The direct telephone line to the Hemostasis Laboratory is 410-614-4724.

Department of Pathology Coagulation Laboratory
Zayed Tower, Level B 1
How to Evaluate a Patient with a Bleeding Problem:

Note I

There is no substitute for a history to assess a bleeding diathesis and in determining what tests should be ordered.

Bleeding History
A. Age of Initial Presentation- early inherited, older acquired

B. Bleeding Presentation
- petechiae, vascular or platelet
- ecchymoses/purpura, defect in primary hemostasis
- spontaneous/deep tissue hemorrhage/delayed onset, bleeding into tissues, joints or body cavities, defect in coagulation factor

C. General Considerations
- does the patient have a bleeding history
- does the family have a bleeding history; sex linked, autosomal dominant/recessive
- what is the patient’s ethnic origin
- has the patient undergone surgery without excessive bleeding
- has the patient had dental procedures without bleeding
- is the bleeding typically from one site or multiple sites
- is the bleeding prolonged, delayed, or recurrent
- what medications may have made the bleeding worse
- does the patient follow any unusual dietary practices
- has the patient required transfusions
- are there are systemic illnesses that may cause hemorrhage, renal disease, liver disease, myeloproliferative disorders, plasma cell dyscrasias, heart murmurs

D. Clinical Situations where bleeding history provides diagnostic clues
- epistaxis; common in normal but also common in von Willebrand disease or qualitative platelet disorder; determine whether spontaneous or post traumatic; bilateral or unilateral; severity best assessed by recurrence, trips to emergency room, cautery or transfusions

- Menorrhagia; not uncommon in normal, but clue to platelet disorder or von Willebrand; severity assessed by number of heavy days, soaking clothes or linens, transfusion, degree of anemia a

- Dental extractions: lack of significant bleeding after extraction think normal hemostasis, severity assessed by need for packing, suturing or transfusion

- Surgical procedures; inquired about circumcision, tonsillectomy, appendectomy and skin biopsies; severity inquired about hematoma, transfusion, reoperation

E. Limited usefulness in documenting bleeding disorder; GI or GU bleeding, hemoptyysis, childbirth!
F. Medications; aspirin and aspirin containing medications, including non steroidal, antibiotics, anticoagulants

G. Diet/ Habits/Life Styles; alcohol, vitamins, herbs, supplements

H. Family History; spontaneous mutations are not uncommon, ask about marrying cousins, many recessive disorders seen in consanguineous marriages. Of course pattern of inheritance important with sex linked in factor 8 or factor 9, autosomal dominant von Willebrand disease
How to Evaluate a Patient with a Thrombotic History:
Note II

A. Age of presentation, infancy to young adulthood, most likely inherited; adults, inherited or acquired; elderly most likely acquired

B. General consideration
   Does patient have a history of venous thrombosis
   Does patient have a family history of venous thrombosis
   Are the thrombotic events recurrent
   Was the thrombosis idiopathic
   Is there a history of cancer
   Has the patient been treated for a clot before
   What is the ethnic origin
   What is the age of the patient

C. Clinical Situations associated with thrombosis
   Past history of venous thrombosis
   Trauma
   Malignancy
   Immobilization
   Inflammatory conditions
   Autoimmune disorders
   Nephrotic syndrome
   Surgery
   Obesity
   Pregnancy
   Hormone use

D. Medications
   Estrogens
   Chemotherapy, including tamoxifen
   Anti thrombotic agents

E. Family History; a negative history for clots does not rule out the presence of an inherited disorder of thrombosis, some times one or more environmental factor is needed
How to Evaluate the Screening Coagulation Tests in a Bleeding Patient:

Note III

**INITIAL LABORATORY EVALUATION OF A BLEEDING PATIENT**

- **n1 PT, ↑ aPTT**
- **↑ PT, n1 aPTT**
- **↑ PT, ↑ aPTT**
- **n1 PT, n1 aPTT**

**Abnormal test(s) repeated with a 50:50 mix of patient plasma and normal pooled**

- Normal 50:50 mix
- Normal 50:50 mix
- Normal 50:50 mix

**Test for factor deficiency**
- Isolated: Factor VIII, IX, IX (Rare)
- Isolated: Factor VII
- Isolated: Fibrinogen Factor II, X, V, (Rare)

**If 50:50 mix is prolonged**

**Test for inhibitor activity**
- Specific: Factors XI, IX, VIII
- Specific: Factor VII
- Specific: Factor X, V, Prothrombin, Fibrinogen (Rare)
- Non-specific: antiphospholipid (Common)
- Non-specific: antiphospholipid (Rare)
- Non-specific: antiphospholipid

- Dysfibrinogenemia
- Factor XIII deficiency
- α2 – Antiplasma deficiency
- Mild isolated factor deficiency (>25% <40%)
- Elevated fibrin degradation products
- Monoclonal gammopathy
- Qualitative or quantitative platelet disorders
- Vascular disorders
Some General Recommendations for Hemostasis Testing

Note IV

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<td>Arterial Thromboembolism</td>
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<tr>
<td>Platelet-type (mucocutaneous) bleeding-defect in primary hemostasis</td>
</tr>
<tr>
<td>Coagulation factor-type bleeding (e.g., hemarthroses, deep tissue hematoma)-defect in secondary hemostasis</td>
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<td>Delayed bleeding</td>
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*Thrombophilia testing generally recommended for patients with 1. Recurrent thromboembolism, 2. First event age 50 or less, 3. Strong family history of thromboembolism (at least 1 first degree relative with TE age < 50 years) and 4. Thromboembolism in unusual sites (e.g., hepatic or mesenteric vasculature, cerebral vasculature).
What You Need to Know about Mixing Studies

Note V

Types of Inhibitors

Non Specific: lupus anticoagulant, FDP, paraproteins

Inhibitors to Specific Factors
   Neutralizing antibodies to VIII, IX, XI, XII, thrombin, fibrinogen
   Non Neutralizing to VIII, IX, FX(amyloid) II

Global Inhibitors
   Heparin, glycosaminoglycans, hirudin, bivalirudin, argatroban,

Trigger to order Mixing Study

Prolonged PT or PTT

When Mix ordered on PTT lab first does Hepzyme:
If PTT normalizes to PTT normal range, no further testing

Important Premises for Mixing Study:

The results of the mix study will allow one to distinguish
Between Factor deficiency or an inhibitor

Theoretically the normal level of coagulation factor
Present in Normal Plasma will be enough to correct the deficit of factor (S)
In patient plasma

PTT or PT reagents should be sensitive to factor levels approximating 30-40% activity

An inhibitor present in patient plasma will act against not only its own coagulation factors but
Also against those in normal plasma.

Detecting inhibitors having various avidities and concentration is dependent upon use of
different mix types 1:1 or 4:1
   4:1 may be more useful when initial PTT is only mildly elevated
effective in picking up weaker inhibitors
may be better to detect the lupus anticoagulant in the presence of warfarin
caveat is that the 4:1 mix may not correct for a mild factor and thereby not differentiate
between this and a mild inhibitor
heparin in the sample produces non predictable mix results in terms of correction

Extending incubation times immediate versus 1-2 hours
   Inhibitors such as Factor V III inhibitors and 15% of lupus anticoagulantas require time to
   exert their effect

Criteria for Interpreting
Historically most common used criterion to assess correction or lack thereof has been the in-house Reference ranges
   Namely correction within 5 seconds of upper limit of lab normal range for dilution and incubation time
   Or greater than 80 percent correction from patient clotting time
What How to Assess a Patient for Thrombosis Using the D-dimer Results

Note VIII
Numerous studies have demonstrated the utility of a variety of D dimer assays in the diagnosis of VTE, particularly when used in conjunction with pre-test probability models such as the Wells Criteria. In general, D dimer tests have been used to exclude VTE in patients judged to have a low pre-test probability of thrombosis, while additional objective radiologic testing is done in patients with a moderate or high pre-test probability of VTE.

| Table 1: Wells pre-test Probability Model for Deep Venous Thrombosis |
|---------------------------------|----------------|
| **Clinical Parameter**          | **Score**     |
| Active cancer                   | +1            |
| Paralysis, paresis, recent casting of leg | +1 |
| Bedridden (>3 days) or major OR (12 weeks) | +1 |
| Entire leg swollen              | +1            |
| Calf swelling (>3 cm) compared to other leg | +1 |
| Pitting edema greater in symptomatic leg | +1 |
| Collateral non-varicose superficial veins | +1 |
| Localized tenderness along deep venous system | +1 |
| Previously documented deep-vein thrombosis | +1 |
| Alternative diagnosis as or more likely than DVT | -2 |

Score: Low PTP: ≤ 0 Moderate PTP: 1 or 2 High PTP: > 2


| Table 2: Wells pre-test Probability Model for Pulmonary Embolism |
|---------------------------------|----------------|
| **Clinical Parameter**          | **Score**     |
| Clinical signs and symptoms of DVT | +3            |
| Heart rate greater than 100/min | +1.5          |
| Hemoptysis (coughing up blood)  | +1            |
| Active cancer                   | +1            |
| Bedridden (≥ 3 days) or major OR (4 weeks) | +1.5 |
| Previous objectively diagnoses DVT or PE | +1.5 |
| PE most likely diagnosis        | +3            |

Score: Low PTP: < 2 Moderate PTP: 2 - 6 High PTP: > 6

# How to use Laboratory to Diagnose von Willebrand Disease

## Note IX

### Assay

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<th>Type 2</th>
<th>Type 3</th>
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<tr>
<td><strong>PFA 100 (Collagen/Epinephrine cartridge)</strong></td>
<td>Normal</td>
<td>N or ↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td><strong>Factor VIII Activity</strong></td>
<td>Normal</td>
<td>N or ↓</td>
<td>N or ↓</td>
<td>N</td>
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<tr>
<td><strong>VWF Antigen</strong></td>
<td>Normal</td>
<td>B or ↓ or ↓↓</td>
<td>B or ↓</td>
<td>B or ↓</td>
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<tr>
<td><strong>VWF Activity - by Ristocetin Cofactor</strong></td>
<td>Normal</td>
<td>B or ↓ or ↓↓</td>
<td>↓↓ or ↓</td>
<td>↓ or ↓↓</td>
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</table>

### VWF Multimer Analysis

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<tr>
<th>Appearance</th>
<th>Normal distribution</th>
<th>Normal distribution</th>
<th>Lack HMWM</th>
<th>Lack HMWM</th>
<th>Normal distribution</th>
<th>Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Appearance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

### Supplemental Tests

- **RIPA High Dose**
  - Normal
  - N or ↓
  - ↓
  - N
  - ↓
  - N
  - NR

- **RIPA Low Dose**
  - NR
  - NR
  - NR
  - ↑
  - NR
  - NR
  - NR

- **FVIII Binding (VWF:FVIIIb)**
  - N
  - N
  - N
  - N
  - N
  - ↓↓↓
  - Absent

N = Normal
NR = non reactive
B = borderline
How to use Laboratory Tests in understanding the results of the DDAVP Challenge Test

Note X

Desmopressin (DDAVP), a synthetic analogue of natural pituitary hormone 8-arginine vasopressin, is a common therapy for VWD as it causes release of endogenous stores of VWF and FVIII. To perform a desmopressin trial, baseline VWF and factor VIII are measured and measurements repeated one and four to six hours following drug administration.

Evaluation for VWF should include a measure of activity and VWF antigen. Once administered, increase in plasma levels should last 8 to 10 hours. Following administration, levels of VWF and FVIII activity generally rise three to five times the baseline levels peaking at about one hour post-infusion and clinical desmopressin response can be defined as peak level of VWF:RCo and FVIII or more than 50 IU/dL.

Evaluation of VWF and FVIII activity at 4 or 6 hours post administration is important to evaluate for increased clearance as may occur in certain VWD variants and indicate the possible need for alternate form of therapy.

In addition to therapy, or to assess potential therapeutic responses, a desmopressin challenge may also assist in the functional characterization of VWD. Desmopressin causes release of VWF into plasma with peak concentrations within 30 minutes that will subsequently decrease with time according to clearance and proteolysis mechanisms including ADAMTS13.

In VWD, the patterns of release and clearance, as differentially detected using different assays can help characterize particular types of VWD. For example, the pattern in mild Type 1 VWD is fairly characteristic, with VWF reaching levels around 2-4x baseline, reducing over time and reaching approximate baseline values within 24 hours. The VWF initially detected by an optimised VWF:CB assay tends to increase more so than that detected by VWF:Ag and VWF:RCo, since optimised VWF:CB assays best reflect the ultra-HMW VWF initially released by desmopressin. Thus, in type 1 VWD post desmopressin the VWF:CB/VWF:Ag ratio tends to rise to a higher level (generally >1.0) than that of VWF:RCo/VWF:Ag ratio. The pattern for type 2A (and 2B) is different, with desmopressin tending to increase VWF:Ag to much higher levels than either VWF:RCo or VWF:CB. Thus, neither the VWF:CB/VWF:Ag or VWF:RCo/VWF:Ag ratio tend to rise, remaining <0.7 for most cases. The pattern for type 2M VWD (GPIb binding defect) is different again, with VWF:CB/VWF:Ag ratios remaining normal post desmopressin, and VWF:RCo/VWF:Ag ratios remaining low (generally <0.7).

Pre- and post-desmopressin evaluation of VWF test parameters can also uncover VWD related defects in VWF clearance, using differential test patterns for VWF:Ag versus the VWF propeptide, for example in vWD Vicenza there is accelerated clearance in 2-4 hours.
How to Evaluate the Coagulation Tests When a Patient is on Anticoagulants

Note XI

<table>
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<tr>
<th>ASSAY</th>
<th>HEPARIN</th>
<th>LOW MOLECULAR WEIGHT HEPARIN</th>
<th>COUMADIN</th>
<th>DIRECT THROMBIN INHIBITOR</th>
<th>COMMENTS</th>
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<tbody>
<tr>
<td>Clot based tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>d-RVVT</td>
<td>No effect</td>
<td>No effect</td>
<td>Elevation that corrects in the mixing study</td>
<td>Elevates Does not correct on mix</td>
<td></td>
</tr>
<tr>
<td>Reptilase time</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td></td>
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<tr>
<td>Protein S or C activity</td>
<td>No effect cause</td>
<td>No effect</td>
<td>Decreases</td>
<td>Spuriously low activity</td>
<td>Direct thrombin inhibitors impair thrombin formation so generally no clot formed</td>
</tr>
<tr>
<td>Assay has neutralizer</td>
<td>Neutralizers present</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC for Factor V&lt;sub&gt;Leiden&lt;/sub&gt;</td>
<td>No effect cause</td>
<td>No effect</td>
<td>No effect</td>
<td>May increase the ratio</td>
<td>Lupus anticoagulant may cause low ratio</td>
</tr>
<tr>
<td>Assay has neutralizer</td>
<td>Neutralizers present</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin Time, including dilute thrombin time</td>
<td>prolongs</td>
<td>May slightly prolong</td>
<td>No effect</td>
<td>prologns</td>
<td></td>
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<tr>
<td>Chromogenic Tests</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT III</td>
<td>Usually decreased</td>
<td>May decrease physiologically</td>
<td>No effect</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Protein C or S</td>
<td>No effect</td>
<td>No effect</td>
<td>decreased</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Factor VIII</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
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<tr>
<td>Immunologic Assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT III</td>
<td>Decreased physiologically</td>
<td>May decrease physiologically</td>
<td>No effect</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Protein C or S</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Protein S decrease with pregnancy and estrogens</td>
</tr>
<tr>
<td>Protein S decrease with pregnancy and estrogens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antiphospholipid Antibodies(ACA, Beta 2 GPI)</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Titters may vary over time</td>
</tr>
<tr>
<td>d-dimer</td>
<td>May physiologically</td>
<td>May physiologically</td>
<td>No effect</td>
<td>No effect</td>
<td></td>
</tr>
</tbody>
</table>
Assess the Risk of DIC:
Does the patient have a disorder known to be associated with DIC, sepsis, trauma, OB complications, malignancy, intravascular hemolysis, liver failure, hemangioma, aneurysm, graft rejection, inflammatory syndromes? If YES use this algorithm; if no, DO NOT USE

Request screening coagulation tests of PT, fibrinogen, platelet count, d-dimer; these are quick and readily available

<table>
<thead>
<tr>
<th>Test</th>
<th>Score</th>
</tr>
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<tr>
<td>Platelet Count &gt; 100000/ul</td>
<td>0</td>
</tr>
<tr>
<td>Platelet Count &lt; or equal to 100000/ul</td>
<td>1</td>
</tr>
<tr>
<td>Platelet Count &lt; 500000/ul</td>
<td>2</td>
</tr>
</tbody>
</table>

Prolonged Prothrombin Time by:
- 3 seconds 0
- > or = 6 seconds 2

Fibrinogen
- >100 mg/dl = 0
- < or equal to 100 mg/dl 1

Add score

Analyze Score
If > or = to 5 compatible with overt DIC
< 5 suggestive
<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Symptoms</th>
<th>Plasma Half-Life</th>
<th>Target Hemostatic Level</th>
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<tbody>
<tr>
<td>Fibrinogen</td>
<td>Umbilical cord, joint and mucosal tract, recurrent miscarriages</td>
<td>2–4 days</td>
<td>50 mg/dL</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>Umbilical cord, joint and mucosal tract</td>
<td>3–4 days</td>
<td>20%–30%</td>
</tr>
<tr>
<td>FV</td>
<td>Mucosal tract bleeding</td>
<td>36 hrs</td>
<td>15%–20%</td>
</tr>
<tr>
<td>FVII</td>
<td>Mucosal tract, joint and muscle</td>
<td>4–6 hrs</td>
<td>15%–20%</td>
</tr>
<tr>
<td>FXI</td>
<td>Posttraumatic/surgery bleeding</td>
<td>40–70 hrs</td>
<td>15%–20%</td>
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<tr>
<td>FX</td>
<td>Hemarthroses, soft tissue bleeds, menorrhagia</td>
<td>~ 40 hrs</td>
<td>10%–15%</td>
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<tr>
<td>FXIII</td>
<td>Umbilical cord, intracranial and joint, recurrent miscarriages, impaired wound healing</td>
<td>11–14 days</td>
<td>2%–5%</td>
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<td>K</td>
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<td>L. Prothrombin Fragment 1+2</td>
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SECTION I Introduction
Overview on Hemostasis

Figure 1 shows the “classical” concept of the coagulation cascade. This depiction of the coagulation cascade is useful clinically as it provides a good illustration of what the Prothrombin Time (PT) and activated Partial Thromboplastin time (PTT) measure.

Figure 1. THE CLASSICAL COAGULATION “CASCADE”

This series of reactions is a convenient way to think about various in vitro, clotting assays. The PTT measures the intrinsic factors, and the PT measures the extrinsic factors, with both measuring the factors of the common pathway. Since the endpoint of the test is a clot, abnormally low fibrinogen levels will prolong both the PT and PTT.
Several points regarding the biosynthesis of proteins involved in hemostasis are: 1) Fibrinogen, Prothrombin, Factors V, VII, IX, X, XII, XIII, and probably XI, as well as prekallikrein, HMWK, protein C and S and antithrombin (III) are synthesized in the liver, and 2) Factors II, VII, IX, and X, as well as Protein C and Protein S require the presence of vitamin K for their synthesis. Maintenance of normal hemostasis depends upon the balance between the pro-coagulant coagulation proteins (e.g., factor IX, etc.) and the endogenous anticoagulant proteins (e.g., protein C, protein S, etc.) and the proteins of the fibrinolytic system such as tissue plasminogen activator and plasminogen.

Before you get overwhelmed just remember that the coagulation cascade generates thrombin. Thrombin converts water soluble fibrinogen to water insoluble fibrin. Too little thrombin generation leads to bleeding and relative abundance to thrombin can lead to thrombosis. How simple!

B. Critical Clinical Information

It cannot be over emphasized that accurate interpretation of coagulation tests can only be made in the context of having information about the patient. The minimal information required is:

1) The nature of the clinical problem (e.g., hemorrhage or thrombosis)

2) a complete list of the patient’s medications, particularly any antithrombotic medications such as unfractionated heparin (UFH), low molecular weight heparin (LMWH), warfarin, direct thrombin inhibitors, antiplatelet drugs, and any hormonal preparations.

3) The patient’s age and gender.

4) The family history of bleeding or thrombosis

5) If bleeding is the clinical issue, the type of bleeding the patient is suffering is important. Mucosal bleeding suggests a platelet disorder or von Willebrand Disease, whereas a hemarthrosis suggests a factor deficiency such as Factor VIII or Factor IX deficiency.

6) If thrombosis is the clinical issue, the location of the thrombosis, whether it is arterial, venous or has occurred in both vascular beds is important. It is also important to determine whether the thrombosis is associated with a transient risk factor or idiopathic.

Some Clinical Caveats

Personal and family histories are the most important assessments of a patient’s individual risk for bleeding and thrombosis with surgery.

Routine screening for a bleeding tendency before surgery (prothrombin time (international normalised ratio), activated partial thromboplastin time, and platelet count) is not recommended.

Patients who have had one or more major haemostatic challenges (surgery or trauma) without excessive bleeding are unlikely to have a clinically important hereditary bleeding disorder.

If a personal or family history suggests a bleeding disorder, normal routine screening results do not exclude this possibility; such patients should be referred to a haematologist.
The extent and nature of surgery are the factors that most strongly influence the risk of developing postoperative venous thromboembolism.

The presence or absence of a familial predisposition to thrombosis rarely influence decisions about the use of venous thromboembolism prophylaxis with surgery, and preoperative testing for such abnormalities is not necessary.

SECTION II. SAMPLE COLLECTION

A. Coagulation tests are performed on 3.2 percent citrated plasma. Sodium citrate is the principal anticoagulant used for specimens submitted for coagulation testing. Sodium citrate inhibits coagulation by complexing free Ca++ ions that are essential for several chemical reactions in the coagulation cascade (see Figures 1 and 2 above). For this reason, virtually all coagulation tests are initiated by adding calcium to the sample (a process called “re-calcification”). For this reason, sample collection is very important, and improper collection can significantly affect test results. Citrated vacutainer tubes (“blue tops”) contain 0.5 milliliters (ml) of anticoagulant (3.2% sodium citrate) and the vacuum is precisely set to draw in 4.5 ml of blood. Anything that disturbs this ratio can alter test results. The two most common situations in which this occurs are: 1) when the vacuum is not used (e.g., manually filling the tube from another syringe, with or without removal of the blue top), and 2) when the patient has an abnormal plasma volume (e.g., with erythrocytosis or severe anemia).

B. Platelets are not included in most coagulation tests – in fact, it is essential that they be removed since they accelerate several key enzymatic reactions. Serum contains no fibrinogen, or factors V, VIII or XIII which remain in the clot. Thus, serum does not reflect the in vivo state of circulating blood.

C. Specimens for Special Coagulation tests should never be obtained from a central line or a heparin lock without extensive flushing (at least 20 ml of blood). A clean venipuncture at a separate site is greatly preferred. Blood should be obtained from the opposite arm when patients are receiving therapeutic heparin infusions.

D. Hemolyzed samples cannot be tested. Free hemoglobin activates platelets and in vitro clotting. Lipemic samples, samples from patients receiving intravenous fat emulsions or from patients with very high bilirubin levels (> 20mg/dL), and those with low fibrinogen concentrations may give erratic results with our routine procedures. Occasionally, some tests will need to be checked by more than one method.

E. Only one test requires sample collection and processing on ice: homocysteine. Do not refrigerate any blood sample sent to us for coagulation testing other than homocysteine.

SECTION III. SCREENING TESTS

A. Prothrombin Time (PT)

This assay is used to evaluate the extrinsic and common pathways of the coagulation cascade (Factors VII, X, V, II and fibrinogen).

INDICATIONS Prothrombin Time (PT):

1. Monitoring warfarin (Coumadin) therapy
2. Assessment of clotting factor deficiency in Vitamin K deficiency states.
3. Diagnosis of congenital and acquired deficiencies of coagulation proteins in the extrinsic (Factor VII) and common (Factor X, V, II ) pathways.
4. Diagnosis of inhibitors to Factors II, V, VII, X.

