Preface

This handbook is intended as a source book for laboratory professionals and physicians who diagnose, treat and manage patients with bleeding and thrombotic disorders. This volume contains information regarding the methods utilized by Esoterix-Colorado Coagulation. The recommendations for further testing are those supported by Esoterix-Colorado Coagulation but may vary by institution. To provide state-of-the-art methods for coagulation testing, Esoterix-Colorado Coagulation periodically revises their test methodologies, panels and related specimen requirements and CPT codes.

Since medicine is a constantly changing science, it is recommended that the reader confirm information contained within this publication with other sources. Future assays and new applications of current coagulation assays, along with the development of better therapeutic agents, will unquestionably alter the practice of laboratory medicine.

All references to medications and dosages are general guidelines and the full prescribing information should be evaluated before utilizing any pharmacologic agent.

Colorado Coagulation is part of the Esoterix, Inc. family of laboratories that provides esoteric testing in numerous disease corridors.
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A carefully conducted patient history is the initial, and most important step in evaluating an individual for hemostatic disorders. The history provides the basis for determining the necessity of testing and for the efficient selection of cost-effective laboratory tests and interpretation of results. The patient and family history can provide important clues as to which components of the hemostatic mechanism are responsible for the clinical aberration. Ample time should be allowed to observe the patient carefully, and listen to their responses. Asking questions and discussing the answers on a level that the patient understands is essential to the process. In the instances of inherited disorders, patients may not interpret their clinical manifestations as abnormal especially when they compare themselves to family members. It is incumbent upon the physician to accurately assess the clinical presentation and information obtained during the patient history.

** Bleeding History **

The following information should be obtained through a patient history when a patient presents with a bleeding episode.

** A. Age of Initial Presentation **

1. Infancy
   a) Acquired (e.g.) Immune Thrombocytopenia Purpura, Neonatal Autoimmune Thrombocytopenia, Vitamin K deficiency are common.

2. Childhood
   a) most likely inherited

3. Early adult/older age
   a) most likely acquired

** B. Bleeding Presentation **

1. Petechiae - usually indicates vascular or platelet disorder

2. Ecchymosis/purpura - typically suggests a defect in primary hemostasis

3. Spontaneous/deep tissue hemorrhage/delayed onset (24-48 hrs.) bleeding, bleeding into joints or body cavities - typically suggests coagulation factor defects

** C. General Considerations **

1. Does the patient have a bleeding history?

2. Does the family have a bleeding history? Sex linked, autosomal, dominant/recessive

3. What is the ethnic origin of the patient?

4. Has the patient had major surgery without excessive bleeding? What about dental extractions?

5. Was the surgery prior to or following the current bleeding event?

6. Is the bleeding typically from a single site or multiple sites?

7. Is the bleeding appropriate for the extent of trauma?

8. Is the bleeding prolonged, delayed, or recurrent?

9. What medications have been given that may have contributed to the bleeding? (Consideration must be given to prescription medications as well as over-the-counter preparations and herbal remedies.)

10. Have there been recent dietary changes that may influence the bleeding?

11. Are other disease states present such as liver disease, renal disease, leukemia, or myeloproliferative disorders that may be associated with hemorrhage?

12. Has the patient ever required transfusion therapy?
D. Clinical situations where bleeding history provides valuable information

1. Epistaxis
   a) Not uncommon in normal individuals; however, also a prevalent symptom of platelet disorders and von Willebrand Disease (vWD)
   b) Severity of symptoms typically decrease in individuals with vWD as puberty approaches, and in or during pregnancy or while on estrogen therapy
   c) Determine whether onsets are traumatic or spontaneous
   d) Determine whether from both nares or unilateral
   e) Factors to consider in evaluating severity
      (i) Are nosebleeds recurrent?
      (ii) Have nosebleeds required emergency room treatment?
      (iii) Was packing or cauterization required
      (iv) Were transfusions necessary?

2. Menorrhagia
   a) Not uncommon in women without bleeding disorders, however, is frequently the presenting complaint in women with platelet disorders and vWD and infrequently in coagulation factor deficiencies
   b) Interpretation may be complicated due to difficulty quantitating menstrual blood loss
   c) Severity is best indicated by number of days of heavy flow and duration of flow
   d) Factors to consider in evaluating severity
      (i) # of pads used?
      (ii) Were bedclothes soiled even when double pads were used?
      (iii) Were large clots passed?
      (iv) Did patient become anemic?
      (v) Was iron usage required?
      (vi) Were transfusions or D&C necessary?

3. Dental extractions
   a) Lack of significant bleeding after molar extractions generally indicates normal hemostasis
   b) Increased bleeding following non-molar tooth removal is frequently observed in platelet disorders and von Willebrand disease
   c) Was packing and/or suturing or transfusion therapy required following the dental extraction?

