbody>
</table>
### 55-yo Male with Atrial Fibrillation

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<th>Result</th>
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<tbody>
<tr>
<td>PTT</td>
<td>159 s</td>
<td>25–35 s</td>
</tr>
<tr>
<td>TT</td>
<td>&gt; 150 s</td>
<td>&lt; 21 s</td>
</tr>
<tr>
<td>PTT/control 1:1 mix immediate</td>
<td>78 s</td>
<td>Control 30 s</td>
</tr>
<tr>
<td>PT/control 1:1 mix immediate</td>
<td>15.2 s</td>
<td>Control 12 s</td>
</tr>
</tbody>
</table>

**What do you recommend?**
If the PT is Prolonged

- Congenital deficiencies of II, V, VII, or X
  - PT and PTT long: II, V, X
  - PT only: VII, skip mixing and go to factor assay
  - Prevalence: 500,000–1:2,000,000
- Vitamin K deficiency: des-carboxy II, VII, and X
- Liver disease: PT prolongs before PTT due to des-carboxy II, VII, and X, reduced factor V
- Anti-Xa direct oral anticoagulants
  - Rivaroxaban, apixaban, edoxaban, betrixaban
Isolated Prolonged PTT: Summary

• Random benign prolongation, 95% CI
• Lupus anticoagulant: prevalence of 1–3%
• Drug reaction producing transient LA
• Unrecorded heparin, dabigatran, oral anti-Xa
• Known hemophilic who fails FVIII concentrate Rx
• Hemorrhage or ecchymoses signal acquired coagulopathy; vitamin K deficiency, liver disease
• Specific inhibitor, anti-FVIII
  – Postpartum, malignancy
  – Autoimmune disorders, > 60-yo

Develop Mixing Study Reliability

• PTT reagent sensitivities to factors and to LA
  — Activator: ellagic acid, silica, kaolin, celite
  — 30–40% FVIII, FIX, FXI
  — Intermediate sensitivity to LA
• NP consistency: ~100% activity for all factors
• Consultation for equivocal patient results
• Employ consistent correction limit
DIY Local Mixing Studies—Why?

• Unexpected isolated prolonged PTT or PT may require immediate therapy
• Local results may immediately direct therapy
• Delayed specimen may deteriorate
• Forward mixing study results to ref lab to direct follow-up, for instance, LA profile or Bethesda titer
Bottom Line at the End (BLEAT)

The participant...

• Listed the clinical applications for a PTT mixing study.
• Listed the steps to perform a PTT mixing study.
• Explained why the mixing study is an acute care assay.
• Correlated mixing study results with lupus anticoagulant and specific inhibitor testing.
Thanks for listening! Ya got any questions?

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