**TEST PRINCIPLE Prothrombin Time (PT):**

A mixture of thromboplastin which contains phospholipid and a source of tissue factor (Factor III) (in the past often derived from rabbit brain, more recently manufactured using recombinant human tissue factor and phospholipids) and calcium, is added to citrated patient plasma. Using photo-optical clot detection technology, the time to clot formation is determined. The clotting time of fresh normal plasma ranges from approximately 9.0 to 11.3 seconds. In the past, we used to grind up rabbit brains to make thromboplastin. In Europe human placenta is still used as a source of commercial thromboplastin.

We now use recombinant human thromboplastin which is supplemented with a high concentration of phospholipid. Recombinant thromboplastins are often called high sensitivity thromboplastins because they are very sensitive to reductions in vitamin K-dependent coagulation factors. As a consequence, these thromboplastins are similar in sensitivity to the World Health Organization reference standard thromboplastin and thus have a low International Standardized Index, generally from 0.98 to 1.1. Our current recombinant thromboplastin is also insensitive to therapeutic concentrations of heparin (0.3 to 0.7 units of heparin/mL) so PT results will not change significantly once heparin is discontinued in patients receiving heparin and warfarin. In addition, this thromboplastin is generally not affected by most antiphospholipid antibodies. In those patients who have an antiphospholipid antibody that prolongs the PT, the addition of normal plasma in a mixing study will not correct the PT.

While many hospitals, including ours, use point of care devices to monitor warfarin, I personally am not enthusiastic about this approach. Too many preanalytical variables can affect the results, and personnel not trained in the laboratory sciences perform the test in the clinic are not necessarily the most capable in troubleshooting problems or in addressing quality assurance issues. At least recently the point of care warfarin meters have started to use high sensitivity thromboplastin, including Innovin.

The results of the PT depend on the concentration of factors VII, X, V, II, and fibrinogen. The PT is most sensitive to small changes in factor levels when they are quite low and least sensitive when they are near normal. It is not influenced by factor XII, XI, IX, VIII or XIII, prekallikrein or high molecular weight kininogen concentrations.

**POSSIBLE RESULTS AND INTERPRETATION Prothrombin Time (PT):**

A prolonged PT indicates a deficiency of Factors VII, X, V, II, or low fibrinogen. An abnormally prolonged PT in conjunction with a normal PTT suggests a deficiency of factor VII. Most often, a prolonged prothrombin time is due to acquired causes: warfarin (Coumadin) therapy, vitamin K deficiency, disseminated intravascular coagulation (DIC), and liver disease. In each of these disorders, the factor VII concentration is low. Congenital causes of a prolonged PT are quite uncommon but include deficiencies of Factors VII, X, V, II and fibrinogen. Antiphospholipid antibodies may be associated with antibodies to prothrombin that can cause reduced factor II levels and significant prolongations of the PT (20-25 seconds). These patients bleed because of the severe hypoprothrombinemia.

Prolongation of both the PT and PTT are generally due to multiple factor deficiencies caused by: severe liver disease, DIC, warfarin, heparin, or severe vitamin K deficiency. Remember that you need to have at least 75 mg/dl of fibrinogen to form a clot; therefore, if the fibrinogen level is markedly reduced both the PT and PTT will be prolonged.

A shortened PT may represent the lower end of the normal range, increased tissue factor concentrations in the sample due to a traumatic venipuncture, or an excessive plasma to anticoagulant ratio (e.g., incorrect sample collection or severe anemia), or an infusion of recombinant Factor VIIa.

**FACTORS AFFECTING TEST RESULTS (FALSE POSITIVES & NEGATIVES) Prothrombin Time (PT):**

Erroneous results can be caused by improper collection, storage (e.g., temperature), and processing. Other conditions (poor nutritional state, congestive heart failure, hepatic disease, hyperthyroidism, fever, steatorrhea, renal failure, antibiotic therapy, prolonged use of narcotics, etc.) can deplete the concentration or interfere with the action...
of one or more clotting factors and prolong the PT. Other drugs and conditions (hypothyroidism, hyperlipidemia, edema, diarrhea, total parenteral nutrition, hereditary resistance to warfarin, etc.) may decrease the PT and reduce the effects of warfarin.

B. International Normalized Ratio (INR)

The INR is used to monitor warfarin therapy and is a contrived measurement that reflects the sensitivity of the thromboplastin to drops in factor VII only. It should not be used to monitor the new oral thrombin inhibitor, dabigatran. The World Health Organization (WHO), along with the International Committee on Thrombosis and Haemostasis has recommended that reporting PT results for patients on oral anticoagulant therapy should use International Normalized Ratio (INR) values. Reported INR results are independent of the reagents and methods used, and are specifically intended for assessing patients stabilized on long-term oral anticoagulant therapy. The INR represents the prothrombin ratio that would have been obtained if the primary WHO reference thromboplastin had been used to perform the PT. The INR is calculated as follows:

\[
\text{INR} = \left( \frac{\text{Patient PT}}{\text{geometric mean PT of normal pooled plasma}} \right)^{\text{ISI}}
\]

The International Sensitivity Index (ISI) for a given thromboplastin preparation is a measure of the responsiveness of that thromboplastin preparation to reductions in vitamin K-dependent coagulation factors. For most commercial rabbit brain thromboplastins used in North America, the ISI values range from 2.0 to 2.6. With the advent of recombinant thromboplastins such as the one we use in the coagulation laboratory, ISIs down to 1.0 have become available.

We have used Innovin from Siemens for our prothrombin testing for the last 10 years. This particular thromboplastin is insensitive to heparin, and to most antiphospholipid antibodies. Each year the lot number of thromboplastin changes and the ISI is subject to small changes. We have noticed very little effect upon test results or the normal range, so our check in of this reagent is relatively easy, although we do pay strict attention to calibration of the ISI for our instruments and that the correct ISI is entered into our computer system for automated calculation errors could be a serious problem. Now that there are FDA approved INR calibrators, the assignment of ISI values will more accurate.

Our critical Action value for INR is 5.0.

C. Partial Thromboplastin Time (PTT)

This Test is Used to Evaluate The Intrinsic Pathway of The Coagulation Cascade (Factors XII, XI, IX, VIII, Prekallikrein And High Molecular Weight Kininogen. The test use to be done without activation, now all PTT tests are done activated. Hence no need to use the old terminology of aPTT.

INDICATIONS Partial Thromboplastin Time (PTT):

1. Screening for hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency) von Willebrand disease and other congenital or acquired deficiency states of the intrinsic pathway (Factor XI, XII, prekallikrein, and high molecular weight kininogen).

2. Monitoring unfractionated heparin therapy

3. Screening for inhibitors of Factors VIII, IX, XI, or XII.

4. Screening for the presence of a lupus anticoagulant (antiphospholipid antibody). This requires a low phospholipid reagent.

TEST PRINCIPLE Partial Thromboplastin Time (PTT):
The activated partial thromboplastin time (PTT) is performed by recalcifying plasma in the presence of a standardized amount of platelet-like phosphatides and an activator of the contact factors of the intrinsic coagulation pathway.

A platelet substitute (cephalin or crude phospholipid = partial thromboplastin), a surface activating agent (actin or kaolin) to activate factor XII, and Ca++ ions are added to plasma. This mixture triggers activation of the intrinsic pathway and subsequently, the common pathway of coagulation. Therefore, the PTT is sensitive to deficiencies of all coagulation factors except factors VII and XIII. Using our present reagent, the PTT clotting time is normally between 23.5 and 34 seconds. The PTT generally becomes abnormal when one of the PTT clotting factors is decreased to less than 25-35% of normal. Consequently, mild factor deficiencies can be present with a normal PTT. Our Coagulation Lab uses a low-phospholipid PTT reagent which is sensitive to the presence of the Lupus Anticoagulant (antiphospholipid antibodies).

### POSSIBLE RESULTS AND INTERPRETATION Partial Thromboplastin Time (PTT):

A prolonged PTT indicates a deficiency or inhibitor of Factors VIII, IX, XI, XII, I (fibrinogen), II, V, X, prekallikrein, or high molecular weight kininogen. Common causes of a prolonged PTT include heparin therapy or contamination, a lupus anticoagulant, DIC, and congenital or acquired deficiencies of Factors VIII or IX. Less common causes of a prolonged PTT include deficiency of factor XI or a specific intrinsic pathway factor deficiency or inhibitor.

An abnormally prolonged PTT in conjunction with a normal PT indicates a deficiency of factors XII, XI, IX (Hemophilia B), or VIII (Hemophilia A), von Willebrand disease, or the presence of heparin.

A shortened PTT may represent the lower end of the normal range, or result from an excessive plasma to anticoagulant ratio (e.g., occurs with incorrect sample collection, severe anemia, a traumatic venipuncture), or rarely, a hypercoagulable state-if repeatedly short!

Prolongation of both the PT and PTT is generally due to multiple factor deficiencies caused by: severe liver disease, hypo-or dys-fibrinogenemia, DIC, warfarin, or severe vitamin K deficiency.

### FACTORS AFFECTING TEST RESULTS (false positives & negatives) Partial Thromboplastin Time (PTT):

Erroneous results can be caused by improper sample collection and processing. Prolongation of the PTT can be seen when blood is drawn from intravenous catheters which have been flushed with heparin or when “under-filled” blue top tubes are sent to the lab.

### D. Hepzyme (Heparin Neutralization test)

This assay tests for the presence of heparin (both UFH and LMWH) in the sample.

### INDICATIONS Hepzyme:

1. To rule out heparin contamination as the cause for an abnormal activated Partial Thromboplastin Time (PTT) and/or Prothrombin Time (PT).

2. To compare the PTT results for heparinized and unheparinized samples when monitoring heparin therapy.

3. To evaluate patients on combined heparin and warfarin therapy.

### TEST PRINCIPLE Hepzyme:

The presence of heparin in a specimen may interfere with the interpretation of various coagulation tests Heparinase cleaves heparin at multiple sites per molecule, including the ATIII binding site, producing oligosaccharides with an
average molecular weight of 1000 daltons that have no antithrombotic activity. Hepzyme can neutralize up to 2 USP units of unfractionated heparin in 1 mL of citrated plasma.

POSSIBLE RESULTS AND INTERPRETATION Hepzyme:

Results are reported as positive, negative, or inconclusive. The difference in test results (in seconds) before and after plasma treatment with Hepzyme is used to identify the presence of heparin in a sample. PTT results should be related to the laboratory’s normal range or to within 15% of the patient’s baseline, if measured, for PTT.

FACTORS AFFECTING TEST RESULTS (FALSE POSITIVES & NEGATIVES) Hepzyme:

As performed, the assay will neutralize up to 2 USP units/ml of unfractionated heparin. Higher concentrations of heparin may not be fully neutralized.

E. Mixing Studies

This Test is Used to Determine Whether a Prolonged PT, PTT or dRVVT is Due to a Factor Deficiency or an Inhibitor.

INDICATIONS (Mixing Studies):

1. Evaluation of a prolonged PT, PTT, or DRVVT

TEST PRINCIPLE (Mixing Studies):

If a screening coagulation test is prolonged, it is essential for the clinician to know whether a factor deficiency or inhibitor is responsible for the abnormal result as these conditions are often managed differently. Mixing studies are based on two principles: 1) the inhibitor is in excess, and if present, it will inhibit normal and patient plasma, and 2) that 50% of any factor is enough to yield a normal test result. In fact, 30%-40% of the normal level of the coagulation factor is generally sufficient to give a normal PT, PTT, or dRVVT. In a 1:1 mix, an equal volume of patient and normal plasma are mixed; in a 4:1 mix, 3 parts patient plasma is mixed with 1 part normal plasma. After mixing, the test is repeated. Correction into the normal range after mixing indicates a factor deficiency. Lack of correction suggests the presence of an inhibitor. Rarely, only the 4:1 mix can deter a weak or low-titer inhibitor. Inhibitors such as the lupus anticoagulant tend to result in prolongation of the PTT immediately after mixing while inhibitors directed against specific factors such as factor VIII require incubation at 37°C for 1-2 hours to fully express their inhibitory properties.

POSSIBLE RESULTS AND INTERPRETATION (Mixing Studies):

The results are interpreted as being consistent with an inhibitor, a factor deficiency or a low titer inhibitor. Depending upon the results, additional studies are performed to identify a specific factor deficiency (e.g., factor levels) or inhibitor (e.g., a Bethesda assay) or confirm the presence of an antiphospholipid antibody (dRVVT, anticardiolipin antibody ELISA, etc.).

FACTORS AFFECTING TEST RESULTS (FALSE POSITIVES AND NEGATIVES) (Mixing Studies):

Heparin contamination must be excluded using Hepzyme in any specimen that demonstrates an inhibitor

F. FIBRINOGEN

This Test Measures the Concentration of Functional Fibrinogen in a Test Sample

INDICATIONS (Fibrinogen):

1. Evaluation of coagulation disorders associated with a prolonged PT, PTT or thrombin time.
2. Evaluation of DIC

3. Evaluation of liver disease

4. Monitoring of patients undergoing thrombolytic therapy.

**TEST PRINCIPLE (Fibrinogen):**

We use the Claus method for measuring fibrinogen. This is a functional fibrinogen assay based on the fact that at high thrombin concentrations (100 NIH units/mL), the thrombin clotting time of dilute plasma is inversely proportional to the fibrinogen concentration of the plasma (i.e., the higher the fibrinogen, the shorter the thrombin clotting time). In normal blood coagulation, fibrinogen is converted to fibrin by the enzyme, thrombin. This conversion of fibrinogen to fibrin is a two-step process. The first step is the thrombin-mediated proteolysis of fibrinopeptides A and B from fibrinogen. After the release of the fibrinopeptides, the resulting form of fibrinogen is termed “fibrin monomer.” In the second step, fibrin monomers polymerize to form fibrin polymer. The formation of the fibrin polymer is recognized in the laboratory as the clotting time end point of the reaction. The fibrinogen value is determined from a standard curve generated by testing known concentrations of fibrinogen.

**POSSIBLE RESULTS AND INTERPRETATION (Fibrinogen):**

Low fibrinogen values may be seen in liver disease, during thrombolytic therapy, in patients with congenital a- or hypo- or dys-fibrinogenemia, in DIC and other consumptive states. In patient suspected of having a dysfibrinogen, an immunologic assay is essential to document the discrepancy between fibrinogen protein levels and function. Dysfibrinogens are characterized by the production of normal or slightly reduced levels of fibrinogen that are functionally abnormal such that tests of fibrinogen functions (e.g., Clauss fibrinogen assay) are much more severely affected than tests measuring the mere presence of fibrinogen protein (e.g., fibrinogen antigen assay). An elevated fibrinogen may be seen in acute and chronic stable liver disease. Severe chronic liver disease can be associated with acquired dysfibrinogenemia.

**Factors affecting test results (false positives and negatives) (Fibrinogen):**

High concentrations of heparin or fibrin degradation products can result in abnormal results. Structurally abnormal fibrinogens can yield falsely low results.

**G. THROMBIN TIME**

*The Thrombin Time (TT) Assay is A Test That Evaluates the Conversion Of Fibrinogen to Fibrin.*

**Indications (Thrombin Time):**

1. Diagnosis of hereditary fibrinogen deficiencies (both a- and hypo- fibrinogenemia and dysfibrinogenemia), in conjunction with fibrinogen antigen and activity assays.

2. Diagnosis of spontaneous thrombin inhibitors

3. Detection of Heparin effect

4. Detection of direct thrombin inhibitor effect, such as dabigatran- see anticoagulation section on dabigatran levels.

**Test Principle (Thrombin Time):**
A standard concentration of Thrombin (10 NIH units/ml) is added to plasma and the clotting time is measured. The Thrombin clotting time may be used as a qualitative measure of the level of functional fibrinogen. Prolonged times may be indicative of either decreased or markedly increased levels of functional fibrinogen; dysfibrinogenemia, or increased levels of certain fibrinogen/fibrin degradation products, heparin or dabigatran.

**Possible Results and Interpretation (Thrombin Time)**

A prolonged Thrombin Time generally means there is a reduction in the amount or function of fibrinogen. This result can occur because of inherited quantitative defects in fibrinogen production (a-fibrinogenemia or hypofibrinogenemia) or inherited qualitative defects in fibrinogen that result in the production of a normal amount (dysfibrinogenemia) or reduced amount of a dysfunctional fibrinogen (hypo-dysfibrinogenemia). Acquired quantitative deficiencies of fibrinogen can result from DIC (during which fibrinogen is consumed) or thrombolytic therapy (during which fibrinogen is destroyed). High levels of fibrin and fibrinogen degradation products (as seen in DIC or thrombolytic therapy) can disrupt fibrin polymerization and result in a prolonged thrombin time. Acquired dysfibrinogenemia is common in patients with severe chronic liver disease in which abnormally glycosylated forms of fibrinogen are produced. Thrombin inhibitors such as those seen after exposure to bovine thrombin or antithrombin-like substances occasionally identified in myeloma proteins or fibrin split products can also result in a prolonged thrombin time. Antithrombotic agents such as heparin, dabigatran, recombinant tissue plasminogen activator can result in an abnormally prolonged thrombin time.

**Factors affecting test results (false positives and negatives) (Thrombin Time):**

The Thrombin Time is markedly prolonged in the presence of heparin or direct thrombin inhibitors such as dabigatran.

**H. REPTILASE TIME**

*This Test is Used to Evaluate The Conversion of Fibrinogen to Fibrin Using Reptilase, A Snake Venom.*

**Indications (Reptilase Time):**

1. Identify heparin or a heparin-like inhibitor as the cause of a prolonged thrombin time
2. Evaluation of patients with quantitative or qualitative fibrinogen disorders.

**Test Principle (Reptilase Time):**

Plasma is incubated with Reptilase and the time to clot formation is measured. Reptilase is a thrombin-like enzyme that is derived from the venom of the South American pit viper, Fer-de-lance or the common lancehead (*Bothrops atrox*). This enzyme differs from thrombin in its specificity and the extent of cleavage of the fibrinogen molecule. Reptilase cleaves only fibrinopeptide A, whereas thrombin cleaves fibrinopeptides A and B. Reptilase is inhibited only slightly, or not at all by heparin, heparinoids, or hirudin, and low levels of fibrin degradation products (FDP), thus making it useful in the differential diagnosis of a prolonged thrombin time.

**Possible results and interpretation (Reptilase Time):**

A prolonged Reptilase Time may be due to decreased fibrinogen levels, dysfibrinogenemia, fibrin degradation products, soluble fibrin monomer complexes, abnormal anti-thrombins (e.g., myeloma proteins), and uremia.

**Factors affecting test results (false positives and negatives) (Reptilase Time):**

Partially clotted samples (micro-clots) will cause misleading results.
I. D-DIMER (An Immunoassay)

This Test is a Rapid Method For Quantifying D-Dimers in Plasma Samples.

Indications (Quantitative D-Dimer):

1. Diagnosis of DIC
2. Assist in the diagnosis of venous thromboembolism

Test Principle (Quantitative D-Dimer):

This is a quantitative immunoassay for Factor XIIIa-cross-linked dimers of the D fibrin degradation product. Thus, there must be activation of both: a) clotting and b) fibrinolysis. Thrombin must be formed to convert fibrinogen to fibrin and activate Factor XIII that cross-links fibrin. Under the action of thrombin, fibrinogen is cleaved to give rise to fibrin monomers. These monomers tend to associate themselves to form intermediate polymers that are subsequently stabilized by thrombin-activated Factor XIII with the introduction of covalent cross-linkages in the region of the D-domain to produce the insoluble fibrin clot. The presence of the thrombin triggers TPA release activating the fibrinolytic system. Plasmin is formed at the site of the fibrin clot attacking the fibrin clot and fibrinogen. However, unlike plasmin’s action on fibrinogen that produces fibrinogen degradation products, plasmin’s action on the fibrin clot leads exclusively to the generation of derivatives of cross-linked fibrin containing D-Dimer.

The rapid quantitative D-dimer assay (Innovance D Dimer, Siemens, Marburg Germany) uses an enhanced latex turbidimetric method to quantify D dimer in plasma samples. Polystyrene particles with a monoclonal antibody against the D-dimer fragment covalently linked to their surface are added to the plasma specimen to be analyzed. Since the epitope against which the antibodies are directed is present twice in the D-dimer fragments, only one antibody is required to cause the latex particles to agglutinate.

Aggregation of the latex particles increases the turbidity of the sample allowing quantification of the D dimer fragment using the Siemens Coagulation Analyzer.

Disseminated Intravascular Coagulation

Disseminated Intravascular Coagulation (DIC) is an acquired coagulopathy characterized by excessive activation of the coagulation cascade that results in thrombocytopenia, coagulation factor deficiencies and secondary fibrinolysis. It is triggered by a wide variety of medical and surgical catastrophes (e.g., sepsis, snake bites, major trauma, amniotic fluid embolism, disseminated cancer, etc.) In the laboratory, the presence of DIC can be identified by a prolonged PTT, PT, and thrombin time, decreased fibrinogen levels and thrombocytopenia. The test of choice for DIC is the D-Dimer assay. D dimers are fragments of cross-linked fibrin that are produced when fibrin clot is digested by plasmin. It is important to remember that D dimer levels are a reflection of not only clot dissolution but also plasma clearance by the liver. Therefore, elevated D dimer levels can occur in the presence of liver disease as well as DIC. Nevertheless, these competing causes can often be differentiated clinically as liver disease in the absence of accelerated fibrin clot formation generally only causes modest D dimer elevations while DIC is associated with moderate to marked elevations of D dimer fragments.

Exclusion of the Diagnosis of Venous or Pulmonary Thromboembolic Disease

Numerous studies have demonstrated the utility of a variety of D dimer assays in the diagnosis of VTE, particularly when used in conjunction with pre-test probability models such as the Wells Criteria. In general, D dimer tests have been used to exclude VTE in patients judged to have a low pre-test probability of thrombosis, while additional objective radiologic testing is done in patients with a moderate or high pre-test probability of VTE. Most d-dimer assays have been standardized to have the same cutoff value for exclusion of thrombosis. This value in our laboratory is less than 0.5 mg/L. We also give the normal range since the test is use to evaluate for DIC. This cut off level (Note: not the normal range) should be used when employing the D dimer in conjunction with a pre-test
probability assessment and objective radiologic testing in the diagnosis of VTE in outpatients. The following are two well known probability assessment scoring systems:

Possible results and Interpretation (Diagnosis of Venous Thromboembolism):

The D-Dimer level is calculated by the analyzer in mg/L using a standard curve. The total measuring range is from 0.4mg/L to 55 mg/L. When used in conjunction with other clinical and radiologic information in the diagnosis of VTE, a cut-off level of 0.5 mg/L should be used.

Factors affecting test results (false positives and negatives) Diagnosis of Venous Thromboembolism):

Levels of plasma lipids greater than 120mg/dL, bilirubin greater than 24 mg/dl, heparin greater than 2.5 IU/ml and rheumatoid factor greater than 900 IU/ml can lead to spurious results. Plasma samples containing heterophil antibodies or mouse monoclonal antibodies used for diagnosis or therapy may lead to inaccurate results. It remains unclear whether human anti-mouse antibodies will interfere with the assay. Fibrinogen degradation fragment D will not interfere with assay in levels up to 20mg/L. Fibrinogen will not influence the assay with levels less than 9 g/L.

K. CRYOFIBRINOGEN

This is a Qualitative Test Used to Identify The Presence of A Cryofibrinogen in A Patient Sample

Indications (Cryofibrinogen):

1. Diagnosis of cryofibrinogenemia.

Test Principle (Cryofibrinogen):

Cryofibrinogens are complexes of fibrinogen, fibrin split products, and plasma globulins, that are precipitable in the cold and can be associated with purpura thrombosis, and/or hemorrhage. Citrated blood must be transported immediately to the coagulation laboratory. The sample must be maintained at 37°C (body temperature). The plasma and blood must be separated at 37°C using heated lab equipment and rapid processing techniques. Once the plasma is separated from the blood, the plasma is divided in half. One half of the plasma is maintained in a 37°C heating block for 24 hours and the other half is placed on ice in the refrigerator for 24 hours. At the end of 24 hours, if the iced sample has gel fibrin-like strands and the 37°C does not, the sample is considered positive for cryofibrinogen. The results may be further confirmed by placing the iced sample in a 37°C incubator for 30 minutes and the gel strands should disappear, confirming the presence of a cryofibrinogen. The absence of this cold precipitable substance in serum provides substantiation that the protein is cryofibrinogen rather than cryoglobulin.