4. Surgical procedures
   a) Surgical procedures and dental extractions usually represent the most significant challenges to hemostasis
   b) Question regarding bleeding associated with circumcision, tonsillectomy, appendectomy, skin biopsies
   c) Obtain details of procedure when possible
      (i) Did the surgeon make comments regarding blood loss?
      (ii) Was a hematoma present?
      (iii) Were blood products required for transfusion?
E. Clinical situations that may be related to a specific bleeding disorder, however, by themselves provide limited usefulness in diagnosis

1. Hematuria
2. Gastrointestinal bleeding
3. Hemoptysis
4. Bleeding associated with childbirth

F. Medications - A detailed history of recent medications is essential. Special detail should be paid to:

1. Aspirin and aspirin containing compounds - NSAIDs. Since aspirin is not considered “medicine” by some patients, care must be taken in how the question is asked. In addition, these agents are found in many over-the-counter preparations. It may be necessary to ask pointedly about the use of these agents as patients may not inherently consider them as medications.
2. Antibiotics
3. Anticoagulants
4. Radiation/chemical exposure

G. Diet/Habits

1. Alcohol
2. Vitamins
3. Herbs
4. Supplements

H. Family history

A positive family history of bleeding problems is helpful in determining the etiology of a patient's bleeding disorder; however, a negative family history does not rule out the possibility of an inherited bleeding disorder. Spontaneous mutations are not infrequent and may be as high as 30% in classical hemophilia. Inquire into all blood related family members.

**Thrombotic History**

The following information should be obtained through a patient history when a patient presents with a venous thrombotic episode.

A. Age of Initial Presentation

1. Infancy - young adulthood - most likely inherited
2. Adults - inherited or acquired
3. Elderly - most likely acquired

B. General Considerations

1. Does the patient have a history of venous thrombosis?
2. Does the patient have a family history of venous thrombosis?
3. What are the anatomic sites of the venous thrombosis?
4. Are the thrombotic events recurrent?
5. Was the thrombosis spontaneous or provoked.
6. Is there a history of 3 or more first trimester pregnancy losses or any 2nd or 3rd trimester loss?
7. Is there a history of severe preeclampsia, intrauterine growth restriction or placental abruption?
8. Is there a history of malignancy?
9. What are the results of previous diagnostic testing for thrombotic evaluation?
   a) Laboratory tests
   b) Venography, scans
10. What underlying disease states are evident in this patient?
11. Did the patient previously receive or is the patient currently receiving antithrombotic therapy?
12. What is the age of the patient?
13. What is the ethnic origin of the patient?
14. What is the patient’s blood type?

C. Clinical situations associated with thrombosis
   1. Past history of venous thrombosis
   2. Trauma
   3. Malignancy
   4. Immobilization
   5. Inflammatory disorders and autoimmune diseases
   6. Myeloproliferative Disorders
   7. Nephrotic Syndrome
   8. Obesity
   9. Surgery
   10. Pregnancy, post partum state
   11. Hormone Use

D. Medications
   1. Estrogen: oral contraceptives, Hormonal Replacement Therapy, Selective Estrogen Receptor Modulator (SERMS) Therapy (e.g. Tamoxifen)
   2. Chemotherapy
   3. Antithrombotic Agents

E. Multiple Hits
   It is not unusual for a patient with thrombosis to have more than one risk factor for thrombosis such as an inherited thrombophilic disorder and an acquired risk factor (e.g. pregnancy).

F. Family History
   A positive family pedigree for inherited thrombotic disorders is useful in evaluating the patient. However, a negative history does not rule out the presence of inherited disorders of thrombosis.
References


Diagnostic Approach to Bleeding Diathesis

*If screening studies are normal, consider FXIII deficiency, plasminogen activator inhibitor-1 deficiency, or alpha 2-antiplasmin deficiency.

**Antiphospholipid antibody is not associated with bleeding diathesis unless there is thrombocytopenia or hypoprothrombinemia.

This schematic is a general guideline for bleeding diathesis evaluation.

(Protocols may vary among institutions)
Diagnostic Approach to Venous Thrombotic Risk

Abnormal Antithrombin, Protein S, and Protein C plasma-based assays should be confirmed and acquired causes of deficiencies excluded before a diagnosis of hereditary deficiency is made.

This schematic is a general guideline for thrombotic risk evaluation. (Protocols may vary among institutions)
SPECIMEN COLLECTION AND PRE-ANALYTICAL VARIABLES

Introduction

The accuracy of hemostasis testing is predicated on the proper collection and management of the blood specimen. The established protocol of an institution must be strictly adhered to if results are to be meaningful. A number of external factors and pre-analytical variables that can be controlled to ensure quality test results are addressed below.