Possible results and interpretation (Cryofibrinogen):

Patient samples are scored as positive or negative. Cryofibrinogenemia has been associated with DIC, myocardial infarction, pregnancy, neo-plastic, thromboembolic, connective tissue, vasculitis, and infectious disorder.

Factors affecting test results (false positives and negatives) (Cryofibrinogen):

Sample must be maintained at room temperature until plasma and red blood cells are separated or the cryofibrinogen may deposit on red blood cells and give a false negative result.

SECTION IV. FACTOR ASSAYS AND VON WILLEBRAND FACTOR STUDIES

A. FACTOR II, V, VII, and X activity assays

These Tests Are Used to Determine The Functional Activity of Coagulation Factors in the Extrinsic and Common Pathways of Coagulation, Namely Factors II, V, VII, and X.
**Test Principle of Extrinsic Factor Assays:**

Factor assays are performed by determining the extent that the patient’s plasma corrects the clotting time of test plasma known to be deficient in the particular clotting factor of interest (e.g., factor VII deficient plasma is used to measure the factor VII activity in patient plasma samples). The prothrombin time (PT) is the coagulation test that is used to measure the functional levels of the coagulation factors in the extrinsic and common pathways of the coagulation cascade. The activated partial thromboplastin time is the coagulation test that is used to measure the functional levels of the coagulation factors in the intrinsic pathway.

Analysis is performed by making serial dilutions of the reference plasma in buffer (with assay values for the factor that needs analysis). Each dilution is then mixed with an equal volume of “substrate” plasma that is known to contain normal levels of all factors, except the factor that is being assayed. A prothrombin time is performed on the serial dilutions.

The results are plotted on a log/log graph (polynomial curve) with percent factor on the abscissa and the time in seconds on the ordinate. The test results of the above dilutions will form a straight line that forms the basis for a standard curve at the connected points which can then be used to determine the activity of that coagulation factor in patient samples. To analyze a factor activity level on an unknown sample, 1:5 (100% activity) and 1:10 (50% activity) dilutions are prepared and mixed with “substrate” plasma. The percent activity is read from the abscissa of the graph by finding where the clotting time obtained for the unknown sample intercepts the standard curve. All of this now is in fact programmed into our analyzers, and graph paper can no longer be found in our laboratory.

**Indications (Factor Assays Studies):**

1. Determination of the functional activity of coagulation factors in the extrinsic and common pathways (Factors VII, X, V and II)

**Possible results and interpretation (Factor Assays Studies):**

Isolated Factor II deficiency may be inherited or acquired. Inherited factor II deficiency is extremely uncommon with an incidence of approximately 1/2,000,000. Rare inherited dysprothrombinemias suggest that factor II antigen and activity assays should be used in the evaluation of patients with prothrombin deficiency. Factor II deficiency is occasionally seen in an acquired form among patients with antiphospholipid antibody syndrome, who develop factor II (prothrombin) antibodies. Acquired inhibitors of factor II may be seen in patients exposed to bovine thrombin, which is used to prepare fibrin glue, a local hemostat employed commonly in cardiac surgery and neurosurgery.

Isolated deficiency of Factor V activity may be inherited (incidence 1/1,000,000) or rarely acquired in patients suffering from amyloidosis. Cases of congenital factor V deficiency associated with the production of a dysfunctional molecule have also been reported. Rare cases of combined factor V and VIII deficiency and V and VII deficiency also have been identified. Mutations in the endoplasmic reticulum Golgi intermediate compartment protein ERGIC-53, have been noted in some families with congenital factor V and VIII deficiency. Inhibitors directed against factor V can be seen in patients exposed to fibrin glue prepared with bovine thrombin.

Note: Factor V deficiency is not synonymous with Factor V Leiden (see hypercoagulable state discussion).

Congenital Factor VII deficiency has an incidence of 1/500,000. Both quantitative and qualitative defects have been identified. Both factor VII activity and antigen are useful in determining which type of defect is present. Rare cases of combined factor V and VII deficiency have also been reported. A continually updated repository of mutations associated with factor VII deficiency is available on the Internet at [http://hadb.org.uk](http://hadb.org.uk)

In common with the other vitamin K dependent coagulation factors (factors II, VII, IX and X as well as endogenous antithrombotic factors protein C and S), factor VII deficiency can occur in association with vitamin K deficiency and therapy with vitamin K antagonists. As the site of synthesis of all the coagulation factors, the liver, when diseased, can result in acquired deficiencies of all the coagulation factors (except factor VIII whose synthesis in hepatic sinusoidal endothelial cells and Kupffer cells persists even in the face of advanced liver dysfunction). Factor VII deficiency is acquired earlier in liver disease due to its short plasma half-life.
Factor X deficiency is rare, its incidence being less than 1/500,000. Mutations resulting in qualitative and quantitative defects have been described. Acquired factor X deficiency is most commonly seen in conjunction with vitamin K deficiency or therapy with vitamin K antagonists. Amyloidosis can also cause acquired factor X deficiency.

Factors affecting test results (false positives and negatives) (Factor Assays Studies):

Factor activity results can be influenced by the type of coagulometer or the reagents used to perform the test. Dilution errors can adversely affect the obtained factor activity results as well. The presence of the Lupus Anticoagulant has been associated with falsely decreased results. This effect of a LA can often be identified and overcome by observing test results for a plateau effect during serial dilutions of the plasma sample to be assayed.

B. Factor VIII, IX, XI, and XII, Prekallikrein and High Molecular Weight Kininogen Activity Assays

These Tests Are Used to Determine The Functional Activity of Coagulation Factors In The Intrinsic Pathways of the Coagulation Cascade, Namely Prekallikrein (Fletcher Factor), High Molecular Weight Kininogen (Fitzgerald Factor) and Factors VIII, IX, XI, And XII.

Indications Factor VIII, IX, XI, and XII, Prekallikrein and High Molecular Weight Kininogen Activity Assays:

1. Diagnosis of deficiency states or inhibitors affecting factors in the intrinsic pathway of the coagulation cascade.

Test Principle Factor VIII, IX, XI, and XII, Prekallikrein and High Molecular Weight Kininogen Activity Assays:

These factor assays are performed by determining the extent to which the patient’s plasma corrects the clotting time of a plasma sample known to be deficient in the particular clotting factor of interest (e.g., factor VIII-deficient plasma is used to perform factor VIII activity assays). The activated partial thromboplastin time is performed by re-calculating plasma in the presence of a standardized amount of platelet-like phosphatides and an activator of the contact factors of the intrinsic coagulation pathway (factor XII, prekallikrein, high molecular weight kininogen). Plasma samples from patients with a deficiency of any factor in the intrinsic pathway (factors VIII, IX, XI, or XII, prekallikrein and high molecular weight kininogen) may have prolonged PTT values. Prolongation of the PTT forms the basis for measurement of factors in the intrinsic pathway of coagulation in patient plasma samples. Analysis is performed by making serial dilutions (1:5 to 1:320 dilutions of calibration reference plasma or factor assay reference plasma in buffer (both with known assay values for the factor of interest)). Each dilution is mixed with an equal volume of “substrate” plasma that is known to contain normal levels of all factors except the factor of interest. An PTT is performed on the serial dilutions. The results are plotted on a log/log graph (polynomial curve) with the percent dilution on the abscissa and the time in seconds on the ordinate. The connected points from the above dilutions form a straight line that is the basis for a standard curve.

This standard curve is then used to measure the factor activity of patient samples with unknown factor content. To analyze the factor activity level on an unknown sample, 1:5 (100% activity) and 1:10 (50% activity) dilutions are prepared and mixed with immunoabsorbed, factor deficient (<1%) plasma. The percent factor activity of the patient sample is read from the abscissa of the standard curve graph by finding where the time obtained on the unknown intersects the standard curve.

Factor VIII activity can also be measured using a chromagenic assay based upon the principle that factor VIII is a cofactor of Factor IX. In this assay, the end point is the amount of a colored product formed by cleavage of a chromagenic substrate by the “tenase” complex (so called because the complex of factor IX and its cofactor, factor VIII cleave and activate factor X in the plasma in vivo) instead of the clotting end point of traditional factor assays designated by the formation of fibrin clot. Since all other components of the chromagenic assay are in excess, the amount of factor VIII provided by the patient sample is limiting and determines the amount of colored product formed that is measured by a spectrophotometer.
This assay is valuable in patients with antiphospholipid antibodies (LA) that can sometimes interfere with the clotting endpoint of traditional factor assays.

**Possible results and interpretation, Factor VIII, IX, XI, and XII, Prekallikrein and High Molecular Weight Kininogen Activity Assays:**

Low Factor VIII activity may be seen in von Willebrand Disease, Hemophilia A, Hemophilia A carrier states and in the presence of an inhibitor. Heparin therapy and acute DIC may also produce an abnormally low Factor VIII activity. Hemophilia A is inherited in an X-linked recessive manner and occurs in approximately 1 in every 10,000 live male births. Factor VIII inhibitors develop in 15-20% of hemophilia A patients due to exposure to exogenous factor VIII. The incidence of spontaneous factor VIII inhibitors is approximately 1/1,000,000. These autoantibodies are associated with autoimmune disorders, malignancies, medications and the peripartum period. High Factor VIII activity may be seen secondary to stress, inflammation, exercise, pregnancy, surgery, and estrogen use because it is an acute phase reactant. Chronically elevated factor VIII levels are associated with a 6-fold increase in the risk of venous thrombosis.

Low Factor IX activity may be seen in Hemophilia B, Hemophilia B carrier states, vitamin K deficiency, severe liver disease, oral anticoagulant therapy, or in the presence of circulating inhibitors. Like hemophilia A, hemophilia B is inherited in an X-linked recessive fashion but its incidence is about 10-fold less common occurring in 1 out of every 50-100,000 male births. Factor IX alloantibodies occur in 2-3% of hemophilia B patients and are more common among Scandinavian patients and those with large gene deletions. These antibodies are associated with anaphylactic reactions among hemophilia B patients when receiving factor replacement. Acquired factor IX deficiency has been rarely associated with amyloidosis. Acquired factor IX autoantibodies are extraordinarily rare. One study suggests that increased factor IX levels are associated with a 2-fold increase in the risk of venous thrombosis.

Decreased levels of Factor XI are seen in hereditary deficiency states (Hemophilia C or Rosenthal’s disease) and severe liver disease. Congenital Factor XI deficiency is rare (mean incidence of 1 per 100,000) except in Ashkenazi Jewish populations among whom the heterozygote gene frequency can be as high as 6-13 percent and homozygotes occur in 0.1-0.3%. Factor XI deficiency is generally a mild to moderate bleeding disorder that generally is symptomatic only with trauma or surgery. Factor XI can be activated by frozen storage leading to spurious increases in the assayed level. Elevated factor XI levels have been identified as a risk factor for venous thrombosis.

Factor XII deficiency is a rare disorder, which despite causing an impressive prolongation of the PTT (> 100 seconds) is not associated with any clinical bleeding diathesis. Although the index patient succumbed to a pulmonary embolism following trauma, factor XII deficiency probably does not result in a significant hypercoagulable state. Low prekallikrein and high molecular weight kininogen levels are rare congenital deficiency states, which similar to factor XII deficiency generally result in no significant physiological consequences for patients.

**Factors affecting test (false positive and negatives), Factor VIII, IX, XI, and XII, Prekallikrein and High Molecular Weight Kininogen Activity Assays:**

Variations in instruments or reagents used to perform assays and dilution errors can cause variations in the obtained factor activity results. The presence of the Lupus Anticoagulant has been associated with falsely decreased results. This effect of a LA can often be identified and overcome by observing test results for a plateau effect during serial dilutions of the plasma sample to be assayed. Factor XI can be activated by frozen storage causing a falsely increased assayed activity level.

**C. Factor XIII Activity Assay**

*This Test is used to Test Factor XIII Deficiency*

**Indications (Factor XIII Test):**
1. Diagnosis of factor XIII deficiency or inhibitors directed against factor XIII. The old clot solubility assay detects only the most severe forms of factor XIII deficiency. The urea stability test is sensitive to levels of 1-5%, while the acetic acid method is sensitive to below 10%. In cases of suspected factor XIII deficiency measurement of levels should be done. Severe factor XIII deficiency is associated with intracranial bleeding, and of course the PT and PTT are normal.

**Test Principle (Factor XIII Screening Test):**

We use the Siemens Berichrom photometric assay for Factor XIII which measures ammonia released in the first step of the transglutaminase reaction. Normal factor XIII activity levels range from 5—220%. Levels between 5-30% are sufficient to prevent spontaneous bleeding. There are other assays available but only the Berichrom is approved in the United States. Of note there are reports of the thromboelastogram being abnormal, with reduced maximum amplitude and strength and increased clot time at 30 minutes.

**Possible results and interpretation (Factor XIII Test):**

Low Factor XIII Levels are seen in:
- Individuals with inherited FXIII deficiency
- FXIII inhibitors - rare but may be seen in association with Isoniazid
- Henoch-Schoenlein purpura (HSP)
- In patients on and following cardiopulmonary bypass
- Chronic inflammatory bowel disease
- Levels fall in pregnancy. Severe inherited FXIII deficiency is associated with recurrent miscarriage
- Excessive activation, as seen in DIC, exposure to some snake venoms and caterpillar toxins [Lonomia Achelous caterpillar Venom]
- Low factor XIII levels have also been reported in several cases associated with the use of sodium valproate. In these cases the FXIII levels return to normal after the drug is withdrawn.

**Factors affecting test results (false positives and negatives) (Factor XIII Test):**

D. Factor VIII and IX Inhibitors

*The Bethesda Assay is Used to Measure the Inhibitory Activity of Factor VIII and IX Inhibitors*

**Indications (Factor VIII and IX Inhibitors):**

1. Diagnosis of factor VIII or IX inhibitors

**Test Principle (Factor VIII and IX Inhibitors):**

Factor inhibitors are quantified by mixing dilutions of the patient’s plasma sample with normal pool plasma which contains a normal factor level and measuring the factor activity levels immediately after the mix and after a 2 hour incubation at 37°C. By comparing the difference in factor activity of the patient incubation mixture and a control mixture, the amount of inhibitor present is calculated in Bethesda units. A modification of the Bethesda assay, the Nijmegen modification, dilutes patient plasma in buffered plasma to eliminate the decrease in factor activity that occurs with pH shifts during incubation. The imprecision introduced with unbuffered plasma is relevant particularly for lower titer inhibitors.

**Possible results and interpretation (Factor VIII and IX Inhibitors):**

Inhibitors are either “not detected” or “positive.” In the latter case, the titer of the inhibitor (in Bethesda units) is reported. Inhibitors to specific coagulation factors can be classified as being either autoantibodies or alloantibodies.
Autoantibody factor inhibitors are antibodies directed at a specific coagulation factor that arise in patients without a previous history of a coagulation disorder. The most common autoantibody inhibitors are directed against factor VIII and have an annual incidence of 1 per 1,000,000. Factor VIII inhibitors are often associated with exposure to certain medications (penicillins, sulfonamide antibiotics) or medical conditions (pregnancy, lymphoproliferative disorders, autoimmune disorders, solid tumors). Immunosuppressive therapy is effective in eradication of these antibodies in 60-70% of patients. Autoantibodies directed against other coagulation factors are extremely rare. In contrast to autoantibodies, alloantibodies develop in patients who lack a particular coagulation factor and generate an alloantibody upon exposure to this factor during treatment with coagulation factor concentrates. Factor VIII alloantibodies are the most common alloantibody inhibitor with an estimated prevalence of 15-25% among patients with severe hemophilia A. The prevalence of inhibitors is much lower among patients with hemophilia B where their occurrence has been estimated to be 2%. The development of an inhibitor in hemophilia A patients is usually suspected due to a lack of clinical response to factor VIII concentrates. In hemophilia B patients the development of an inhibitor is often heralded by developing allergic reactions to factor infusions as well as poor hemostatic responses to factor IX concentrates.

Factor VIII and IX alloantibodies are classified by their Bethesda Unit titer in low titer (5 Bethesda units or less) and high titer inhibitors (>5 Bethesda units) because the Bethesda unit titer influences the selection of treatments for bleeding episodes. Patients with low titer inhibitors can be effectively treated with factor concentrates by doubling or tripling the dose. The advantage of this approach is that factor activity levels can be followed to objectively assess the adequacy of therapy. The ability to measure factor levels can be very useful in situations in which objective confirmation of the adequacy of replacement therapy is important such as during major surgical procedures or treatment for life-threatening bleeds. The disadvantage of this approach is that further exposure to factor VIII or IX concentrates may result in an increase in Bethesda unit titers in subsequent days/weeks complicating future therapy.

Factors affecting test results (false positives and negatives) (Factor VIII and IX Inhibitors):

The inhibitor assay should not be performed on patients with normal factor activity levels (i.e., you should not look for a Factor VIII inhibitor, if the Factor VIII activity is normal). Samples drawn post-infusion of Factor VIII or Factor IX concentrates during prolonged treatment may be associated with transient saturation of the inhibitor by the infused factor and result in artifically reduced levels. This assay cannot distinguish between specific factor inhibitors and fibrin degradation products, heparin, or heparin like inhibitors that may also lead to a positive mixing test result.

E. Von Willebrand Disease

Von Willebrand factor (vWF) plays two key roles in hemostasis; it participates in platelet adhesion and aggregation and it serves as a carrier protein for factor VIII, protecting it from inactivation by activated protein C. The most common form of vWD is type I. Approximately 75% of patients with vWD have this form. In type I vWD, there is a modest and equivalent reduction of both vWF activity and protein levels as reflected in the ristocetin cofactor and vWF antigen assays. FVIII activity may also be reduced because of its shortened half-life in the absence of sufficient vWF. Analysis of vWF multimer sizes (agarose SDS gel electrophoresis) in patients with type I vWD demonstrates a normal pattern with all sizes of vWF multimers being present.

Whereas type 1 vWD is an illness characterized by a mild quantitative deficiency of vWF, type 2 vWD is an illness in which mutations result in the production of qualitatively defective vWF molecules. Four major type 2 variants are recognized: type 2A, type 2B, type 2M and type 2N von Willebrand disease. Type 2A results from mutations in the vWF molecule, which prevent normal assembly of vWF multimers or result in vWF multimers that are unusually susceptible to in vivo proteolysis. The result is a loss of large and intermediate vWF multimers, the fractions that are the most active in platelet adhesion. In type 2B vWD, mutations results in an increased affinity of vWF for platelets producing spontaneous platelet aggregation, the selective loss of the high molecular weight vWF multimers and often mild to moderate thrombocytopenia. Recent studies indicate the thrombocytopenia associated with Type 2B vWD is also due to reduced platelet production secondary to abnormal megakaryocyte function. Mutations affecting platelet and endothelial interaction characterize Type 2M vWD.
Each of these type 2 disorders is distinguished from type 1 vWD by much larger reductions in vWF activity (ristocetin cofactor activity) than in vWF protein levels (vWF antigen). In addition, type 2A and 2B are characterized by the loss of the large and intermediate or large vWF multimer fractions, respectively, on vWF multimer analysis. Platelet aggregation studies using low doses of ristocetin are useful in demonstrating the hyper responsive phenotype of type 2B vWD.

Type 2N vWD results from mutations in the factor VIII binding site on vWF. As a result, type 2N vWD behaves much like a mild autosomal form of hemophilia A, with factor VIII activity levels in the 5-15% range. vWF protein (vWF antigen) and activity levels (ristocetin cofactor assay) and multimer patterns (vWF multimer analysis) are normal.

The most severe form of vWD is type 3 vWD. It is an autosomal recessive form in which one abnormal gene is inherited from both parents. These individuals have undetectable levels of vWF protein and function with markedly reduced FVIII levels:

**F. Ristocetin Cofactor Assay**

*The Ristocetin Cofactor Assay Measures the Functional Level of von Willebrand Factor in Plasma*

**Indications (Ristocetin Cofactor Assay):**

1. Diagnosis of von Willebrand Disease.

**Test Principle (Ristocetin Cofactor Assay):**

Von Willebrand Disease (vWD) is associated with a decrease in von Willebrand Factor (vWF) protein. The Ristocetin Cofactor Activity is based upon the observation that the antibiotic, ristocetin can induce platelet agglutination in a vWF-dependent fashion. The Ristocetin Cofactor Assay is performed by agglutinating a standardized suspension of platelets in the presence of von Willebrand Factor (provided by the patient plasma) using the antibiotic, Ristocetin. Although the platelets play a passive role in such agglutination, there is an absolute requirement that the Ristocetin-dependent receptor be intact. Levels of Ristocetin Cofactor activity are determined by the ability of the test plasma and Ristocetin to induce aggregation in a standardized platelet suspension. Following reconstitution, lyophilized platelets are treated with Ristocetin in the presence of dilutions of normal standardized human plasma with a known amount of Ristocetin Cofactor Activity. A standard curve is prepared, after which patient plasma is then used as a source of Ristocetin Cofactor Activity. The Ristocetin Cofactor Activity of the patient sample is interpolated from the standard curve. Although the Ristocetin Cofactor Assay is considered by many investigators to be the single most important assay for von Willebrand Disease, a complete diagnostic evaluation for vWD requires the determination of the von Willebrand Factor antigen level, Factor VIII activity, bleeding time, vWF multimer analysis and family history. Recently, the platelet function analyzer (PFA-100) has also been demonstrated to provide useful diagnostic information in the evaluation for von Willebrand disease.

**Possible results and interpretation (Ristocetin Cofactor Assay):**

vWF activity is reduced in type 1 and 2 von Willebrand Disease and absent in type 3 (severe, homozygous) von Willebrand Disease. In type 1 vWD, there is typically an equivalent reduction in the ristocetin cofactor activity and vWF antigen level reflecting the fact that Type 1 vWD is due to a mutation that reduces the production of an otherwise structurally normal vWF molecule. In contrast, type 2 vWD is caused by a mutation in the coding region of the vWF gene producing a vWF molecule that functions abnormally (see above discussion of vWD for more details). As a consequence, type 2 vWD is usually associated with significantly greater reductions in ristocetin cofactor acidity than vWF antigen levels.

This difference between Type 1 and Type 2 vWD is reflected in the Ristocetin Cofactor: vWF Antigen ratio that is reported with Ristocetin Cofactor and vWF Antigen test results. In Type 1 vWD, the Ristocetin Cofactor: vWF Antigen ratios is ≥ 0.6, while in Type 2 vWD the Ristocetin Cofactor: vWF Antigen ratio is 0.5 or less. One exception is Type 2N vWD, in which ristocetin cofactor activity and vWF antigen levels are both normal because the mutation in the vWF molecule is isolated to the FVIII binding pocket so the only abnormality is in the factor
VIII activity of the patients plasma and the factor VIII binding activity of vWF (see above section on vWD). In Type 3 vWD, in which homozygous mutations in the vWF gene lead to severely reduced or absent production of vWF, both ristocetin cofactor activity and vWF antigen levels are extremely low. To differentiate between the various types of vWD, a complete von Willebrand evaluation is recommended for accurate, comprehensive diagnosis of von Willebrand Disease, including family history, Factor VIII activity, vWF antigen, ristocetin cofactor activity, bleeding time and/or PFA-100 and von Willebrand Factor multimer analysis.

Factors affecting test results (false positives and negatives) (Ristocetin Cofactor Assay):

Larger vWF multimers are able to mediate platelet adhesion more effectively than smaller multimers. Consequently, patients who have selective loss of the larger more active vWF multimers (e.g., Type 2 vWD) are likely to have greater reductions in vWF function (and thus ristocetin cofactor activity) than predicted by their vWF protein level (as reflected in their vWF antigen levels). This loss of higher molecular weight multimers will be reflected in an abnormal distribution of multimers (i.e. a predominance of low molecular weight multimers, with high molecular weight multimers decreased or absent) rather than an absolute deficiency of vWF. Prolonged transport of samples to the laboratory or inadequate sample preparation (e.g., failure to remove platelets from the patient sample) can be associated with false positive results because of platelet binding of vWF. Alternative functional assays of vWF function such as a collagen binding assay should be considered in the event of borderline ristocetin cofactor activity results.

G. Von Willebrand Factor (vWF) Antigen

This Test Measures the Quantity of Von Willebrand Protein in Patient Plasma

Indications (Von Willebrand Factor (vWF) Antigen):

1. Diagnosis of von Willebrand Disease

Test Principle (Von Willebrand Factor (vWF) Antigen):

A plastic support coated with specific rabbit anti-human vWF antibodies captures the vWF to be measured. Next, a rabbit anti-vWF antibody coupled with peroxidase binds to the remaining free antigenic determinants of vWF, forming the antibody-antigen antibody “sandwich.” The amount of bound antibody (i.e. the amount of vWF in the sample) is determined by the activity of the enzyme peroxidase during a specified period of time on the substrate ortho-phenylenediamine in the presence of hydrogen peroxide. After stopping the reaction with dilute sulfuric acid, the color intensity measured spectro-photometrically bears a direct relationship with the vWF concentration in the patient sample.

Possible results and interpretation (Von Willebrand Factor (vWF) Antigen):

vWF antigen is absent or greatly diminished in patients with severe (type III) von Willebrand Disease. In type 1 von Willebrand disease, there is a mild to moderate reduction in the amount of this protein. Levels of vWF antigen may be normal or low in type II variants of von Willebrand Disease. The amount of vWF protein and the functional level of vWF can be used to differentiate between the different types of vWD (see above).

Factors affecting test results (false positives and negatives) (vWF) Antigen:

vWF levels are influenced by ABO blood type (see Table below). Therefore, physicians should take into account a patient’s ABO blood type, clinical symptoms and laboratory test results when making a diagnosis of vWD. In general, patients should have clinical symptoms of bleeding as well as abnormal laboratory testing results to consider a diagnosis of vWD, particularly in patients with ABO blood group O who tend to have lower vWF levels. The following conditions may elevate vWF and mask the diagnosis of von Willebrand Disease: pregnancy, oral contraceptives, liver disease, inflammation, exercise, stress, traumatic venipuncture, post-operative state, and fainting. In extremely rare instances, anti-rabbit antibodies that can lead to aberrant test results in affected individuals.
Table 3: Influence of ABO Blood Group on vWF Antigen Values in Volunteer Blood Donors

<table>
<thead>
<tr>
<th>ABO Type</th>
<th>N</th>
<th>Geometric Mean</th>
<th>Geometric Mean ±2 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>456</td>
<td>74.8</td>
<td>(35.6 – 157.0)</td>
</tr>
<tr>
<td>A</td>
<td>340</td>
<td>105.9</td>
<td>(48.0 – 233.9)</td>
</tr>
<tr>
<td>B</td>
<td>196</td>
<td>116.9</td>
<td>(56.8 – 241.0)</td>
</tr>
<tr>
<td>AB</td>
<td>109</td>
<td>123.3</td>
<td>(63.8 – 238.2)</td>
</tr>
</tbody>
</table>

The Groups Were Statistically Significantly Different From Each Other As Follows: 0 vs. A, AB, p < .01; A vs. AB p < .01; B vs. A p < .05.

H. Von Willebrand Factor Multimer Analysis

*This Test Allows Quantification of the Different von Willebrand Factor Multimer Fractions*

**Indications (vWF Multimer Analysis):**

1. Diagnosis of von Willebrand Disease.

**Test Principle (vWF Multimer Analysis):**

Sub-classification of von Willebrand Disease (vWD) is based on the distribution of vWF multimers in patient plasma. vWF multimers are detected using SDS-agarose gel electrophoresis with radiolabeled antibody detection or trans-blotting and enzyme-linked antibody detection. Type 1 vWD has all normal multimer sizes present while type IIa is associated with the selective loss of large and intermediate multimers. Type 2B vWD is characterized by loss of large molecular weight multimers. Type 2N vWD is associated with a normal vWF multimer pattern while type 2M is characterized by a normal multimer pattern or an accumulation of ultra-large vWF multimers in the Vicenza subtype of Type 2M vWD. Type 3 vWD is associated with the complete absence of multimers given the severity of vWF deficiency. Standard gels resolve the smaller MW multimer bands into 3-5 satellite bands, but are less sensitive to differences in the high MW multimers. Low resolution gels will detect, 1) loss of the high MW multimers seen in some variants of vWD (Type 2B), 2) acquired loss secondary to DIC, HUS, acute TTP or cardiac defects such as VSD and ASD, and 3) larger than normal multimers seen in newborns, post DDAVP infusion, recurrent TTP during remission and in the vWD Vicenza variant. Platelet contamination of the plasma will show an artificial loss of high MW multimers.
SECTION V. TESTS TO EVALUATE HYPERCOAGULABLE STATES

A. Activated Protein C (APC) Resistance Assay

This Test is Used to Screen Patients For The Presence of Factor V Leiden

Indications (Activated Protein C (APC) Resistance Assay):

1. Diagnosis of the hypercoagulable state associated with resistance to activated Protein C caused by the Factor V Leiden gene mutation.

Test Principle (Activated Protein C (APC) Resistance Assay):

Resistance to activated Protein C (APC) is the most common inherited risk factor for thrombosis, occurring in 5% of Caucasian European Americans, 20% of unselected patients with venous thromboembolism and 40% of selected VTE patients with early onset VTE (before age 50 years), a positive family history of VTE, recurrent VTE or idiopathic VTE. It is often associated with other inherited risk factors for thrombosis. The APC Resistance assay is performed prior to genetic testing as it is highly sensitive and specific for Factor V Leiden and much less expensive.

The addition of activated Protein C to normal plasma results in the prolongation of the PTT clotting time, due to the inactivation of Factor Va and Factor VIIIa. The APC resistance phenotype is in more than 90% of the cases, due to a mutation in the Factor V gene, resulting in a replacement of arginine with glutamine in the Factor V protein. The selectivity of the assay for Factor V Leiden or other mutations in the Factor V gene rendering the protein resistant to inactivation by APC is increased by normalizing the concentrations of other plasma proteins involved in formation and regulation of thrombin. Hence, by performing the PTT based APC resistance assay in the presence of an excess of Factor V deficient plasma, the sensitivity and specificity for the Factor V Leiden mutation is significantly increased. Further, this modification allows for the analysis of plasma from patients who are on oral anticoagulant (OAC) therapy.

Possible results and interpretation (Activated Protein C (APC) Resistance Assay):

A value less than 2.2 indicates there is a high likelihood of true APC resistance and DNA based testing for Factor V Leiden, should be performed.

Factors affecting test results (false positives and negatives) (Activated Protein C (APC) Resistance Assay):

No significant differences are noted between fresh and frozen samples. The sensitivity and specificity of the assay for activated protein C resistance is unaffected by plasma samples obtained from patients on warfarin. The prescribed assay procedure allows for the analysis of plasma from heparinized patients at heparin levels < 1U/mL plasma (un-fractionated and low molecular weight heparins). Although the 1:4 pre-dilution strongly decreased interference, the assay may give misleading results in patients with high titer inhibitors (lupus anticoagulants). In such cases, increasing the dilution (e.g. 1:8) may correct the test result. As for any PTT-based assay, care should be taken to avoid contact activation of samples, since this may lead to activation of Factor VIII and Factor V. Elevated Factor VIII levels do not interfere with the B. Factor V Leiden

This is a DNA-Based Assay for Factor V Leiden

Factor V Leiden is a mutation that may be associated with as many as 40% of unexplained venous thromboses in selected patient populations from thrombosis clinics. By way of comparison, deficiencies of Antithrombin III, Protein S and Protein C each account for no more than 5% of patients with a hypercoagulable state. The mutation is
located at one of the sites in Factor V where it is cleaved and inactivated by activated Protein C (APC). Hence, the presence of this mutation is responsible for the vast majority of cases of activated Protein C resistance.

**Indications (Factor V Leiden):**

1. To confirm the presence of Factor V Leiden in patients with an abnormal APC Resistance Assay.

**Test Principle (Factor V Leiden):**

Although the presence of the factor V Leiden mutation may be identified by restriction endonuclease digestion of PCR amplified genomic DNA, we use the Invader™ Assay Fluorescence Resonance Energy Transfer (FRET) detection format (Third Wave Technologies, Inc.). Labeled wild type and mutant oligonucleotide probes and invader wild type and mutant probes are added to paired samples of patient DNA in thin walled PCR tubes. The cleavase enzyme recognizes this complex and release the 5’ flap from the WT probe. This flap then binds to the Fluorescence Resonance Energy Transfer (FRET) probe included in the reaction mixture. This complex allows the cleavage and release of a fluorescein tag, which can be detected by a fluorescence plate reader. If the mutant factor V sequence is present instead of the wild type sequence then only the mutant probes form a complex and only this sample generates a fluorescence signal. Likewise if both mutant and wild type sequences are present (the heterozygous states), both probes generate a signal. Unlike PCR, which depends on target amplification, the FRET detection format results in signal amplification that occurs if the target DNA is present in the sample. An excess of the probe and invader oligonucleotide (relative to the target DNA) in the first reaction and an excess of the FRET detection probe (relative to the flap released from the cleavage event in the first step) in the second step ensure massive signal amplification. Wild type and mutant controls are included on each run to assist in signal interpretation.

**Possible results and interpretation (Factor V Leiden):**

Patients can be negative for the Factor V Leiden mutation, heterozygous or homozygous for the mutation. The presence of Factor V Leiden is a potent risk factor for venous thrombosis, with heterozygotes and homozygotes having a 5-8-fold and 50-fold, respectively, increased risk of thrombosis. There is a substantial variation in the Factor V Leiden gene frequency according to the ethnic background of the individual.

**Factors affecting test results (false positives and negatives) (Factor V Leiden):**

The assay only detects the presence of the mutation in the DNA; it says nothing about whether or not the mutant gene is expressed.

C. Factor II G20210A Gene Mutation

*This is A DNA-Based Assay For The Prothrombin Gene G20210A Mutation*

Factor II G20210A is a mutation in the 3’-untranslated region of the prothrombin gene that is associated with a 2-5 fold increased risk of venous thromboses. There is also an association between this mutation and an increased risk of cerebrovascular disease among young individuals. Recent evidence suggests that this point mutation improves the efficiency of RNA processing resulting in elevated prothrombin levels, the presumed cause of the hypercoagulable state.
Indications (Factor II G20210A Gene Mutation):

1. To test for the relatively common hypercoagulable state due to the prothrombin mutation, Factor II G20210A.

Test Principle (Factor II G20210A Gene Mutation):

We use the Invader™ Assay Fluorescence Resonance Energy Transfer (FRET) detection format (Third Wave Technologies, Inc.) to identify the prothrombin G20210A mutation. The details of the method are given above in the description of the Factor V Leiden DNA assay.

Possible results and interpretation (Factor II G20210A Gene Mutation):

Calculation of WT to mutant ratios allows patient to be identified as being either wild type, heterozygous or homozygous for the prothrombin G20210A mutation. The presence of the Factor II 20210 mutation increases the risk of thrombosis 2-3 fold for both sexes and all ages. The mutation is found in 18% of patients with thrombotic family history. The risk of thrombosis increases 20-25 times normal when this mutation occurs in conjunction with Factor V mutation. Due to the genetic nature of the disorder, family counseling may be appropriate. Like Factor V Leiden, there is likely to be substantial variation in the Factor II 20210 gene frequency, according to the ethnic background of the individual.

Factors affecting test results (false positives and negatives) (Factor II G20210A Gene Mutation):

The same potential variables mentioned in the description of Factor V Leiden DNA assay can also influence results for the prothrombin G20210A gene mutation DNA assay.

D. Homocysteine

This Test Measures The Concentration Of Homocysteine in A Patient Sample

Indications (Homocysteine):

1. Diagnosis of hyperhomocysteinemia

Test Principle (Homocysteine):

The most commonly used method for the measurement of homocysteine is high performance liquid chromatography (HPLC) with fluorescence detection. The essential steps are as follows: 1) Conversion of homocysteine disulfides to free homocysteine with borohydride reduction; 2) conjugation of homocysteine with monobromobimane and 3) Separation of homocysteine-bimane from other plasma thiol-bimane adducts by reverse-phase HPLC; and IV) Detection and quantification of homocysteine-bimane by fluorometry.

Possible results and interpretation (Homocysteine):

Homocysteine is a thiol-containing amino acid produced during the metabolism of methionine that can be remethylated back to methionine by methionine synthase (cobalamin and folate are cofactors) or converted to cysteine by cystathionine-beta-synthase (pyridoxine is a cofactor), in a trans-sulfuration step. Homocysteine has numerous effects on various components of hemostasis, but the mechanism by which it promotes thrombosis is not completely clear. Hyperhomocysteinemia has been associated with a 2-3 fold increased risk of venous and arterial thrombosis. Markedly elevated levels of homocysteine (can be several hundred micromol/L or more) can be seen in patients with the autosomal recessive metabolic disorder, homocystinuria. Cause of hyperhomocysteinemia may occur as a result of inherited disorders that alter the enzyme activity in the remethylation and transulfuration pathways or by nutritional deficiencies of cobalamin (vitamin B12), folate or pyridoxine (vitamin B6). Plasma homocysteine is increased in patients with renal failure.
Factors affecting test results (false positives and negatives) (Homocysteine):

The assay is sensitive and specific with no interfering reagent peaks. However, differences in sample handling can cause significantly different results in homocysteine measurement. WHOLE BLOOD SAMPLES NEED TO BE KEPT ON ICE AFTER COLLECTION. Serum or plasma is separated from blood cells as soon as possible. The homocysteine in plasma/serum samples is stable for at least several days in room temperature, for several weeks at 4°C and for years at -20 °C. This test is not affected by anticoagulant therapy.

Antiphospholipid Antibody Syndrome Testing

E. Activated Partial Thromboplastin Time (Described above in the Screening test section)

F. Dilute Russell Viper Venom Time (dRVVT)

The dRVVT Measures The Direct Activation of Factor X And is Useful In The Diagnosis of A Lupus Anticoagulant (Antiphospholipid Antibodies)

Antiphospholipid antibodies such as the lupus anticoagulant are autoantibodies that are directed against negatively charged phospholipids (cardiolipin, phosphatidylserine, etc. or phospholipid binding proteins such as β2-glycoprotein I, annexin V or prothrombin. Although the presence of these antibodies can prolong phospholipid-based coagulation tests in vitro, these antibodies are associated with an increased risk of thrombosis and fetal loss in vivo. Clinical conditions associated with these antibodies include infections (syphilis, HIV infection), exposure to certain drugs (phenothiazines), neoplasms, and autoimmune diseases (SLE, primary antiphospholipid antibody syndrome). These antibodies can be detected using ELISA assays against phospholipids or phospholipid binding proteins (anti-cardiolipin antibodies, anti-beta-2-glycoprotein 1 ELISA) or phospholipid-dependent coagulation assays (PTT-using a low phospholipid reagent dRVVT, kaolin clotting time, dilute Prothrombin Time).

Indications (dRVVT):

1. Diagnosis of Antiphospholipid antibody syndrome

2. Distinguishing between factor VII and factor X deficiency (with an isolated prolonged PT).

Test Principle (dRVVT):

The Russell viper (species Vipera russelli) is a common Asian pit viper whose venom can act as a partial thromboplastin that directly activates factor X to factor Xa. An extract of this venom is added to the patient’s plasma with a platelet substitute and with calcium and the time to clotting is measured. This direct activation of factor X bypasses the Intrinsic and Extrinsic pathways in the coagulation cascade thereby excluding interferences from deficiencies of Factors VII, VIII, IX, XI and XII and their respective inhibitors. A modified test – the dilute RVVT – has been developed as a sensitive screening test for the presence of a lupus anticoagulant. By using a more dilute amount of phospholipid than in the RVVT, the dRVVT significantly increases the sensitivity of the test for the presence of LA. The dRVVT is also useful for diagnosis of LA in patient samples that have a normal PTT, as the dilution and type of phospholipid contained in the dRVVT reagent makes it more sensitive that the PTT for LA.

Possible results and interpretation (dRVVT):

If the test plasma gives a dRVVT that is within the established normal reference range, the test is negative for LA. An abnormally prolonged dRVVT may be caused by a Lupus Anticoagulant, a deficiency of factors X, V, II or fibrinogen, liver disease, vitamin K deficiency, or during warfarin therapy which is the most common reason for an abnormal dRVVT in our hospital. If the dRVVT is prolonged, the dRVVT Confirm test and mixing studies with normal plasma are performed to determine if the prolongation of dRVVT is due to a LA or another cause.
Factors affecting test results (false positives and negatives) (dRVVT):

The dRVVT contains agents that neutralize heparin up to and including 1.0 unit/mL. Plasmas containing heparin levels greater than 1.0 Unit/mL may give elevated results using the dRVVT and should not be evaluated with this test. If the dRVVT is performed on suspected LA plasmas that contain inhibitors to factors II, V or X, the reagent may not identify the presence of a LA inhibitor, due to the prolongation of the dRVVT due to these factor inhibitors.

G. dRVVT Confirm Assay

This Test Confirms the Presence of A Lupus Anticoagulant

Indications (dRVVT Confirm Assay):

1. Evaluation of an abnormal dRVVT.

Test Principle (dRVVT Confirm Assay):

The dRVVT Confirm is specifically designed for use in conjunction with the dRVVT. It compares the results of a dRVVT performed with or without concentrated phospholipids. Test plasma with a prolonged dRVVT result (e.g., greater than 2 S.D. of the established normal reference mean) is retested using the dRVVT Confirm. The dRVVT Confirm reagent is supplemented with excess phospholipids that reverse (correct) the inhibition of anticoagulation produced by antiphospholipid antibodies that have LA properties. The results of the two tests are interpreted by dividing the clotting time of the dRVVT (in seconds by the clotting time result of the dRVVT Confirm (in seconds). This equation generates a ratio result that corresponds to the magnitude of correction of the dRVVT with the addition of concentrated phospholipids.

Possible results and interpretation (dRVVT Confirm Assay):

The results are interpreted by the following ratio:  

\[
\frac{\text{dRVVT (seconds)}}{\text{dRVVT Confirm (seconds)}}
\]

If the ratio result is greater than the established normal reference ratio range (>1.4), then the test is positive for a LA. If the ratio results are within the established laboratory normal reference ratio range (≤1.4), then the test is negative for a LA. The dRVVT and the dRVVT Confirm may be prolonged in patients with deficiencies of Factors II, V, or X or fibrinogen. However, the final ratio result will be normal, unless a LA is present. If a LA is present, the dRVVT Confirm will shorten significantly with the addition of phospholipids, producing an abnormal dRVVT/dRVVT Confirm ratio. Coumadin and other Vitamin K antagonists may prolong both the dRVVT and the dRVVT Confirm time; however the ratio result will be normal if no LA is present.

The present of a factor deficiency or warfarin can also be identified by reviewing the results of mixing studies of the dRVVT performed with normal plasma. In patients with a factor deficiency or on warfarin therapy, the dRVVT will correct with a 1:1 mix and 4:1 mix with normal plasma (patient:normal plasma). In contrast, if a LA is present, the 1:1 mix and 4:1 mix with normal plasma will not correct.

Factors affecting test results (false positive and negatives) (dRVVT Confirm Assay):

The dRVVT Confirm contains agents that neutralize heparin up to, and including a 1.0 Unit/mL. Plasmas containing heparin levels greater than 1.0 Unit/mL may give elevated results using dRVVT and should not be evaluated with this test. If the dRVVT Confirm is performed on suspected LA plasmas that may contain inhibitors to Factors II, V or X, the reagent may not identify the presence of an LA inhibitor, due to the prolongation of the dRVVT Confirm by these factors inhibitors.
I. Quantitative IgG, IgA, and IgM Anticardiolipin Antibody ELISA

This Assay Measures The Amount of IgG, IgA and IgM Antibodies in A Sample

Acquired antibodies directed against anionic phospholipids or phospholipid-binding proteins are associated with autoimmune disorders (systemic lupus erythematosus, rheumatoid arthritis), infections (syphilis, HIV infection), neoplasms, drugs, (phenothiazines, procainamide) and a primary disorder, the antiphospholipid antibody syndrome. Antiphospholipid antibodies have been associated with an increased risk of venous and arterial thrombosis and fetal loss. The presence of these antibodies can be confirmed by coagulation tests (such as the PTT and the dRVVT) and by ELISA assays for anticardiolipin antibodies and beta-2-glycoprotein.

Indications Quantitative IgG, IgA, and IgM Anticardiolipin Antibody ELISA:

1. Diagnosis of the antiphospholipid antibody syndrome

Test Principle Quantitative IgG, IgA, and IgM Anticardiolipin Antibody ELISA:

We use the QUANTA Lite™ IgG, IgA, and IgM Anticardiolipin ELISA method (INOVA Diagnostics, Inc., San Diego, CA). Serum samples are incubated in microtiter plate wells coated with anticardiolipin. After washing away non-specifically bound protein, an anti-human IgG, IgA, or IgM secondary antibody labeled with alkaline phosphatase (AP) is added. After a second wash step to remove excess antibody, an AP substrate is added which generates a colored product in the presence of AP. The amount of colored reaction product formed is measured photometrically at 405 nM and directly corresponds to the amount of antibody present in the patient serum sample.

Possible results and interpretation Quantitative IgG, IgA, and IgM Anticardiolipin Antibody ELISA:

Samples with antibody levels less than 10 GPL, 10 MPL and 20 APL are considered negative. Samples with antibody levels 10-19 GPL, MPL, or APL units are considered to be low positive results. Samples with antibody levels 20-79 GPL, MPL or 20 APL units are considered moderately positive. Samples with antibody levels greater than 80 GPL, MPL or APL units are considered to be strongly positive. Although the correlation between anticardiolipin antibody results and clinical events has varied from study to study, low positive results have generally not been associated with an increased risk of clinical events. Moderately increased antibody levels and particularly high antibody levels have been associated with clinical symptoms. Clinical studies indicate that elevated IgG antibodies levels are most consistently associated with an increased risk of thrombosis while elevated IgM levels have been less consistently associated with clinical sequelae. IgA antibodies do not appear to be associated with an increased risk of thromboembolism in most studies.

The results of the quantitative ELISA for IgG, IgA, and IgM need to be interpreted in light of other clinical information. While anticardiolipin antibodies have been associated with a variety of different illnesses, their presence must be integrated with clinical information for optimal clinical decision-making. A complete evaluation for antiphospholipid antibody syndrome usually includes an PTT, mixing studies (if the PTT is prolonged), a dRVVT performed with and without purified phospholipids and an anti-beta glycoprotein I antibody ELISA. The diagnosis of antiphospholipid antibody syndrome requires clinical and laboratory evidence of the disease. Patients with positive test results who are asymptomatic do not fulfill criteria for the antiphospholipid antibody syndrome and thus, should not be treated. Since transient anticardiolipin antibodies can be seen in conjunction with many viral infections, patients should have positive test results confirmed by repeat testing. Syphilis and HIV infection are associated with antiphospholipid antibodies; therefore, patients with positive tests should be evaluated for these infections.

Factors affecting test results (false positives and negatives) Quantitative IgG, IgA, and IgM Anticardiolipin Antibody ELISA:

Rheumatoid factor may interfere with measurement of IgM anticardiolipin antibodies.
J. Quantitative β2-Glycoprotein 1 ELISA

This Test Measures The Amount of β2-Glycoprotein 1 Antibody in The Serum

In the early 1990s, several investigators demonstrated that β2-Glycoprotein 1, a phospholipid-binding protein, was the antigenic target of antiphospholipid antibodies in patients with autoimmune disorders. β2-Glycoprotein 1 may function physiologically as an antithrombotic agent providing one potential explanation why patients with these antibodies are at increased risk of developing thromboses.

Indications Quantitative β2-Glycoprotein 1 ELISA:

1. Diagnoses of the antiphospholipid antibody syndrome.

Test Principle Quantitative β2-Glycoprotein 1 ELISA:

We use the QUANTA Lite™ β2-Glycoprotein 1 (β2-GPI) ELISA (INOVA Diagnostics, Inc., San Diego, CA). Patient serum is added to microtiter plate wells precoated with β2-GPI. A secondary antibody against human immunoglobulin labeled with horseradish peroxidase (HRP) is added next. Washes before and after the addition of the secondary antibody eliminates excess unbound protein and antibody. The amount of β2-GPI antibody in the patient sample is determined by the amount of colored product generated after the addition of the peroxidase substrate.

Possible results and interpretation Quantitative β2-Glycoprotein 1 ELISA:

Samples with values greater than 20 Standard β2-GPI IgG Units (SGU) are considered positive. The clinical significance of positive test results must be interpreted in conjunction with the patient’s clinical findings. Treatment should not be based upon test results alone. Patients with a positive β2-GPI ELISA may not always have positive antiphospholipid ELISA test results and vice versa. The presence of β2-GPI IgG antibodies is felt to be a more potent risk factor for thrombosis with IgM antibodies and many studies have not been able to demonstrate clinical significance for IgAβ2-GPI IgG antibodies. A complete evaluation for the antiphospholipid antibody syndrome usually includes an PTT, mixing studies (if the PTT is prolonged), a dRVVT performed with and without purified phospholipids and mixing studies and antiphospholipid antibody testing.

Factors affecting test results (false positives and negatives) Quantitative β2-Glycoprotein 1 ELISA:

Rheumatoid factor may interfere with measurement of IgM β2-Glycoprotein 1 antibodies.

K. Protein S Activity

PROTEIN S — Protein S (PS), a vitamin K-dependent glycoprotein, is a cofactor of the protein C system. It is synthesized by both hepatocytes and megakaryocytes and circulates in two forms: 40 to 50 percent as the free form, and the remainder bound to the complement component, C4b-binding protein (C4b-BP); only the free form has activated protein C cofactor activity In the presence of PS, activated protein C inactivates factor Va and factor VIIIa, resulting in reduced thrombin generation. PS also serves as a cofactor for protein C enhancement of fibrinolysis, and can directly inhibit prothrombin activation via interactions with other coagulation factors.

Three phenotypes of PS deficiency have been defined on the basis of total PS concentrations, free PS concentrations, and activated protein C cofactor activity.

Type I — The classic type of protein S deficiency is associated with approximately 50 percent of the normal total S antigen level, and more marked reductions in free protein S antigen and protein S functional activity (ie, a quantitative defect)
Type II — Type II protein S deficiency is characterized by normal total and free protein S levels, but diminished protein S functional activity (ie, a qualitative defect).

Type III — Type III deficiency is characterized by total protein S antigen measurements in the normal range and selectively reduced levels of free protein S and protein S functional activity to less than approximately 40 percent of normal.

DIAGNOSIS — Protein S deficiency is the most difficult of the hereditary thrombophilias to document with certainty. Free protein S antigen is probably the better than the functional protein S activity assay, which has a larger coefficient of variation and occasional false positives when the factor V Leiden mutation is present.

Total protein S — Total protein S antigen is measured by various types of immunoassay techniques. These tests generally involve dilution of plasma samples, which favors dissociation of the protein S-C4b-binding protein complexes.

Free protein S — Original free protein S assays used polyethylene glycol precipitation to remove protein S-C4b-binding protein complexes from plasma; free protein S was then measured by immunoassay of the supernatant fractions. It is now possible to measure free protein S specifically using monoclonal antibody-based assays and ligand sorbent assays, which eliminate the PEG precipitation step.

Functional assays — Functional assay methods are based upon the ability of protein S to serve as a cofactor for the anticoagulant effect of activated protein C. However, some of these assays are not specific for protein S since they are also sensitive to the defect characterized by resistance to activated protein C. As a result, their use can lead to an erroneous diagnosis of functional protein S deficiency in a patient with other causes of activated protein C resistance. A strategy of first screening plasma samples for APC resistance prior to performance of functional protein S assays can obviate this potential problem.

Plasma concentration — The average plasma concentration of total protein S antigen in normal adults is 23 microg/mL. Levels increase with advancing age and are significantly lower and more variable in females than males. The increase with age is seen with total protein S but not free protein S; the latter finding is explained by an association between beta-chain containing C4b-binding protein antigen levels and age.

In one series of 150 patients, plasma total protein S antigen concentration was associated with serum total cholesterol, rising 10 percent as total cholesterol increased from the 5th to the 95th percentile. A similar rise in triglycerides was associated with an even larger increase in mean free protein S antigen.

The range in protein S levels in the normal population is wider than that for protein C or antithrombin. Additionally, functional protein S assays have a higher coefficient of variation than antigenic assays. In practice, it is therefore necessary to perform repeat testing and perform family studies to firmly establish the diagnosis of hereditary protein S deficiency.

In general, levels of total or free protein S antigen <60 to 65 IU/dL are considered to be in the deficient range. However, in a prospective family cohort study involving 1143 relatives with various thrombophilic defects, only young relatives with free protein S levels less than the 5th percentile (<41 IU/dL) or less than the 2.5th percentile (<33 IU/dL) were at higher risk of a first venous thrombosis compared with those in the upper quartile (>91 IU/dL).

Timing of screening and effect of warfarin — An important consideration in the laboratory evaluation of patients with a suspected deficiency of antithrombin, protein C, or protein S is the timing of testing. Erroneous diagnoses can
be made due to the influence of acute thrombosis, comorbid illness, or anticoagulant therapy on the concentrations of these plasma proteins. Interpretation of protein S measurements is particularly difficult in individuals treated with oral anticoagulants, which substantially lower both antigenic and functional levels of the protein. It has been proposed that a reduction in the ratio of protein S antigen to prothrombin antigen can be used to infer a diagnosis of the classic type of protein S deficiency state in this setting.

In practice, it is preferable to investigate patients suspected of having protein S (or protein C) deficiency after oral anticoagulation has been discontinued for at least two weeks and to perform family studies. If it is not possible to discontinue warfarin due to the severity of the thrombotic diathesis, such individuals can be studied while receiving heparin therapy, which does not alter plasma protein S concentrations.

If, however, plasma levels of protein S are obtained at presentation and are well within the normal range, then a deficiency of this protein is essentially excluded. A low concentration, on the other hand, must be confirmed by repeat testing after anticoagulation is discontinued.

Newborns — Total protein S antigen values in healthy newborns at term are 15 to 30 percent of normal while C4b-binding protein is markedly reduced to less than 20 percent. Thus, the free form of the protein predominates in this setting and functional levels are only slightly reduced as compared with those in normal adults. As methodologies for measurement of protein S differ among laboratories and the concentration is substantially lower in normal newborns and young infants compared with adult values, it is important to use age-based norms for the specific laboratory performing the test.

Protein S Activity:

1. Diagnosis of Protein S Deficiency

Test Principle (Protein S Activity):

The Protein S activity assay measures the ability of Protein S to function as a cofactor, for Protein C. Dilutions of normal plasma are mixed with Protein S depleted plasma. A reagent containing Factor Xa, activated Protein C, and phospholipid is then added to activate the mixed plasma. After a five-minute activation time, clot formation is initiated by the addition of calcium chloride. Under these conditions, the prolongation of the clotting time is directly proportional to the concentration of Protein S in the patient plasma. The use of Factor Xa as the activator minimizes the potential interference by high levels of Factor VIII that can be found in some patients. The value for Protein S of a patient sample is determined by comparing the clotting time of the patient sample with the time obtained for dilutions of pooled plasma used to construct a standard curve.

Possible results and interpretation (Protein S Activity):

Protein S levels of 55% to 65% are consistent with either a deficiency state or the lower end of the normal distribution. Protein S deficiency may occur in hereditary deficiency states, liver disease, vitamin K deficiency, therapy with warfarin, L-asparaginase, or during an acute thrombotic event. In addition, Protein S activity and free Protein S antigen are reduced in inflammatory disease or during estrogen therapy or pregnancy where the levels of C4b binding protein are elevated. Protein S bound in C4b binding protein cannot complex with protein C and thus cannot function as a cofactor. Reductions of free functional protein S associated with elevated levels of C4b binding protein contribute to the thrombophilic state associated with pregnancy, estrogen therapy and inflammation. Hereditary Protein S deficiency is a heterozygous disorder that results in half-normal plasma levels of protein S. It is associated with a 10-fold increased risk of venous and perhaps arterial thrombotic events and may occur in as many as 0.1% of the general population. Family studies may be diagnostically useful.
Since the results of protein S activity assays can be influenced by multiple pre-analytical variables, some laboratories have begun to use total and free protein S antigen testing preferentially for diagnosis of protein S deficiency.

**Factors affecting test results (false positives and negatives) (Protein S Activity):**

The presence of heparin greater than 1.2 USP unit/ml or Lupus-type anticoagulants may interfere with the assay results by prolonging the clotting time and giving an artificially high Protein S value. A decrease in Protein S activity does not necessarily indicate a decrease in plasma concentration, since it is nonfunctional when bound to C4b binding protein. A decreased Protein S activity should generally be evaluated further with a Protein S antigen study.

**L. Protein S Antigen**

*This Test Measures The Concentration of Protein S Protein in a Patient Sample*

**Indications (Protein S Antigen):**

1. Diagnosis of Protein S Deficiency

**Test Principle (Protein S Antigen):**

The internal wall of a plastic micro-titer plate well is pre-coated with a monoclonal antibody to Protein S. A second Protein S monoclonal antibody that is coupled to peroxidase is added to the pre-coated microtiter well at the same time as the plasma sample. The total Protein S antigen in the plasma sample is simultaneously captured by the first monoclonal antibody that is anchored to the micro-titer plate well and by the second monoclonal antibody-peroxidase conjugate forming a “sandwich” in a one-step reaction. After rinsing away excess secondary antibody, the quantity of the bound peroxidase tag is determined by its activity during a specified period on the substrate ortho-phenylenediamine in the presence of hydrogen peroxide. The reaction is terminated by the addition of dilute sulfuric acid. The intensity of the colored reaction product bears a direct relationship with the total amount of Protein S concentration in the plasma sample.

**Possible results and interpretation (Protein S Antigen):**

Low Protein S (PS) antigen indicates a reduction in the total Protein S in the plasma sample. To diagnose a hereditary deficiency; family studies can be useful. Acquired PS deficiency is seen in liver disease, with warfarin treatment, DIC, and acute thrombosis. Reductions in free protein S, which can result in reduced protein S activity in the presence of normal levels of total PS antigen, can occur in conjunction with inflammation, pregnancy and estrogen use. These conditions increase levels of the acute phase reactant, C4b binding protein causing an increase in bound “inactive” PS and an acquired reduction in free “active” Protein S. Newborns have low levels of C4b BP so that more of their Protein S is in the free form resulting in normal levels of protein S activity despite reductions in total Protein S.

**Factors affecting test results (false positives and negatives) (Protein S Antigen):**

C4b binding protein has a very high affinity for free protein S. This test is not influenced by acute phase reactants, rheumatoid factor, protein C, hemoglobin, bilirubin, fibrinogen, unfractionated heparin, or low molecular weight heparin.

**N. Protein C Activity**

*This Test is Used to Measure The Activity of Protein C in A Patient Sample*

Protein C is a vitamin K-dependent plasma protein that regulates hemostasis with both anticoagulant and profibrinolytic effects. Protein C is a serine protease that circulates in plasma in an inactive form until activated by
the endothelial bound thrombin-thrombomodulin complex. Activated protein C complexes with protein S to proteolytically inactivate FVa and FVIIIa, down-regulating activation of the coagulation cascade. Activated protein C plays a minor role in the regulation of the fibrinolytic cascade by inactivating plasminogen activator inhibitor 1 (PAI-1). Congenital heterozygous protein C deficiency leads to a 10-fold increased risk of venous thrombosis. The prevalence of heterozygous protein C deficiency is 0.2%. Nevertheless, protein C deficiency has a low prevalence in thrombosis clinics where it accounts for only 4% of patients. Homozygous deficiency in neonates is associated with very severe thrombotic disorder known as “purpura fulminans.” An acquired deficiency may be due to vitamin K deficiency (e.g., as a result of absorption disturbances or oral anticoagulant therapy). In vitamin K deficiency, other vitamin K dependent coagulation factors are also diminished in activity and therefore the risk of thrombosis under these conditions is small. Due to the short half-life of Protein C, the induction of oral anticoagulant therapy (particularly with large loading doses) without concomitant heparin therapy in patients with protein C deficiency may lead to very low levels of Protein C activity and precipitate a prothrombotic disorder known as coumarin skin necrosis which is caused by thrombosis of dermal blood vessels.

**Indications (Protein C Activity):**

The determination of the Protein C activity is indicated in the following cases:

1. For the detection of congenital or acquired Protein C deficiency.

2. In conjunction with other methods (antigenic determination, Protein C coagulometric method) for the differential diagnosis of different Protein C deficiency states.

3. For monitoring replacement therapy with Protein C concentrates in congenital Protein C deficiency

**Test Principle (Protein C Activity):**

We use the Berichrom Protein C method for detection of protein C activity. Berichrom Protein C detects the amidolytically active portion of activated Protein C, including the non-carboxylated molecules synthesized in vitamin K deficiency. Thus, in conditions of vitamin K deficiency, a higher Protein C activity is found with Berichrom Protein C than when using the coagulometric method. To obtain a complete picture of the cause of a Protein C deficiency, it is therefore advisable also to use the coagulometric method and antigenic determination technique. In the Berichrom Protein C method, a specific Protein C enzyme isolated from the venom of the Southern copperhead (*Agkistrodon contortrix contortrix*) is used to activate the protein C in the patient sample. The resulting Protein C activity is determined by its enzymatic activity on a chromogenic substrate that can be measured spectrophotometrically by an increase in absorbance at 405 nm. The assay is based on the following reactions:

**Possible results and interpretation (Protein C Activity):**

Protein C levels of 55% to 70% are consistent with either a deficiency state or the lower end of the normal distribution. Protein C deficiency may occur in hereditary Protein C deficiency states, liver disease, DIC, acute thrombosis and post-operatively. Although vitamin K deficiency and warfarin therapy can cause reductions in protein C activity when measured using coagulometric assays, our current assay is not affected by these conditions. Hereditary Protein C deficiency is a heterozygous disorder that results in half-normal plasma levels of protein C. It is associated with an increased risk of venous thrombosis and may occur in as many as 0.2-0.4% of the general population. Consequently, family studies may be diagnostically useful. Homozygous deficiency in neonates is associated with very severe thrombotic manifestations (“purpura fulminans”).

**Factors affecting test results (false positives and negatives) (Protein C Activity):**

The assay is not affected by heparin when present in a concentration of less than 1U/mL of plasma. Higher levels of heparin may result in over-estimation of Protein C levels. Warfarin therapy can cause decreased Protein C antigen levels. Therefore, if possible, discontinue warfarin at least 2 weeks before testing. Abnormal proteins produced during therapy with vitamin K antagonists result in Protein C activity levels of <5-30% in normal individuals.
O. Protein C Antigen

This Test Measures The Concentration of Protein C in A Patient Sample

**Indications (Protein C Antigen):**

1. To confirm a low Protein C activity or, in conjunction with the Protein C activity, to identify a dysfunctional Protein C molecule.

**Test Principle (Protein C Antigen):**

A plastic support coated with specific rabbit anti-human Protein C antibodies captures the Protein C to be measured. Next, rabbit anti-Protein C antibody coupled with peroxidase binds to the remaining free antigenic determinants of Protein C, forming a “sandwich.” The bound enzyme peroxidase is then measured by its activity during a predetermined time on the substrate ortho-phenylenediamine, in the presence of hydrogen peroxide. After stopping the reaction with a sulfuric acid, then intensity of the color produced bears a direct relationship with the Protein C concentration initially present in the plasma sample.

**Possible results and interpretation (Protein C Antigen):**

Decreased Protein C antigen may occur in hereditary Protein C deficiency states, liver disease, DIC, acute thrombosis, vitamin K deficiency, therapy with warfarin, and post-operatively. Hereditary Protein C deficiency has been described in some patients with recurrent venous thrombosis; family studies may be diagnostically useful. Patients with heterozygous deficiencies of Protein C here approximately half normal levels.

**Factors affecting test results (false positives and negatives) (Protein C Antigen):**

In extremely rare instances, anti-rabbit antibodies in certain subjects can lead to aberrant results.

P. Antithrombin (III) Activity

This Test is Used to Measure The Activity of Antithrombin (III) in Patient Samples

Antithrombin III (AT III) is synthesized in the liver and functions as an inhibitor of thrombin. Its activity is dramatically enhanced by heparin. It also inhibits factor Xa and, to a lesser extent factors IXa, Xla, XIIa, plasmin, urokinase kalikrein and trypsin.

**Indications (Antithrombin (III) Activity):**

1. Diagnosis of Antithrombin III (AT III) deficiency.

**Test Principle (Antithrombin (III) Activity):**

Antithrombin (III) has powerful and immediate anti-thrombin activity in the presence of heparin. The test procedure consists of two steps. First, the test plasma is incubated with a known excess of thrombin in the presence of heparin. The residual thrombin is quantified by its amidolytic action on a synthetic chromogenic substrate. Since the quantity of thrombin that is neutralized in the first reaction step is proportional to the AT (III) level present in the plasma being tested, it follows that the residual thrombin in the second reaction step is inversely proportional to the AT (III) level of the tested plasma.

**Possible results and interpretation (Antithrombin (III) Activity):**

Decreased AT III activity may occur in hereditary deficiency states, liver disease, acute thrombosis, protein wasting states, DIC, and in some patients receiving heparin or estrogen. Levels of 60% to 80% are consistent with either a
deficiency state or the lower end of the normal distribution. Hereditary heterozygous ATIII deficiency is associated with a 20-fold increased risk of VTE and has an estimated prevalence of 1 in 2-5,000 in the general population. AT (III) deficiency is found in approximately 4% of patients in thrombosis clinics. AT deficiency is inherited in an autosomal dominant fashion and affected patients typically have half-normal levels. Some patients have mutations which result in a quantitative defect that is characterized by equivalently decreased functional and antigen levels. Others produce a defective molecule with reduced function but normal antigen levels. Acquired ATIII deficiency can be seen in liver disease, the post-operative state, nephrotic syndrome (usually with >4 grams of protein/day), DIC, extensive thrombosis, women using oral contraceptives and during heparin or L-asparaginase therapy.

**Factors affecting test results (false positives and negatives) (Antithrombin (III) Activity):**

The test procedure described here is not affected by therapeutic doses of heparin. It is therefore suitable for testing plasmas collected from patients receiving heparin therapy. Icteric plasma (bilirubin > 400µmol/1) will interfere with the assay. Please call the laboratory for special arrangements if this applies to the patient’s sample.

**P. Antithrombin (III) Antigen**

*This Test Measures The Concentration of AT III Protein in A Patient Sample*

**Indications (Antithrombin (III) Antigen):**

1. Diagnosis of AT (III) deficiency. A normal antigen level does not mean that the protein is functional so the interpretation of AT deficiency requires also measuring a AT activity

**Test Principle (Antithrombin (III) Antigen):**

Quantitative determination of Antithrombin III (ATIII) antigen can be by the measurement of light absorbance produced by a suspension of microlatex particles coated with specific anti-ATIII antibodies. When a beam of monochromatic light is allowed to traverse a suspension of microlatex particles to which specific antibodies have been attached by covalent bonding and the wavelength of the light is much greater than the diameter of the latex particles, it can pass through the latex suspension unabsorbed. However, in the presence of the antigen being tested, the antibody-coated latex particles agglutinate to form aggregates of diameters greater than the wavelength of the light and the later is absorbed. There is a direct relationship between the observed absorbance value and the concentration of the antigen being measured.

**Possible results and interpretation (Antithrombin (III) Antigen):**

AT deficiency can be due to quantitative (decreased production of a normally functioning protein) and qualitative defects (production of a dysfunctional protein). In conjunction with AT III activity levels; AT III antigen determination can help determine if AT III deficiency is due to decreased production of synthesis of a dysfunctional protein.

**Factors affecting test results (false positives and negatives) (Antithrombin (III) Antigen):**

Cloudy or lipemic samples may lead to an over-estimation of ATIII levels. Interference by rheumatoid Factor (RF) cannot formally be excluded.

**R. Elevated Factor VIII, IX, and XI Levels and Thrombophilia**

Elevated levels of factors VIII, IX and XI have been noted in case-control studies such as the Leiden Evaluation of Thrombophilia Study to be risk factors for the development and recurrence of venous thromboembolism. Several studies have documented that elevated factor VIII activity levels greater than 2 standard deviations above the population mean are associated with an increased risk of VTE. One large single center cohort study found that levels of factor VIII activity increase the risk of recurrent VTE by 6-fold. Elevated factor VIII levels have been demonstrated to be genetically determined in family studies but the molecular mechanisms responsible remain to be
completely clarified. Since factor VIII is an acute phase reactant, clinical studies have generally measured factor VIII levels at least 6 months after episodes of VTE and in the absence of infectious or inflammatory disorders. These strict requirements and the high prevalence of elevated factor VIII levels in thrombosis clinic populations (~20-25%) have limited the application of factor VIII activity levels to decision-making on duration of anticoagulation. The LETS study also identified elevated factor IX and XI levels as being risk factors for VTE with each being associated with a 2-fold increase in risk. Confirmation of these findings and integration of this information into clinical decision-making on anticoagulation has yet to be performed.

**Indications (Elevated Factor VIII, IX, and XI Levels and Thrombophilia):**

1. Diagnosis of thrombophilia.

**Test Principle (Elevated Factor VIII, IX, and XI Levels and Thrombophilia):**

Factor VIII, IX and XI activity levels are performed as outlined above in the sections on coagulation factor assays.

**Possible results and interpretation (Elevated Factor VIII, IX, and XI Levels and Thrombophilia):**

Factor VIII, IX and XI activity levels above the 95th percentile are elevated and considered to represent an independent risk factor for VTE. Since factor VIII, IX and XI activity levels can be influenced by heparin, direct thrombin inhibitor, or thrombolytic therapy samples should not be drawn in patients receiving these medications. Factor IX levels are reduced by treatment with vitamin K antagonists such as warfarin and therefore accurate levels cannot be measured in patients receiving these medications. Factor VIII is an acute phase reactant so it should not be measured in the setting of acute thrombosis, inflammation or infection.

**Factors affecting test results (false positives and negatives) (Elevated Factor VIII, IX, and XI Levels and Thrombophilia):**

Variations in instruments or reagents used to perform assays and dilution errors can cause variations in the obtained factor activity results. The presence of the Lupus Anticoagulant has been associated with falsely decreased results. This effect of a LA can often be identified and overcome by observing test results for a plateau effect during serial dilutions of the plasma sample to be assayed. Factor XI can be activated by frozen storage causing a falsely increased assayed activity level.

**S. Dysfibrinogenemia**

Dysfibrinogenemia is characterized by the synthesis of an abnormal fibrinogen molecule that can lead to a predisposition to abnormal bleeding, thrombosis or both. Congenital Dysfibrinogenemia is a rare cause of thrombophilia that accounts for less than 1% of patients seen in thrombosis clinics. Dysfibrinogenemia should always be considered in any patient who appears to have a predisposition to form venous and/or arterial thromboses. Laboratory testing for dysfibrinogenemia should include a thrombin time, fibrinogen, fibrinogen antigen level and a Reptilase time. Samples should not be collected during therapy with heparin, direct thrombin inhibitors or thrombolytic agents. Refer to the sections covering these tests above for more information.

**VI Tests for Evaluating The Fibrinolytic System:**

**A. Plasminogen Activity**

*This Test is Used to Measure the Activity of Plasminogen, the Fibrinolytic Enzyme, in Plasma Samples*

Plasminogen is an important regulatory protein in the fibrinolytic system. Plasminogen is converted to plasmin, a serine protease that digests fibrin.

**Indications (Plasminogen Activity):**
This assay may be useful in the evaluation of unusual hemorrhagic or thrombotic disorders. During thrombolytic therapy, it may be of interest to measure the circulating plasminogen level in order to monitor hepatic regeneration.

**Test Principle (Plasminogen Activity):**

The procedure comprises two steps 1) an excess of streptokinase is added to the test sample whose plasminogen is to be determined. The streptokinase and the plasminogen form a complex that has plasmin-like activity and 2) the quantity of streptokinase-plasminogen formed is assessed by its action on a synthetic substrate. Quantification is achieved by the amount of p-nitroaniline released, measured at 405 nm. Under these conditions, the reaction is insensitive to plasmin inhibitors.

**Possible results and interpretation (Plasminogen Activity):**

A low plasminogen activity may be seen in liver disease. DIC, primary fibrinolysis, after cardiopulmonary bypass, or during therapy with streptokinase, urokinase, or tissue plasminogen activator. Hereditary plasminogen deficiency may be: 1) Quantitative or type I: these deficiencies are identified by a simultaneous decrease of both functional and antigenic levels of plasminogen. Or more rarely 2) Qualitative or type II; these deficiencies are characterized by a functional level that is significantly lower than the antigenic level. Whether a deficiency of plasminogen is associated with an increased risk for venous thromboembolic disease is controversial. Some feel that plasminogen deficiency in isolation is a risk factor for thromboses. Conversely, others suggest that this deficiency is not by itself thrombogenic, but it constitutes a factor that enhances the clinical risk associated with other hemostatic disorders.

**Factors affecting test results (false positives and negatives) (Plasminogen Activity):**

The plasminogen activity assay is insensitive to plasma inhibitors and to fibrin- or fibrinogen degradation products.

B. Tissue Plasminogen Activator (TPA) Antigen

*This Test Measures The Concentration of Tissue Plasminogen Activator (TPA) in A Sample*

Tissue plasminogen activator (TPA) is an endogenous activator of plasminogen that is released from endothelial cells in response to numerous physiological stimuli including thrombin, epinephrine and venous occlusion.

**Indications (TPA):**

This test may be useful in the evaluation of unusual hemorrhagic or thrombotic disorders.

**Test Principle (TPA):**

The TPA ELISA assay utilizes the double antibody principle. The plasma sample or a standard containing TPA is added to a microtiter plate test well that is coated with goat-anti-TBA IgG and contains soluble non-immune goat IgG. After incubation sufficient to allow > 95% of the TPA to bind to the goat antibodies, HRP-labeled anti-TPA Fab fragments are added. These are allowed to react with bound TPA. The wells are emptied and washed to remove unbound conjugate after peroxidase substrate is added. The amount of yellow color developed is directly proportional to the amount of TPA present in the sample.

**Possible results and interpretation (TPA):**

Abnormally elevated TPA activity may be seen following venous occlusion, exercise, or DDAVP infusion. Levels may increase during hepatic disorders, since the liver eliminates TPA. High plasma levels of TPA have been found in some patients with bleeding tendencies. Decreased plasma levels of functional TPA may be associated with a predisposition to venous thrombosis.

**Factors affecting test results (false positives and negatives) (TPA):**
Traumatic venipuncture or prolonged stasis may invalidate results.

C. Plasminogen Activator Inhibitor-1 (PAI-1 Activity)

PAI-1 is a fast-acting inhibitor of TPA that is released into circulation by endothelial cells. The concentration of PAI-1 is normally much higher than TPA activity levels. An increased plasma level of PAI-1 can lead to impaired fibrinolytic function. However, it is unclear whether elevated PAI-1 is associated with thrombotic disease.

Indications (PAI-1 Activity):

This test may be useful in the evaluation of unusual hemorrhagic or thrombotic disorders.

Test Principle (PAI-1 Activity):

This assay utilizes a monoclonal antibody specific for the active form of PAI-1. Active TPA is lyophilized while immobilized on the surface of the microtiter plate test well. After reconstitution, plasma samples containing PAI-1 are added and active PAI-1 reacts with the bound TPA. Simultaneously, and HRP-conjugated monoclonal antibody to PAI-1 is added. After incubation, unbound sample and conjugated antibody are washed away and the bound PAI-1 quantified with an HRP-sensitive substrate. The amount of color developed is directly proportioned to the concentration of active PAI-1 in the sample.

Possible results and interpretation (PAI-1 Activity):

Elevated PAI-1 can be seen post-thrombosis persisting for months. (Clinical Chemistry 35:1544), pregnancy, post-surgery, and in inherited states. There is no clear association between the elevated PAI and thrombosis (Circ 85:1822, '92; Thromb Haemost 62:673, '89). PAI-1 behaves as an acute phase reactant protein. Rapid increases in PAI-1 occur following major surgery. MI, severe trauma, and sepsis. PAI-1 also gradually increases with age and during pregnancy. Postpartum PAI-1 rapidly returns to pre-pregnancy levels. Diurnal variation has been reported.

A low PAI-1 activity may be seen in liver disease, amyloidosis, or rarely, as a congenital deficiency, and may be associated with clinically apparent bleeding.

Factors affecting test results (false positives and negatives) (PAI-1 Activity):

PAI-1 levels will be undetectable and inaccurate in patients Tissue Plasminogen Activator, Streptokinase, or Urokinase. Extremely hemolyzed or icteric samples may also interfere with the assay.

D. α₂ Antiplasmin (α₂-PLASMIN INHIBITOR)

This Test Measures α₂-Antiplasmin Activity in A Sample

α₂ Antiplasmin (α₂-AP), a fast-acting inhibitor of plasmin, is an important regulator of fibrinolysis. It reacts with plasmin to form a 1:1 stoichiometric complex. α₂-AP can also form complexes with plasminogen and bind to fibrin through the mediation of factor XIIIa.

Indications (α₂-Plasmin Inhibitor):

This assay can be useful in the evaluation of unusual hemorrhagic or thrombotic disorders. Measurement of α₂-antiplasmin levels also may be useful in monitoring fibrinolytic treatment. Elevated α₂-AP levels may be associated with reduced efficacy of thrombolytic agents.

Test Principle (α₂-Plasmin Inhibitor):

The assay is a quantitative determination of α2-Antiplasmin (α2-AP) using a synthetic chromogenic substrate. In the presence of excess plasmin, α2-Antiplasmin (α2-AP) rapidly inhibits plasmin activity. Unbound active plasmin
cleaves a chromogenic substrate containing p-nitroaniline (pNA). The residual quantity of plasmin (as measured by the pNA release) is inversely proportional to the α2-AP level in the sample. The assay is carried out in two steps:

1) α2-AP (sample) + plasmin (excess) -> α2-AP/plasmin (inactive) + plasmin (active)

2) Plasmin + chromogenic substrate -> Peptide + pNA (yellow)

Possible results and interpretation (α2-Plasmin Inhibitor):

A low α-2 anti-plasmin may be seen in DIC and liver disease, during thrombolytic therapy, or rarely, as a congenital deficiency, and may be associated with clinically apparent bleeding. Elevated levels are observed during the post-operative period. Severe bleeding is manifested in a homozygous deficiency of α2-Antiplasmin. Heterozygotes characteristically have half normal levels of the inhibitor.

Factors affecting test results (false positives and negatives) (α2-Plasmin Inhibitor):

Several cases of congenital α2-AP deficiency have been reported in the literature. Homozygous α2-AP deficiency is associated with clinically significant bleeding. Patients with the heterozygous deficiency tend to have less severe bleeding manifestations.

The antiplasmin activity tested by the amidolytic method is closely related to α-2 antiplasmin or fast-acting antiplasmin. However, in some therapeutic cases, a slight interference by α-2-macroglobulin (a slower acting plasmin inhibitor) cannot be precluded (e.g. cases where the α-2 antiplasmin level is greatly decreased or in the presence of a high α-2-macroglobulin level.)

Plasma collected in sample tubes containing either aminocaproic acid, aprotinin or heparin must be precluded, since the concentrations of these substances are at levels that will interfere with the test, whereas therapeutic does have little or no effect.

Lipemic plasma and plasma with increased bilirubin or hemoglobin may interfere with the assay.

A frequent concern in interpreting diagnostic coagulation testing is the effect of anticoagulants on the test value. Directly below is a table that points out the caveats in interpreting the above tests when a patient is on anticoagulants.

SECTION VI. Qualitative Platelet Dysfunction Testing

A. Platelet Count

This measurement is generally performed on an automated particle counter and is very accurate. An estimate of the platelet count can be made from the peripheral blood smear. Although lacking accuracy, the latter is useful in detecting extremely high or low platelet counts and should correlate with the electronic counter. Platelet morphology may also be helpful.

B. Bleeding Time

The bleeding time measures the time it takes to stop bleeding after a standard cut is made in the skin. It is a measurement of the platelet and vascular components of hemostasis.

The bleeding time is normal in coagulation factor deficiencies except for von Willebrand Disease. It is prolonged when the platelet count is less than 75,000/µL or when there is a qualitative platelet abnormality. The long bleeding time in von Willebrand Disease is due to the deficiency of von Willebrand Factor, which is required for normal platelet adhesion. It is not due to an intrinsic platelet abnormality. The bleeding time is the only test that can be
correlated with a susceptibility to bleeding and is often prolonged in patient’s taking aspirin, uremia, severe liver disease, and several inherited hemostatic disorders. However, it is notoriously inaccurate in predicting surgical bleeding.

C. Platelet Aggregation and Secretion

We now use light transmission platelet aggregation, whereas for several years we did impedance aggregation. The change to light transmission aggregation, was based upon our frequent need to do aggregation studies on patients with low platelet counts, standardization of normal range, and technologist preference. As aggregation occurs, ATP is released. This release reaction is measured by chemiluminescence. In general we follow the guidelines published by Institute of Laboratory standardization, of which I was a co-author.

ATP release is measured by a visible light range luminescence technique in platelet rich plasma (PRP and whole blood). The basic principle of the Lumi-aggregometer is to measure secretion by a sensitive luminescent (firefly luciferin-luciferase) assay for extra-cellular ATP, in combination with the simultaneous measurement of aggregation.

Specimens should be collected into sterile evacuated tubes with a non-wetable lining and 1/10 volume of 3.8% buffered sodium citrate. Subjects for whole blood aggregation tests should be resting but need not be fasting. They should be non-smoking. They should avoid taking any prescription or over the counter medications known to affect platelet function for ten days to two weeks prior to the test.

Interpretation of aggregation studies is based upon the above normal controls and appearance of the curves. These results will be assessed in conjunction with patient history and other laboratory findings. Thus the “rule of thumb” for interpreting aggregation and secretion data is as follows:

1) Absent or decreased secretion of ATP to thrombin may be indicative of storage pool deficiency or a secretion defect. Absence of a secondary wave in collagen aggregation and failure to respond to ADP are also present.

2) No aggregation and no secretion to arachidonic acid may confirm impaired thromboxane synthesis (aspirin administration);

3) Aggregation, but no secretion to arachidonic acid may be indicative of storage pool deficiency or a secretion defect.

4) Von Willebrand Disease is characterized by a deficiency of a plasma cofactor (vWF) required for platelet adhesion. The antibiotic Ristocetin induces platelet adhesion in the presence of vWF and GP1b receptor. Thus, failure to aggregate in response to Ristocetin may be indicative of von Willebrand Disease or absence of GP1b receptor (Bernard-Soulier Syndrome). No aggregation in response to Ristocetin may be indicative of von Willebrand Disease. Repeating Ristocetin aggregation with the addition of normal plasma or concentrated vWF will correct the aggregation abnormality in von Willebrand Disease, but not in Bernard-Soulier Syndrome. In some cases, a prolonged lag time may be the only indication of abnormality. Hyper-responsiveness to Ristocetin may be seen in type 2B and platelet von Willebrand Disease and can be confirmed by testing at a final concentration of 0.3mg/mL ristocetin.

5) Glanzmann’s Thrombasthenia, an abnormality of platelet GP11b-111a (the fibrinogen receptor), is characterized by abnormal aggregation and secretion to all agonists with the exception of ristocetin.

D. Platelet function analyzer 100 (PFA-100)™

This Test is An In Vitro Whole Blood Assay Of Platelet Function

Indications (Platelet function analyzer):
1. This is a useful whole blood screening test for disorders of platelet function.

**Test Principle (Platelet function analyzer):**

The PFA-100™ (Dade Behring, Inc., Newark, DE) is a an instrument that stimulates and measures *in vitro* the process of platelet adhesion and aggregation following a vascular injury. The PFA-100™ test system consists of 1.) Test cartridges with a sample reservoir, a capillary and a membrane with a central aperture that is coated with collagen and either ADP or epinephrine through which the citrated whole blood sample is aspirated, and 2.) An analyzer that measures the time required for the membrane aperture to become occluded with a platelet plug (the closure time). After citrated whole blood is deposited into the sample cup, it is aspirated from the sample reservoir through the capillary and the membrane aperture under high shear flow conditions. To simulate the subendothelium in vivo, the membrane in the sample reservoir is coated with collagen. An additional physiological platelet agonist, either ADP or epinephrine is also present on the membrane. As whole blood is aspirated through the membrane aperture, platelets adhere, become activated and secrete their granule constituents. Subsequently, additional platelets adhere forming aggregates that occlude the membrane aperture arresting the flow of blood. This event is sensed by the analyzer and recorded as the closure time (CT) (the time elapsed from the start of the test until aperture occlusion occurs).

**Possible results and interpretation (Platelet function analyzer):**

The CT is an indicator of platelet function in the analyzed whole blood sample. Consequently, the CT is affected by thrombocytopenia as well as congenital or acquired disorders of platelet function including Bernard-Soulier syndrome, Glanzmann’s thrombasthenia, storage pool disorders, von Willebrand disease, uremia and exposure to anti-platelet drugs such as aspirin, nonsteroidal anti-inflammatory agents clopidogrel, ticlopidine, etc. Deviations of the hematocrit from the normal range can also influence the assay. The Collagen/ADP (COL/ADP) Test Cartridge is primarily used to detect platelet dysfunction induced by intrinsic platelet defects, von Willebrand Disease or exposure to platelet inhibiting agents. The Collagen/Epinephrine (COL/EP) Test Cartridge is used to indicate if an abnormal test obtained with the COL/ADP Test Cartridge may have been caused by the effect of the ASA or medications containing ASA.

<table>
<thead>
<tr>
<th></th>
<th>Mean Closure Time (sec)</th>
<th>Reference Range (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen/Epinephrine</td>
<td>132</td>
<td>94-193</td>
</tr>
<tr>
<td>Collagen/ADP</td>
<td>92</td>
<td>71-118</td>
</tr>
</tbody>
</table>

**Factors affecting test results (false positives and negatives) (Platelet function analyzer):**

Microthrombi or particulate matter in the test sample can result in a flow obstruction causing automatic cancellation of the test. Since samples in the Collagen/ADP and Collagen/Epinephrine cartridges are tested sequentially it is conceivable that in specimens with high sedimentation properties some settling may occur in the cartridge that is analyzed last which may influence the closure time. The manufacturer recommends that such samples be tested in each cartridge separately. Hemolysis, by reducing the hematocrit and increasing the ADP content of the patient sample may interfere with accurate testing.

As with any test of platelet function, a thorough medication history is essential to avoid misleading results due to medications that influence platelet function. Likewise, platelet counts less than 150,000/µL and hematocrit values less than 35% may influence closure times. The influence of hematocrit values above 50% or platelet counts greater than 500,000/µL on closure times remain unknown.

Certain fatty acids and lipids found in various human diets are known to inhibit platelet function and physicians may wish to advise patients to refrain from fatty foods prior to testing.

**Section VII Diagnostic Testing for Thrombotic Thrombocytopenic Purpura**
Thrombotic Thrombocytopenic Purpura (TTP) is characterized by the pentad of 1. Microangiopathic hemolytic anemia, 2. Thrombocytopenia, 3. Neurologic dysfunction (e.g., headache, transient ischemic episodes, confusion, seizures, coma), 4. Renal disease and 5. fever. Before 1998, our understanding of the pathophysiology of TTP was limited. In that year, adults with idiopathic TTP were reported to have acquired autoantibodies that inhibited a von Willebrand factor (vWF) cleaving protease, which is normally present in plasma. In 2001, the vWF cleaving protease was purified and cloned by several groups and shown to be a new member of the “a disintegrin and metalloprotease with thrombospondin type 1 repeats” family, and named ADAMTS13. Thanks to these discoveries, we now have a plausible model for the pathogenesis of TTP. Autoantibodies inhibit the activity of ADAMTS13. The absence of ADAMTS13 allows vWF and platelets to accumulate unchecked and form microvascular thrombi, which lead to platelet consumption, microangiopathic hemolysis, and microvascular occlusion. Plasma exchange is efficacious because it removes the pathogenic autoantibodies and replenishes the missing ADAMTS13 protease, restoring the normal regulation of vWF-dependent platelet adhesion.

Subsequent studies have begun to demonstrate the potential and also the limitations of ADAMTS13 testing. Modest decreases in plasma ADAMTS13 activity occur in a variety of acute and chronic illness, but rarely are the values < 25% of normal. ADAMTS13 is synthesized mainly in the liver, and severe ADAMTS13 deficiency (<5%) has occurred with hepatic failure due to various causes. Severe ADAMTS13 deficiency appears to be more frequent in sepsis-induced DIC. In a retrospective study of 109 patients, ADAMTS13 levels were <5% in 17 patients and <20% in 51 patients.

In contrast, studies of patients with thrombotic microangiopathy have confirmed the specificity of severe ADAMTS13 deficiency (undetectable or < 5%) for idiopathic TTP, although the sensitivity remains controversial. Severe ADAMTS13 deficiency rarely occurs in “secondary” TTP associated with cancer, hematopoietic stem cell or solid organ transplantation, preeclampsia, systemic infections, drug toxicity, or other predisposing conditions. In addition, severe ADAMTS13 deficiency is almost unheard of in diarrhea-associated hemolytic uremic syndrome caused by Shiga toxin-producing E coli (D+HUS) or other thrombotic microangiopathy accompanied by oliguric renal failure (“atypical” HUS). The Oklahoma TTP+HUS registry provides representative data: none of 92 patients with these varieties of secondary TTP or D+HUS had severe ADAMTS13 deficiency.

On the other hand, when patients with idiopathic TTP are stratified by plasma ADAMTS13 activity level, the incidence of severe ADAMTS13 deficiency (< 5% of normal pooled plasma) has varied from 33% to 100% with intermediate values of 52% to 94% in other studies. Some of this variation probably reflects differences in case definitions for idiopathic TTP or assay methodology. In any case, some patients diagnosed with idiopathic TTP do not have severe ADAMTS13 deficiency, at least in vitro. Whether the short-term prognosis differs for idiopathic TTP with or without ADAMTS13 deficiency is not yet clear. One study suggests that the initial response to plasma exchange is similar for both groups.

In our laboratory, we measure the functional activity of the cleaving protease using a recombinant von Willebrand protein that has a fluorescent tag that is quenched as long as the protein remains intact. With ADAMTS13 mediated proteolysis, the fluorescent tag is released allowing precise measurement of enzyme activity.
SECTION VIII. HEPARIN INDUCED THROMBOCYTOPENIA

A. Platelet Factor 4 ELISA for Heparin-induced Thrombocytopenia Antibodies (PF-4 ELISA)

This Test Is Used In The Diagnosis of Heparin Induced Thrombocytopenia
Any positive test result is a critical action value at our Hospital.

Heparin Induced Thrombocytopenia (HIT)

Patients not uncommonly develop thrombocytopenia during the administration of heparin. Two different forms of heparin-induced thrombocytopenia (HIT) have been described. Type 1 HIT is characterized by the development of minimal thrombocytopenia (platelet nadir generally no less than 100,000/µL) during the first few days of intravenous heparin administration that spontaneously resolves with continued therapy. It is non-immunologic and due to the platelet aggregating properties of heparin when administered in high intravenous doses. Type II HIT typically develops 4 - 14 days after initiation of heparin therapy (unless the patient has been previously sensitized) and can be associated with the administration of small or large doses of heparin by intravenous or subcutaneous routes. Platelet counts typically fall below 100,000/µL or by 50% from their baseline (median platelet count, 60,000/µL). Patients with this form of HIT often have antibodies to platelet constituents such as platelet factor 4 and are at an extremely high risk for venous and/or arterial thrombosis unless heparin is promptly discontinued and alternative anti-thrombin agents are employed. The platelet factor 4 ELISA assay has proven useful in the diagnostic evaluation of patients with HIT type II.

Indications (Diagnosis of HIT):

The GT1-PF4 ELISA Assay is a qualitative solid phase enzyme linked immunosassay (ELISA), which is used to detect antibodies reactive with platelet Factor 4 (PF4) when it is complexed to polyanionic compounds, such as Polvyvinyl Sulfonate (PVS). These antibodies are found in some patients undergoing heparin therapy and are associated with thrombocytopenia and an increased risk for venous and arterial thrombosis. The antibody class that our assay detects is IGG, and not IGA or IGM. By detecting IGG only, the number of false positives has decreased.

Test Principle (Diagnosis of HIT):

The GT1 platelet factor 4 (PF4) ELISA assay is based upon the knowledge that antibodies associated with HIT Type II are directed against the complex of heparin and platelet factor 4, a constituent of platelet alpha granules, which is released upon platelet activation and secretion. In this assay, PF4 is immobilized upon polyvinyl sulfonate (PVS) in microtiter plate wells. Test serum is added to the wells. After washing away unbound immunoglobulin an alkaline phosphatase linked anti-human immunoglobulin is added to the wells and then incubated. A second wash procedure removes unbound secondary antibody and then substrate PNPP (P-nitrophenyl phosphate) is added. Following 30 minutes of incubation, the reaction is stopped by the addition of sodium hydroxide solution and the optical color intensity is measured by spectophotometry at 405nm. The amount of color generated is proportional to the amount of PF4 antibody present in the sample.

Possible results and Interpretations (Diagnosis of HIT)

Test results showing mean OD values equal to or greater than 0.400 are regarded as positive. This assay is intended to be used as a screening test for HIT. The sensitivity of the PF4 Elisa for the diagnosis of HIT type II is as high as 85%. Therefore, not all patients with HIT type II will have positive tests and if clinical suspicion is high for this entity, the patient should be treated accordingly despite negative assay results. In some cases, repeat testing in several days is useful to confirm cases of HIT when antibody titers are initially too low for detection. Some antibodies that recognize sites on the PF4-heparin complex may not be reactive using this assay. In addition, occasional patients with HIT type II have antibodies directed at other antigens such as IL-8 or neutrophil activating peptide 2 (NAP-2). Conversely, a positive PF4 ELISA is not definitive evidence of HIT type II without the characteristic clinical picture. Surveilliance studies of patients receiving heparin have demonstrated that as many as 61% of cardiac surgery patients develop PF4 heparin antibodies without any clinical evidence of HIT. Therefore PF4 ELISA testing should only be performed for patients when HIT type II is clinically suspected. Positive or
negative results obtained using this assay are to be used in conjunction with clinical findings and other laboratory
data.

**Factors affecting test results (false positives and negatives) (Diagnosis of HIT)**

Erroneous results can occur from bacterial contamination of test materials, inadequate incubation periods, inadequate washing of test wells, or omission of test reagents or steps. The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased non-specific binding and produce false-positives in this assay. GTI-PF4 ELISA Assay is designed to detect antibodies directed against complexes of platelet Factor 4 and Polyvinyl Sulfonate.

**B. 14C-Serotonin Release Assay**

*This Serotonin Release Assay is a Sensitive and Specific Functional Assay for HIT Antibodies*

**Indications 14C-Serotonin Release Assay:**

1. Diagnosis of Heparin-induced Thrombocytopenia

**Test Principle 14C-Serotonin Release Assay:**

The Serotonin Release Assay is a functional assay for the presence of antibodies associated with heparin-induced thrombocytopenia. The SRA has similar sensitivity but much greater specificity than the HIT PF-4 ELISA for the presence of HIT antibodies. Consequently, the Special Coagulation Laboratory sends patient samples that test positive for the presence of HIT antibodies in the HIT PF4 ELISA on a reflex basis for SRA testing. Serum samples suspected to have heparin-dependent antibodies are tested with the 14C-Serotonin release assay. Donor platelets are labeled with 14C-serotonin and allowed to incubate with patient serum, in the absence and presence of low and high concentration of heparin. The amount of 14C released into the supernatant is measured and if >20% at 0.1 u/ml heparin and <20% at 100 u/ml heparin, the sample is reported as positive for heparin antibodies, supporting a diagnosis of heparin-induced thrombocytopenia. The inhibition of serotonin release at a high concentration of heparin is consistent with an immune complex-mediated disorder. A second sample may be required to confirm the absence of heparin-dependent antibodies if the clinical findings indicate heparin-induced thrombocytopenia or if the initial test result is a borderline reaction.

**SECTION IX. Intraoperative Global Hemostasis Testing by Thromboelastography**

*The Thromboelastograph is an In Vitro Test that Can Measure the Speed of Clot Formation, Clot Strength and Clot Lysis.*

*This test is to facilitate the use of blood products in the Operating Rooms and those Patients on ECMO or Ventricular Assist Device*

**Indications thromboelastograph:**

1. Diagnosis of disorders of coagulation, platelet function and fibrinolysis

**Test Principle thromboelastograph:**

The thromboelastograph is one of two clinically available viscoelastic tests that can characterize the speed of blood clot formation, clot strength, clot retraction and clot lysis over time. Thromboelastographs have been used principally as a real time measure during liver transplant and cardiac surgery to guide blood product replacement during these long operations associated with a significant risk of iatrogenic coagulopathy. The Computerized Thrombelastograph® (TEG®) Coagulation Analyzer (Haemoscope Corp., Skokie, IL) is a small instrument capable
of running two samples simultaneously and is easy to set up. It is connected to a computer (running the TEG® Analytical Software) through an A/D interface box. The Thromboelastograph® Coagulation Analyzer coagulation profile is displayed on the computer screen as an outline with the range of normal values displayed as dotted lines. The test is performed as follows: A sample of celite activated whole blood (0.36 ml) is placed into a prewarmed cuvette. A suspended piston is then lowered into the cuvette which moves in rotation of a 4.5 degree arc backwards and forwards. The normal clot goes quite fast through an acceleration and strengthening phase. The fiber strands which interact with activated platelets attach to the surface of the cuvette and the suspended piston. The clot forming in the cuvette transmits its movement onto the suspended piston. A “weak” clot stretches and therefore delays the arc movement of the piston, which is graphically expressed as a narrow thromboelastogram. A strong clot in contrary will move the piston simultaneously and proportionally to the cuvette’s movements, creating a thick thromboelastogram.

Insert Figure 1: Principles of TEG® here

The strength of a clot is graphically represented over time as the characteristic cigar shaped figure. There are five parameters of the TEG® tracing: R, k, alpha, angle, MA and MA60, which measure different stages of clot development:

R: is the period of time from the initiation of the test to initial fibrin formation
K: is a measure of time from the beginning of clot formation until the amplitude of thromboelastogram reaches 20 mm. This represents the dynamics of clot formation.
alpha angle: is an angle between the line in the middle of the TEG® tracing and the line tangential to the developing “body” of the TEG® tracing. The alpha angle represents the acceleration (kinetics) of fibrin build up and cross-linking
MA = Maximum amplitude. It reflects the strength of the clot which is dependent on the number and function of platelets and their interaction with fibrin.

Insert Figure 2: Normal TEG®
Thromboelastogaph evaluation of clot formation during heparinization (i.e., cardiopulmonary bypass) is now available. Recently, heparinase (an enzyme that digests heparin) has been introduced into the TEG® technology, allowing identification of abnormal coagulation in “heparinized” patients, prior to heparin reversal with protamine. This test may prove particularly useful during long cardiac bypass pump runs, deep hypothermia, use of ventricular assist devices or complicated major vascular cases (i.e.: repair of thoracoabdominal aneurysms). The test will also deterct if more protamine is needed to fully reverse heparin.

Other reagents that may be added to the whole blood sample are antifibrinolytic agents such as Epsilon-Aminocaproic Acid, Tranexamic acid and Aprotinin. This will test their effectiveness in treatment of excessive fibrinolysis.

Insert Figure 3: Overview of TEG® interpretation.
SECTION X. USE AND MONITORING OF ANTICOAGULATION AND FIBRINOLYTIC THERAPY

A. Unfractionated Heparin (UFH)

Unfractionated heparin consists of a heterogeneous mixture of glycosaminoglycans harvested from porcine intestinal mucosa whose molecular weight varies from 3,000 to 30,000 daltons. The diverse population of molecular weights and tendency of UFH to bind to plasma and tissue proteins and cell surfaces makes monitoring of heparin therapy essential to minimize unnecessary thrombotic or bleeding complications. UFH produces its antithrombotic effect by dramatically accelerating the inhibitory activity of antithrombin (III) against factors IIa, Xa and to a lesser extent I Xa, XIa, and XIIa. UFH is most conveniently and inexpensively monitored by using the PTT. When using the PTT to monitor UFH therapy, it is essential to determine the sensitivity of each batch of PTT reagent against known standardized concentration of heparin, using protamine titration or chromagenic anti-Xa assays to determine heparin concentrations. In this manner, the PTT values that correspond to therapeutic concentration of UFH can be determined and an PTT therapeutic range can be determined.

It cannot be emphasized strongly enough that fixed PTT ratio therapeutic ranges (PTT ration 1.5-2.5) should not be used for monitoring heparin therapy. For this reason, some medical centers have begun using chromagenic anti-Xa assays for UFH monitoring. While this may eliminate the extra step of constructing a standard curve with each batch of PTT reagent, this approach is more expensive and there is considerably less information about the significance of particular heparin levels and clinical outcomes. It is important to remember that the relationship between the PTT and the concentration of heparin is curvilinear such that at high heparin concentration (PTT values greater than 100-120 seconds, plasma levels above 0.8 units/ml) is probably unreliable. One patient group in whom UFH therapy at conventional doses can be complicated by excessively prolonged PTT values is patients with lupus anticoagulants. In these patients, initial therapy can be adjusted to achieve an PTT ratio value that is approximately 2-fold the baseline value. However, this practice is imperfect because of the characteristics of the PTT assay. Therefore, anti-Xa assays (see below) should be used to confirm that this PTT ratio value is therapeutic.

B. Unfractionated Heparin Dosage

Numerous studies have determined that weight-based nomograms should be used when initiating and titrating UFH. Weight-based heparin nomograms have been developed for the treatment of venous thromboembolism, acute coronary syndromes and acute cerebrovascular disease. When managing UFH in patients you are encouraged to use the disease specific nomograms provided by the Medical Intensive Care Unit/Medical Progressive Care Unit, Coronary Care Unit/Coronary Progressive Care Unit and the Neurocritical Care Units that are available in the Nursing Protocols section of the Johns Hopkins Intranet at http://www.insidehopkinsmedicine.org/nursing/cnp/clinical_nursing_practice.html
Note this link does not work any more

C. Low Molecular Weight Heparin (LMWH)

Although still used less than unfractionated heparin in the inpatient environment, the use of LMWH is certain to continue to increase over the next few years as generic products become available and acquisition costs decrease. The disadvantage of increased acquisition costs are counter-balanced by better bioavailability and the absence of any requirement for routine monitoring. In situations where monitoring is advantageous (e.g., renal insufficiency, pregnancy), the anti-Xa assay is used. One must indicate that is LMWH and not UFH that is being assessed.

D. Low Molecular Weight Heparin Dosing

The dosage of enoxaparin (Lovenox™, sanofi-aventis, Bridgewater, NJ) for VTE and ACS is 1 mg/kg sc q12h for adults 50-150 kg. When using enoxaparin in patients undergoing surgical procedures, the last dose should be given 24 hours before the scheduled procedure. Consider using anti-Xa levels to monitor enoxaparin therapy in lower or higher weight individuals particularly when extended durations of therapy are contemplated (> 5-7 days). Enoxaparin should be used with caution in patients with renal dysfunction. (Creatinine clearance < 30 ml/min) as
accumulation has been noted in patients with reduced renal function. The FDA approved regimen for patients with reduced renal function is 1 mg/kg sc qDay. Enoxaparin should not be used in patients with fluctuating renal functions prior to invasive procedures as accumulation may occur and result in abnormal hemostasis. The dosage of dalteparin (Fragmin™, Eisai, Inc., Woodcliff Lake, NJ) for VTE is 200 International Units/kg sc qDay. Dosing guidelines for renal insufficiency have not been developed. Doses should probably not be capped for large body weights. In the event of life-threatening bleeding, LMWH are partially reversible (60-80%) with protamine in a dose of 1 mg/mg of enoxaparin (or per 100 units of dalteparin). If it has been more than 8 hours, the dose should be reduced to 0.5 mg of protamine per mg of enoxaparin (or 100 units of dalteparin).

E. Heparin Anti-Xa Assay

This is A Functional Assay For The Presence of Heparin, Either Un-Fractionated or Low Molecular Weight

**Indications (Heparin Anti-Xa Assay):**

1. Monitoring the anti-thrombotic activity of unfractionated or low molecular weight heparin.

**Test Principle (Heparin Anti-Xa Assay):**

In this assay, a known amount of Factor Xa (FXa) an excess of antithrombin III, and a chromagenic substrate for FXa are added to patient plasma. If heparin is present in the plasma, it will form a complex with antithrombin III (AT). The concentration of the [heparin*AT] complex is dependent on the availability of AT. In order to obtain a more constant concentration of AT, purified AT is added to the test plasma. The remaining FXa acts on its substrate to yield a color that is then read photometrically.

1. Heparin + AT (excess) -> [heparin* AT]
2. Heparin* AT] +FXa (excess) -> [heparin* AT] + FXa (remaining)

In this system, the amount of FXa activity is a function of the amount of heparin. If no heparin is present, there will be abundant FXa available to act upon the substrate. This results is inversely proportional to the heparin concentration, so that the more heparin present, the lower the reading (since less FXa is available). For ease of interpretation, assay results are reported in units of UFH or LMWH per mL.

**Possible results and interpretation (Heparin Anti-Xa Assay):**

An increased anti-Xa activity implies low amounts of heparin. A low anti-Xa activity implies increased amounts of heparin. Each lab must establish accepted therapeutic values for anti-Xa activity.

**Factors affecting test results (false positives and negatives) (Heparin Anti-Xa Assay):**

No drug interference has been reported. The sample must be immediately placed on ice and delivered to the laboratory.

**Interpretation of LMWH (Anti Xa) assay results**

The LMWH (Anti-Xa) assay is useful for measuring the level of functional heparin activity in a plasma sample. Nevertheless, this assay has a number of limitations that should be kept firmly in mind when interpreting test results. Although one would expect that anti-Xa levels correlate precisely with the antithrombotic and hemorrhagic effects of heparin therapy, this correlation has not been well demonstrated in clinical studies. An explanation for this seemingly paradoxical result lies in the diverse effects of heparin preparations on the many other participants in hemostasis including platelets, endothelial cells and proteins such as thrombin and TFPI. Additional variability is introduced by the varying effects of the different LMWH preparations on Xa and IIa activity and poor comparability between different anti-Xa chromagenic assays. These limitations make it imperative that clinicians interpret anti-Xa assay results in a LMWH, assay and equipment specific manner.

**Summary**
Consideration of these factors and of the good results obtained in clinical trials where LMWH has been administered without monitoring as venous thromboprophylaxis and treatment leads to the conclusion that routine monitoring by anti-Xa assay is not currently indicated for most patients. Furthermore, while anti-Xa assays may provide some clue to LMWH pharmacokinetics in individual subjects, such as the obese, the underweight, pregnant women and infants, only limited information on antithrombotic effect and bleeding risk can be deduced from this measurement. If anti-Xa assay is employed for monitoring in these clinical situations the limitations of the information generated must be borne in mind.

E. Pentasaccharides

The first synthetic pentasaccharide anticoagulant, fondaparinux (Arixtra™ GlaxoSmithKline, Research Triangle Park, NC) is available in The Johns Hopkins Hospital pharmacy for the prevention of VTE in patients undergoing hip and knee arthroplasty and hip fracture surgery and in patients who are HIT antibody positive. It also has been approved by the FDA for treatment of VTE and ACS and VTE prevention in patients undergoing major general surgery. Fondaparinux is a synthetic anticoagulant that consist of the 5 sugar residues in UFH and LMWH that bind to heparin binding site in antithrombin (III) so like these anticoagulants; its antithrombotic effect is mediated by accelerating the inactivation of factor Xa by antithrombin (III). Because of its small size it does not mediate inhibition of any other serine proteases by AT III. It binds tightly to AT III so its half-life is 17-21 hours. It is renally cleared so it should not be used in patients with a creatinine clearance less than 30 ml/min and should probably be used cautiously in patients with creatinine clearances below 50 ml/min. The fondaparinux dose for VTE prevention is 2.5 mg sc qDay. For treatment of VTE, patients with a body weight less than 50 kg should receive 5 mg sc qDay while patients 51-100 kg should receive 7.5 mg sc qDay and patients over 100 kg should receive 10 mg sc qDay. Although HIT antibodies have been identified in patients treated with fondaparinux, these antibodies do not seem to be associated with platelet activation or clinical HIT. Therefore, fondaparinux has been used in a limited number of patients (< 100) to treat HIT with favorable outcomes thus far. Consequently, fondaparinux can probably be used in patients with positive HIT antibody assays without triggering clinical HIT. Direct thrombin inhibitors are still the treatment of choice for HIT, given the much greater clinical experience with these medications in the patient population.

F. Direct Thrombin Inhibitors

Direct thrombin inhibitors (DTI) are anticoagulants that directly bind to and inhibit thrombin without need to interact with antithrombin (III). Three DTI are available on the US market and at The Johns Hopkins Hospital; argatroban (Novastan™ GlaxoSmithKline, Research Triangle Park, NC), lepirudin (Refludan™, Bayer Healthcare Pharmaceuticals, Inc., Wayne, NJ) and bivalirudin (Angiomax™, The Medicines Company, Parsippany, NJ). Argatroban and lepirudin are approved for the treatment of HIT while bivalirudin is approved for percutaneous coronary intervention.

Argatroban is a synthetic reversible direct thrombin inhibitor that binds to the thrombin active site. It is metabolized in the liver and has a half-life of 45 minutes. The PTT is used to monitor argatroban therapy and the target PTT ratio range is 1.5-3.0. The standard dose of argatroban is a 2 micrograms/kg/min continuous infusion. In patients with hepatic impairment and critically ill patients, the dose should be reduced by at least 75% to 0.25 to 0.5 micrograms/kg/min. Given its short half life, the PTT should be followed every 4-6 hours until a stable PTT has been obtained. When the PTT is too low, dose increases of 20-25% should be considered while excessive PTT ratios should prompt a dose reduction of at least 25-50%. Argatroban strongly influences the INR during warfarin co-therapy so an INR of 4 must be achieved to ensure a therapeutic INR of at least 2 once argatroban is discontinued. The PTT and INR should be followed every 4-6 hours during discontinuation of argatroban to ensure that an adequate level of anticoagulation with warfarin has been achieved. When treating patients with HIT, warfarin therapy should not be initiated until the platelet count has normalized to ensure that unnecessarily abbreviated course of argatroban are not used. There is no antidote or reversal agent available for argatroban should life-threatening bleeding occur. Recombinant human Factor VIIa (NovoSeven) has been used to treat life-threatening bleeding in patients on DTI.
Table 6: ARGATROBAN DOSING IN HEPATIC INSUFFICIENCY

<table>
<thead>
<tr>
<th>Total bilirubin</th>
<th>AST/ALT</th>
<th>% of standard infusion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8-3.6 mg/dL</td>
<td>150-600 IU/L</td>
<td>25</td>
</tr>
<tr>
<td>&gt; 3.6 mg/dL</td>
<td>&gt; 600 IU/L</td>
<td>AVOID, using lepirudin if possible combined renal/hepatic failure consider bivalirudin</td>
</tr>
</tbody>
</table>

Lepirudin is a recombinant form of the irreversible direct thrombin inhibitor derived from the saliva of the medicinal leech. It is a bidentate DTI that binds irreversibly to the thrombin active site and the fibrinogen binding site on thrombin. It is cleared renally and has a half-life of 80 minutes in patients with normal renal function. The PTT is used to monitor lepirudin therapy and the target PTT ratio range is 1.5-2.0.

Previously a higher target range of 1.5-2.5 had been used for therapy but expert clinical experience suggests that a lower PTT ratio target range be used for therapy to minimize bleeding complications. For patients with normal renal function, lepirudin should be initiated at a dose of 0.1 mg/kg/hr without a bolus. Although previous recommendations had recommended use of a bolus (0.4 mg/kg) and a higher infusion rate (0.15 mg/kg/min), cohort studies have indicated that lower doses are preferable and associated with fewer bleeding complications. Lepirudin doses should be significantly reduced in patients with compromised renal function (see Table below). Antibodies to lepirudin develop in almost 50% of patients within the first week of use that most often increase the circulation time of the drug. Therefore, close surveillance of PTT values is warranted so that appropriate dose adjustments can be made. These antibodies have been rarely associated with anaphylaxis so repeat exposure to lepirudin within several months of use is discouraged. Lepirudin does not appear to significantly increase the INR in patients during co-therapy with warfarin. Similar to argatroban, lepirudin is not reversible by conventional means but hemodialysis using high-flux polysulfone membranes and hemofiltration has been shown to increase clearance anecdotally.

Table 7: LEPIRUDIN DOSING FOR RENAL INSUFFICIENCY

<table>
<thead>
<tr>
<th>Creatinine clearance (ml/min)</th>
<th>% of standard infusion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>45-60</td>
<td>50</td>
</tr>
<tr>
<td>30-44</td>
<td>25</td>
</tr>
<tr>
<td>15-29</td>
<td>10</td>
</tr>
<tr>
<td>&lt;15</td>
<td>Use argatroban</td>
</tr>
</tbody>
</table>

Bivalirudin is a synthetic DTI derived from the saliva of the medicinal leech that contains the thrombin active site and fibrinogen binding site moieties of lepirudin linked by a synthetic polypeptide linker. It is currently only FDA-approved for PCI. The recommended dose for PCI is 0.75 mg/kg IV bolus followed an IV infusion of 1.75 mg/kg/hr with the dose adjusted to an ACT of at least 225 seconds. Bivalirudin has a half-life of 25 minutes and is cleared primarily by hydrolysis in the circulation by proteases such as thrombin. It is also cleared renally to a small extent. Its unique mode of elimination suggests that it may be a useful drug for treatment of HIT in patients with impaired hepatic and renal function. Further studies are warranted to confirm its efficacy in this population. No reversal agent is available for bivalirudin.

G. Oral Anticoagulant Therapy

Oral anticoagulants are compounds structurally similar to vitamin K that function therapeutically as antagonists of vitamin K, inhibiting gamma carboxylation of the coagulation Factors II (prothrombin), VII, IX, X, Protein C, Protein S and Protein Z, resulting in decreased synthesis of effective clotting factors. To achieve antithrombotic effects without inordinate risk of bleeding levels of the vitamin K-dependent factors should be reduced to approximately 20 percent of normal. Since the rate of decay of each clotting factor is determined by its biologic half-life, the level of the individual factors will differ during the period immediately following institution of oral anticoagulant therapy, until a somewhat steady state is achieved. While some anticoagulant effect begins to occur
within 24 hours after the initiation of oral anticoagulant therapy (due primarily to reductions in factor VII activity which has a half-life of 6 to 7 hours (peak anticoagulant activity is delayed for 72 to 96 hours because of the longer plasma half-lives of factors II, IX, and X. Therefore, at least 5-7 days of heparin therapy is usually required until therapeutic levels of anticoagulation are achieved with vitamin K antagonists. Heparin therapy should be continued until a therapeutic INR value greater than 2 has been achieved. Premature discontinuation of heparin therapy (i.e. before at least 5 days of therapy) have been associated with early recurrent VTE.

Since peak anticoagulant activity due to warfarin requires at least 5-7 days of therapy to become manifest, physicians should avoid using loading doses of warfarin (> 7.5 mg per day) when initiating therapy. Instead, initial warfarin dose should be guided by what maintenance dose the physician thinks the patients will require. For most patients, 5 mg of warfarin is a reasonable initial dose. In post-operative patients, patients age 75 years or older, patients with liver disease or an initial INR that is elevated, malnourished patients or patients taking medications known to interact with warfarin, an initial dose of 2.5 mg is preferable. Daily INR should be obtained. Generally, one does not see the INR increase until 2-3 days after the first warfarin dose. Identification of earlier increases in the INR or INR increases greater than 0.5 INR unit in a 24 hour period are often a warning that warfarin dose is too large and the next smaller warfarin dose should be chosen.

The usual INR target for therapy of venous thromboembolism, prevention of systemic embolism in atrial fibrillation, with tissue heart valves or bileaflet (e.g., St Jude) aortic mechanical valves, myocardial infarction or valvular heart disease is 2.0 – 3.0. For mitral or dual bileaflet mechanical valves, (e.g., St Jude etc.) or in patients with mechanical valves with atrial fibrillation, myocardial infarction, left atrial enlargement, endocardial damage or low ejection fraction (< 30%) or history of systemic embolism, the usual target INR range is 2.5 to 3.5. Aspirin 81 mg per day is usually added as well. A higher INR range (3.0 to 4.0) has been recommended for patients with the antiphospholipid antibody syndrome who have experienced recurrent thromboembolism despite an INR of 2-3. The latest INR recommendations for the ACCP were published in September 2004 [Chest 2004 126 (Suppl. 1)].

### Tables 8 and 9 Medications that influence warfarin therapy

<table>
<thead>
<tr>
<th>Table 8</th>
<th>Table 9</th>
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<tbody>
<tr>
<td><strong>SIGNIFICANT INCREASE IN THE INR</strong></td>
<td><strong>MODERATE INCREASE IN THE INR</strong></td>
</tr>
<tr>
<td>AMIODARONE</td>
<td>Cimetidine</td>
</tr>
<tr>
<td>Anabolic Steroids</td>
<td>Ciprofloxacin</td>
</tr>
<tr>
<td>BACTRIM (TMP/SMZ)</td>
<td>Clarithromycin</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Delavirdine</td>
</tr>
<tr>
<td>Disulfiram</td>
<td>Efavirenz</td>
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<tr>
<td>FLUCONAZOLE</td>
<td>Erythromycin</td>
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<tr>
<td>Isoniazid</td>
<td>Itraconazole</td>
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<tr>
<td>METRONIDAZOLE</td>
<td>Lovastatin</td>
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<tr>
<td>Miconazole</td>
<td>Omeprazole</td>
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<tr>
<td>Phenylbutazone</td>
<td>Propafenone</td>
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<tr>
<td>Quinidine</td>
<td>Ritonavir</td>
</tr>
<tr>
<td>Sulfinpyrazone</td>
<td></td>
</tr>
<tr>
<td>Sulfisoxazole</td>
<td></td>
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<tr>
<td>Tamoxifen</td>
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Table 9

<table>
<thead>
<tr>
<th>SIGNIFICANT INCREASE IN THE INR</th>
<th>MODERATE INCREASE IN THE INR</th>
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<tbody>
<tr>
<td>Amobarbital</td>
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<tr>
<td>Aprepitant</td>
<td>Efavirenz</td>
</tr>
<tr>
<td>Butabarbital</td>
<td>Nafcilil</td>
</tr>
<tr>
<td>CARBAMAZEPINE</td>
<td>Ritonavir</td>
</tr>
<tr>
<td>Dicloxacillin</td>
<td></td>
</tr>
<tr>
<td>Griseofulvin</td>
<td></td>
</tr>
<tr>
<td>Methimazole</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
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<tr>
<td>Phenytoin</td>
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</tr>
<tr>
<td>Primidone</td>
<td></td>
</tr>
<tr>
<td>Propylthiouracil</td>
<td></td>
</tr>
<tr>
<td>Rifabutin</td>
<td></td>
</tr>
<tr>
<td>RIFAMPIN</td>
<td></td>
</tr>
<tr>
<td>Secobarbital</td>
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</tr>
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</table>

The most common cause of drug-warfarin interactions noted at The Johns Hopkins Hospital are highlighted in the table above.

Management of supratherapeutic anticoagulation depends upon at least 3 factors: 1.) The INR 2.) The presence or absence of bleeding and its severity and 3.) The patient’s risk of bleeding. Detailed guidelines for managing patients who are over anticoagulated have been published by the American College of Chest Physicians [Ansell et al. Chest 2001 119 (Suppl. 1):22S-38S] and presented at the American Society of Hematology meetings [Crowther M.A. Hematology 2001:ASH Education Program Book p. 341] In general, for an INR above the therapeutic range but less than 5 in the absence of bleeding, lowering the dose of warfarin by up to 20% of the weekly dose or omitting a dose and lowering the dose by up to 20% of the weekly dose and continued monitoring of the INR are the only intervention required. Any drug interactions should be noted and alternative agents should be used if possible. If the INR is greater than 5 and less than 9 in an asymptomatic patient, warfarin should be held and the INR should be monitored daily. Once the INR is falling toward the upper end of the therapeutic range, warfarin may be restarted at a lower dose (at least 20% lower than previous dose). For patients at high risk of bleeding 1 -2.5 mg of oral vitamin K1 can be considered. For patients with an INR above 9 and no evidence of significant bleeding, higher doses of oral vitamin K1 (1 -5 mg) should be considered combined with cessation of warfarin and close monitoring of the INR. Use of FFP or prothrombin complex concentrates may be worthwhile considering in patients at high risk of bleeding. Use of parenteral vitamin K1 (Aquamephyton) and/or prothrombin complex concentrates or FFP (15 to 20 ml/kg) should be considered in any patient who has significant bleeding and requires rapid reversal of anticoagulation. Intravenous vitamin K1 should be administered slowly (no faster than 1 mg/min) with close monitoring, as anaphylactic reactions have been reported following intravenous injection of this agent (incidence ~ 1 in 3000).

H. Aspirin Resistance: Are we ready to Perform Routine Testing?

Aspirin is one of the most important and cost effective drugs for the secondary prevention of cardiovascular disease, reducing the rate of acute arterial thrombotic events in high risk patients by at least 25%. The antithrombotic effect of platelets is to decrease platelet activation. Despite aspirin administration, acute arterial thrombosis may recur, and the term aspirin resistance has been applied. The pharmacologic effect upon platelets is readily measurable by laboratory tests. Since a variety of laboratory tests exist to measure platelet function, there has been much interest in applying them to assess risk for future arterial thrombosis and to adjust aspirin therapy, similar to the approach of adjusting oral anticoagulants in venous thrombosis.

The key questions in formulating the role of platelet function testing to monitor the antiplatelet effect of aspirin is whether the arterial event related entirely to a failure of aspirin (pharmacologic resistance) to inhibit platelet activation or whether some other clinical factor (treatment failure) contributed to the vascular event. The challenges for practitioners and laboratory investigators will be to determine what laboratory measurements of platelet activation to use that are predictive of a vascular event and to standardize and validate their application in diverse populations being treated with aspirin.
The purpose of this section is to understand platelet function, the effect of aspirin upon platelet tests, to define the clinical conditions associated with arterial thrombosis as confounders in interpreting test results, to describe the laboratory tests that measure aspirin effect and to give recommendations appropriate laboratory testing.

Aspirin Resistance

With the above concepts of pharmacologic versus clinical failure in mind, one may consider a variety of definitions for aspirin resistance. In some author’s view aspirin resistance should be defined strictly on the basis of aspirin to exert an effect upon a marker of platelet activation. For example, aspirin resistance may be defined as the failure of aspirin to inhibit thromboxane A2 production or inhibit some other aspect of platelet function such as thrombin generation. This form of resistance may be called laboratory resistance. This thinking parallels clinician’s practice of labeling someone as heparin resistant who fails to increase their PTT tests to an expected level.

This is in contrast to clinical aspirin resistance which is the failure of aspirin to prevent clinical arterial events in patients who are prescribed aspirin and are compliant. This situation is referred to as aspirin failure events, or clinical resistance. The clinical diagnosis of aspirin resistance is typically made retrospectively after an arterial event and may not be specific. It should be emphasized that arterial events are multifactorial in causation, and rather than resistance to aspirin, a patient may have experience a treatment failure. The many causes of these are listed in Table 1.

Platelet Biology

In order to understand the antiplatelet effect of aspirin, one must understand platelet function. Vascular injury, whether trauma or atheromatous changes, result in endothelial disruption. This leads to exposure of collagen and the release of tissue factor. The former starts the process whereby platelets adhere to the sub endothelium which is further accomplished by von Willebrand factor binding to platelets. The increase in tissue factor at the site of injury promotes the activation of the clotting cascade, which ends up generating thrombin, which converts fibrinogen to fibrin. Thrombin also is one of the most platelet agonists. Following adhesion of the platelet, platelet activation begins and is promoted by the release of ADP, epinephrine and generation of thrombin from the clotting cascade. These platelet agonists activate receptors on the platelet membrane that leads to the mobilization of intracellular platelet calcium. This promotes platelet degranulation or secretion and activates phospholipase A2. This enzyme cleaves arachidonic acid from membrane phospholipids and provides the substrate for the generation of prostaglandin H2, which is catalyzed by cyclooxygenase. Prostaglandin H2 is modified to produce a variety of prostaglandins and thromboxane A2. Thromboxane A2 promotes further platelet activation and platelet aggregation at the vascular injury site. Nearby endothelial cells generate prostacyclin, nitric oxide, and carbon dioxide which down regulate platelet reactivity in the vicinity of the clot.
Aspirin and the Possible Causes of Pharmacologic Aspirin Resistance

Aspirin irreversibly inactivates the cyclooxygenase (COX) activity of the platelet enzyme prostaglandin H-synthase 1 (also referred to as COX1). This enzyme catalyzes the conversion of arachidonic acid to prostaglandin H2, which is the first committed step in prostanoid biosynthesis. Prostaglandin H2 is an intermediate compound and a substrate for several downstream isomerases that generate different bioactive prostanoids, including thromboxane A2, the main product of arachidonic acid metabolism in human platelets. Aspirin first binds to an Arg120 residue and acetylates a Ser529 residue located in the narrowest section of the channel, just below the catalytic pocket. Acetylation of Ser529 prevents arachidonic acid from gaining contact with Tyr385, which would normally be the first step in its cyclooxygenation.

Aspirin is considered a relatively weak antiplatelet agent at low doses typically used since it inhibits only thromboxane dependent activation and aggregation. Thromboxane A2 increases expression of fibrinogen receptors on the platelet membrane and acts in an autocrine fashion to trigger the activation of other platelets by activating the thromboxane receptor on the platelet membrane. Thromboxane A2 is also a potent vasoconstrictor. Once daily low dose aspirin (30 mg) suppresses thromboxane A2 by 95% after 5 days of treatment. Aspirin treated platelets still respond to collagen, epinephrine and thrombin, all of which may play a role in activation of platelets in acute coronary syndromes and stroke. Although newly formed platelets express both COX1 and COX2, mature platelets express only COX1. While COX1 is highly sensitive to low doses of aspirin COX2 is inhibited only by doses high enough to have analgesic or anti-inflammatory effects. A variable effect upon drug target, as implied by the term aspirin resistance, could involve COX1 polymorphisms affecting Arg120, Ser529, or both. These polymorphisms could explain a fixed percentage of drug resistance in any given population, dependent on prevalence, but would not be expected to change over time as a function of drug exposure. Although genetic variability in COX1 has been described both increased and diminished responses to aspirin have been associated with the same COX1 haplotype. This haplotype, which is carried by 12% of the population, contains the minor allele of the promoter variant A842G and is in complete linkage disequilibrium with C50T variant in the signal peptide.

Alternatively, enhanced platelet regeneration and an increased proportion of newly formed platelets expressing COX-2 may lead to increase fraction of platelets that may be able to form TXA2 or both a COX-1 and COX-2 dependent manner. In this situation the antiplatelet effect may be decreased with standard daily dosing. Note that increased platelet turnover, which can be seen during coronary artery bypass graft surgery, infection and inflammation can result in an increase proportion of younger platelets that elevates COX-2.

Assessment of Platelet Function

Platelet Function Tests

Early definitions of aspirin’s antiplatelet effect were based upon prolongation of the bleeding time and optical platelet aggregometry. Since these early days of platelet function testing, a variety of devices have been developed to measure platelet function. The vast majority of studies reporting the occurrence of aspirin ‘resistance’ in different clinical settings have relied on ex vivo measurement of platelet function with one or more of the following techniques: light transmittance or impedance-lumi aggregometry in platelet-rich plasma or whole blood; the platelet function analyzer PFA-100® (Dade International Inc., Deerfield, IL), which was developed as a bedside, rapid, whole-blood assay; and the VerifyNow® Rapid platelet Function Assay (Accumetrics, Inc., San Diego, CA), which is also a bedside, whole-blood assay. Thromboelastograph Hemostasis Analyzer (Haemoscope, Niles, IL) a device standardized to test for both aspirin and clopidogrel resistance tests the platelets’ response to an aggregating stimulus, such as arachidonic acid, added at variable concentrations.

Optical aggregometry in citrated platelet rich plasma is regarded by many as the gold standard of platelet function. Optical aggregometry measures the increase in light transmission through platelet rich plasma preparation when platelets are aggregated by a platelet agonist. For determining the effect of aspirin, arachidonic acid is the best platelet agonist for detecting inhibition of thromboxane formation since it is the precursor of thromboxane. Other platelet agonists, such as collagen and ADP, induce platelet aggregation through mechanisms that are less dependent upon thromboxane formation. Despite this technique having been developed fifty years ago, there is little standardization of the technique between laboratories. This lack of standardization is problematic if result from one laboratory is to be compared to those of another laboratory. Several pre-analytical and analytic factors are important
including preparation of platelet rich plasma, final concentration of platelet count and platelet agonists used to
stimulate platelets. Typically when 0.50 mM arachidonic acid is used as an agonist, greater than 90% of platelet
inhibition is observed. When 1 µg/ml of collagen or 5 µM of epinephrine is used greater than 70% inhibition is
observed. It is important to note, platelet aggregation results are affected by race, sex, diet and collection technique.

An alternative to optical platelet function is whole blood aggregometry based upon impedance change when
platelets adhere to an electrode probe of platinum. This technique circumvents the need for centrifugation and
permits testing in whole blood which may be more reflective of in vivo platelet function since interactions of all cell
types are involved with the process being tested. Both optical and whole blood impedance aggregometry are
precise, but require technical skill and interpretation.

Because of this, there is much interest in the dedicated devices such as the PFA-100 or the Verify Now devices that
are well standardized. The PFA-100 device has proven useful for monitoring the pharmacologic effect of aspirin
upon platelets and for identifying aspirin resistant patients in some clinical trials. In this device, a collagen coated
membrane with either ADP or epinephrine is used to coat an aperture thru which citrated whole blood is aspirated
under high shear stress. A platelet plug gradually forms and occludes the aperture. The time for this to occur is
called the closure time, which is the measure of platelet activity. In this testing, epinephrine induced closure time is
prolonged when the patient is taking aspirin. Abnormal ADP closure time reflects other causes of platelet
dysfunction. The PFA-100 may be influenced by the hematocrit value and the platelet count as well as the von
Willebrand factor concentration. These considerations may account for the poor agreement between PFQ-100 and
platelet aggregation.

The Verify Now platelet function analyzer is a turbidimetric based optical system which measure platelet induced
aggregation as an increase in light transmission. Fibrinogen coated micro particles are used to measure platelet
aggregation in response to a novel platelet agonist, propyl gallate or arachidonic acid. Fibrinogen coated micro
particles agglutinate in whole blood in proportion to the number of unblocked platelet glycoprotein IIB-IIIa
receptors. Results are automatically calculated and expressed as aspirin resistance units. The test is easy to perform
and is precise. In the manufacturer determined criterion for aspirin resistance a cut-off value was determined by
comparison to platelet aggregation in response to administration of 325 mg dose of aspirin and epinephrine induced
platelet aggregation. This validation of the cutoff has been questioned since epinephrine is not a specific measure of
aspirin effect and many normal patients have variable response to this agonist. As with the PFA-100 the device has
only been studied in relatively small populations. We await further validation in larger populations.

Other platelet function laboratory tests have been proposed including thromboelastographic analysis, core and plate
analyzer of platelet function, and flow cytometry to measure p-selectin activation. In concept all of these assays
may be potentially useful but standardization and clinical validation studies are too few or too small to draw any
recommendations for their utility.

Biochemical Assessment of Platelet Inhibition

Thromboxane A2 has a short half life in plasma and is rapidly hydrolyzed to Thromboxane B2. Thromboxane B2 is
metabolized to 11-dehydro thromboxane B2, 11-dehydro 2, 3 dinor Thromboxane B2 and a number of other minor
thromboxane B2 metabolites which are excreted by the kidney. In the serum or plasma one can measure using a
variety of ELISA assays available, thru manufacturers of research reagents. Urinary 11-dehydro-thromboxane B2
excretion provides a noninvasive, time-integrated index of whole-body thromboxane A2 production. The assay of
the urine metabolites require a variety of preparatory steps before the metabolite can be measured. Recently one
cogulation manufacturer (Corgenix, Broomfield, Co.) has developed a clinical assay that measures urinary 11-
dehydrothromboxane B2 directly without preparatory steps and promises to improve availability of measurements of
urinary 11-dehydrothromboxane B2 that meets clinical needs. Moreover, standardization of the testing and careful
proficiency testing thru a network of laboratories organized by Aspirin Works, Inc. promises to allow a laboratory to
apply cutoff values verified from carefully performed clinical trials for interpretation of an individual’s test result.

Aspirin-insensitive thromboxane A2 biosynthesis has been described in patients with unstable angina as well as in
patients with post-stroke dementia. The clinical relevance of this aspirin-insensitive activity has been explored by
Eikelboom et al., who performed a nested case-control study of baseline urinary 11-dehydro-thromboxane B2
excretion in relation to the occurrence of major vascular events in aspirin-treated, high-risk patients enrolled in the
Heart Outcomes Prevention Evaluation (HOPE) trial. After adjustment for baseline differences, the odds for the composite outcome of myocardial infarction, stroke, or cardiovascular death increased with each increasing quartile of 11-dehydro-thromboxane B2 excretion, with patients in the upper quartile having a 1.8-times higher risk than those in the lower quartile. This study also pointed out another important point to deal with in aspirin resistance. They showed that increasing levels of the thromboxane metabolite and increasing risk of arterial events is a continuum rather than categorical (positive or negative outcome). The implications that aspirin resistance is a continuum and the association between higher levels of aspirin resistance and increasing adverse vascular events is linear or log-linear thus resembling other risk factors as obesity, blood pressure, and hyperlipidemia and risk of arterial events.

It should be noted that platelets are not the only source of thromboxane A2 biosynthesis and, therefore, the urinary excretion of 11-dehydro-thromboxane B2 is reduced incompletely by 60-80% following aspirin administration. The underlying mechanisms include COX-2 expression in inflammatory cells endowed with thromboxane synthase and a higher percentage of COX2-expressing platelets. Moreover, signal-dependent de novo synthesis of COX-1 occurring over time in aspirin-treated platelets after persistent activation has been suggested to provide an additional source of aspirin-insensitive thromboxane biosynthesis.

**Recommendations and Summary on Testing**

For laboratory practitioners, aspirin resistance will likely be thought of in terms of effect upon a laboratory test or tests of platelet activation. It is clear that there is a variable antiplatelet effect to aspirin in different people and the effect is likely to be highly variable and broad. This presents problems in establishing cutoff values unless large numbers of individuals are tested using highly standardized assays. Until this is done determining the predictive value for a test or combination tests is highly uncertain. For these reasons, clearly the recommendations of the International Society of Hemostasis and Thrombosis, Scientific and Standardization Committee should be followed. These authorities unanimously recommended that no test be performed to assess the antiplatelet effect of aspirin individual patients. This statement parallels the recommendations found in the seventh American Conference on Antithrombotic therapy. Future research is needed to develop and evaluate valid quantitative measures of platelet inhibition that correlate with patient outcomes using well designed studies.

For users of our laboratory, we offer a variety of tests for clinical investigation, but for patient testing start with the PFA-100 and urinary thromboxane.

| Table 11: POSSIBLE CAUSES OF RECURRENT ARTERIAL EVENTS IN PATIENTS TAKING ASPIRIN |
|---------------------------------|---------------------------------|
| Pharmacologic Considerations    |                                  |
| Non-compliance                  |                                  |
| Under dosing                    |                                  |
| Inadequate absorption           |                                  |
| Drug Interference-non steroidal anti-inflammatory drug | |
| Genetic                         |                                  |
| COX-1, COX-2, thromboxane synthase polymorphisms | |
| Platelet Glycoprotein Polymorphisms-GP1a/IIa, IB/IX, and IIa/IIIb receptors | |
| Genetic hyper-responsiveness to epinephrine | |
| Factor XIII polymorphisms       |                                  |
| Prothrombin Gene Mutation, and enhanced thrombin formation | |
| Enhanced Platelet Function       |                                  |
| Greater percentage of newly formed platelets with enhanced COX-2 levels | |
| Inflammation and macrophage activation | |
| Stress, smoking, hypercholesterolemia | |
| Endovascular devices, such as grafts, prosthetic valves, stents | |
| Red cell induced platelet activation | |
| Non-atheromatous Causes of Vascular Events | |
| Embolism from the heart (prosthesis, tumor, vegetation) | |
| Arteritis                       |                                  |
| Thrombus from extremities with coexistent patent foramen ovale | |
**Hemoclot® Thrombin Inhibitor Assay for Dabigatran (Pradaxa)**

The Hemoclot® Thrombin Inhibitor Assay is a quantitative, clotting-based assay for the measurement of Dabigatran. Since we have a very stable and accurate calibrators for Dabigatran the assay is very accurate. The assay involves mixing pre-diluted test plasma [the precise dilution depending upon the predicted concentration of the DTI e.g. High concentration then dilute 1:20, low concentration then dilute 1:8] with a normal human plasma pool. Clotting is then initiated by adding a fixed but excess of α-thrombin and measuring the clotting time. The time to clot formation is directly proportional to the concentration of the direct thrombin inhibitor present in the plasma. A calibration curve is constructed from a series of plasma calibrants of known DTI [e.g. Dabigatran] concentration. On linear graph paper, the concentration of the DTI is plotted on the X-axis and the clotting time in seconds, on the Y-axis. From this graph the concentration of the DTI can be determined.

We do not report a therapeutic range but only the concentration since no established therapeutic range is defined. The assay should only be used for measuring Dabigatran and no other direct thrombin inhibitor.

**IX. NORMAL RANGES**

**NOTE:** Normal ranges for studies may vary slightly over time. For the most up-to-date values, see the computer generated report.

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>9.6 – 11.1 seconds</td>
</tr>
<tr>
<td>PTT</td>
<td>23.5 – 35.4 seconds</td>
</tr>
<tr>
<td>dRVVT</td>
<td>27.0- 45.0 seconds</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>150-450 mg/dl</td>
</tr>
<tr>
<td>Thrombin time</td>
<td>15.6-20.7 seconds</td>
</tr>
<tr>
<td>Reptilase Time</td>
<td>13.7-22.9 seconds</td>
</tr>
<tr>
<td>Quantitative D-Dimer</td>
<td>043-2.24 mg/L (for VTE diagnosis cut off is 1.6 mg/L)</td>
</tr>
<tr>
<td>Heparine</td>
<td>negative</td>
</tr>
<tr>
<td>Prothrombin fragment 1+2</td>
<td>0.5-3.0 nM</td>
</tr>
<tr>
<td>Factor II, V, VII, IX, X, XI, XII</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>clot stable after 24 hours</td>
</tr>
<tr>
<td>Factor Inhibitors</td>
<td>&lt; 0.5 Bethesda Units</td>
</tr>
<tr>
<td>Ristocetin Cofactor Assay</td>
<td>50-150%</td>
</tr>
<tr>
<td>vWF antigen</td>
<td>50-150%</td>
</tr>
<tr>
<td>vWF multimers</td>
<td>normal pattern and distribution</td>
</tr>
<tr>
<td>DDAVP challenge</td>
<td>(see individual tests)</td>
</tr>
<tr>
<td>APC resistance</td>
<td>ratio 2.2-4.0</td>
</tr>
<tr>
<td>Factor V Leiden</td>
<td>negative</td>
</tr>
<tr>
<td>Homocysteine</td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>4.6-12.1 µmole/L (&lt; 60 years old)</td>
</tr>
<tr>
<td>male</td>
<td>5.0-15.6 µmole/L (&lt; 60 years old)</td>
</tr>
<tr>
<td>Protein S Activity</td>
<td>65-140%</td>
</tr>
<tr>
<td>Protein S Antigen (total)</td>
<td>60-150%</td>
</tr>
<tr>
<td>Protein C Activity</td>
<td>70-180%</td>
</tr>
<tr>
<td>Protein C Antigen</td>
<td>60-150%</td>
</tr>
<tr>
<td>Test</td>
<td>Value</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Antithrombin III Activity</td>
<td>80-120%</td>
</tr>
<tr>
<td>Antithrombin III Antigen</td>
<td>68-128%</td>
</tr>
<tr>
<td>Heparin anti-Xa (LMWH)</td>
<td>0.6-1.0 units/ml</td>
</tr>
<tr>
<td>Plasminogen Activity</td>
<td>80-120%</td>
</tr>
<tr>
<td>TPA Antigen</td>
<td>0.5-14.0 ng/ml</td>
</tr>
<tr>
<td>PA1-1 Activity</td>
<td>4.0-43.0 1U/ml</td>
</tr>
<tr>
<td>α-2 antiplasmin</td>
<td>80-120%</td>
</tr>
<tr>
<td>Cryofibrinogen</td>
<td>negative</td>
</tr>
<tr>
<td>Platelet count</td>
<td>150-350,000/mm³</td>
</tr>
<tr>
<td>Bleeding Time</td>
<td>&lt; 8 minutes</td>
</tr>
<tr>
<td>Platelet aggregation</td>
<td>(needs interpretation)</td>
</tr>
<tr>
<td>Clot retraction</td>
<td>63-96% of original plasma volume</td>
</tr>
<tr>
<td>PF 4 (HIT) ELISA</td>
<td>negative</td>
</tr>
<tr>
<td>Serotonin release assay</td>
<td>negative</td>
</tr>
</tbody>
</table>
### X. NORMAL RANGES ACCORDING TO AGE*†

<table>
<thead>
<tr>
<th>TEST</th>
<th>1-7 DAYS</th>
<th>1-4 WEEKS</th>
<th>1-3 MONTHS</th>
<th>3-6 MONTHS</th>
<th>&gt; 6 MONTHS (ADULT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>10.0-15.9</td>
<td>10.0-15.9</td>
<td>10.0-14.9</td>
<td>10.0-14.9</td>
<td>11.2-13.6 seconds</td>
</tr>
<tr>
<td>PTT</td>
<td>28.0-50.0</td>
<td>22.4-56.8</td>
<td>29.0-52.2</td>
<td>25.1-47.1</td>
<td>23.8-35.0 seconds</td>
</tr>
<tr>
<td>Factor II</td>
<td>26-127</td>
<td>33-140</td>
<td>34-144</td>
<td>45-159</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor V</td>
<td>34-193</td>
<td>50-187</td>
<td>48-179</td>
<td>48-176</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor VII</td>
<td>28-191</td>
<td>35-191</td>
<td>39-191</td>
<td>39-191</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>50-239</td>
<td>50-211</td>
<td>50-210</td>
<td>50-168</td>
<td>50-200%</td>
</tr>
<tr>
<td>vWF</td>
<td>50-287</td>
<td>50-254</td>
<td>50-240</td>
<td>50-200</td>
<td>50-150u/ml</td>
</tr>
<tr>
<td>Factor IX</td>
<td>15-111</td>
<td>15-108</td>
<td>21-139</td>
<td>25-169</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor X</td>
<td>12-104</td>
<td>20-114</td>
<td>31-141</td>
<td>35-155</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor XI</td>
<td>10-137</td>
<td>23-137</td>
<td>27-153</td>
<td>41-211</td>
<td>50-200%</td>
</tr>
<tr>
<td>Factor XII</td>
<td>11-113</td>
<td>11-101</td>
<td>17-133</td>
<td>25-140</td>
<td>50-200%</td>
</tr>
<tr>
<td>AT III</td>
<td>39-93</td>
<td>39-93</td>
<td>41-108</td>
<td>50-120</td>
<td>73-120%</td>
</tr>
<tr>
<td>PC</td>
<td>17-90</td>
<td>20-90</td>
<td>21-112</td>
<td>28-112</td>
<td>80-120%</td>
</tr>
<tr>
<td>PS</td>
<td>12-88</td>
<td>22-105</td>
<td>33-133</td>
<td>54-134</td>
<td>65-140%</td>
</tr>
</tbody>
</table>

For all other Special Coagulation studies, normal values have not been established in this laboratory for these ages.

† Normal values have not been established in this laboratory for premature infants.