Patient History

A thorough patient history and complete physical examination are necessary to accurately interpret coagulation studies and to ascertain if there are medications or conditions that may affect coagulation test results. Herbal preparations, whether ingested as pills, teas or applied topically, may interfere with hemostasis especially in those individuals on anti vitamin K therapy. Numerous over-the-counter drugs contain aspirin and other non-steroidal anti-inflammatory agents can suppress platelet function for up to seven days. Anticoagulant therapy, including warfarin, heparin and direct thrombin inhibitors typically prolong the prothrombin time, activated partial thromboplastin time, and thrombin clotting time. Anticoagulant therapy also affects the accuracy of protein C, protein S, and antithrombin assays. Patients who have suffered a significant recent bleeding or thrombotic episode are likely to have decreased coagulation protein levels as a result of consumption. This acquired decrease may lead to an incorrect interpretation as an inherited deficiency.

Specimen Collection

Specimens ideally should be collected at a consistent time of day (usually early morning), to eliminate the effects of circadian variation of certain coagulation proteins. Specimens may be collected in an evacuated tube system or by syringe technique. Twenty and 21 gauge needles provide a reasonable amount of comfort for the patient and do not mechanically disrupt platelets or induce hemolysis.

When specimens are collected using an evacuated tube system, the tube must be made of non-reactive or non-wettable surface such as siliconized glass or plastic. Tubes with borosilicate, soda lime glass, or soft glass cannot be used as these materials may activate coagulation factors. Immediately following phlebotomy, the specimen should be gently inverted to adequately mix the blood with the anticoagulant. The number of inversions recommended by the tube manufacturer may vary depending on whether the evacuated tube is plastic or glass. The evacuated tube system may cause mechanical disturbances during collection and is not appropriate for platelet function studies.

Syringes are often employed with butterfly needles. This method produces less turbulence to the specimen than evacuated tubes but is more costly. Blood must be transferred from the syringe to an evacuated tube immediately after collection. A straight needle is affixed to the syringe and used to puncture the cap of the evacuated tube. When blood is transferred to the tube, it should be allowed to flow gently down the side to avoid turbulence. This transfer should occur immediately after collection, and the specimen gently inverted with the anticoagulant. If the stoppered top is removed, care must be taken not to overfill the tube. NCCLS H18-A3 requires transfer from syringe by puncturing the cap, thus allowing for accurate tube fill. The syringe technique is both costly and time consuming and thus is not generally used for routine collection.

Although not preferred, some circumstances require blood collection through indwelling catheters. In this case, the tubing must first be flushed with 5 mL of saline, then 5 mL or six times the catheter volume of blood must be collected and discarded before the coagulation specimen is obtained.

Collection techniques should minimize tissue trauma at the site of collection. A free-flowing puncture technique is optional. Multiple punctures or patting of the venipuncture site can result in an unacceptable specimen. Additionally, the activated partial thromboplastin time (APTT) can shorten following traumatic or repeated punctures.

Likewise, the tourniquet should be applied loosely and application time should be limited to one minute or less to avoid excess tissue thromboplastin release or hemolysis. Prolonged application of the tourniquet can also slow venous circulation and elevate coagulation factors such as factor VIII, PF 1+2, and tissue plasminogen activator. In some circumstances, the tourniquet should be removed before the sample is obtained.
**Anticoagulant**

When filling a vacuum system tube, the tube should be filled to the proper level. This ensures that the whole blood to anticoagulant ratio is 9:1. 90% fill is ideal but with certain reagents, a 70% fill volume is acceptable. Unless studies are performed locally to determine minimum fill volume, a 90% volume should be used. Overfilling a tube is not acceptable and to avoid this, the stopper should not be removed whenever possible to fill the evacuated tube. The recommended anticoagulant for coagulation testing is buffered sodium citrate at a concentration of 3.2% although 3.8% sodium citrate is also available. This anticoagulant maintains the pH near physiologic values and binds calcium, preventing clot formation. If the patient's hematocrit is above 55%, the amount of anticoagulant should be adjusted using the following formula:

\[
C = 1.85 \times 10^{-3} \times (100-H) \times V
\]

Where:
- \(C\) = volume of sodium citrate in milliliters
- \(H\) = hematocrit in percent
- \(V\) = volume of whole blood in milliliters

Blood specimens drawn from patients receiving thrombolytic therapy, i.e. urokinase, Streptokinase or tissue plasminogen activator (t-PA), contain free plasmin. Once plasmin is generated, it proteolytically degrades fibrinogen, factors V (FV) and VIII (FVIII), and platelets. This plasmin activity continues in vitro in the collection tube. Therefore, it is recommended that specimens collected for evaluation of possible fibrinolytic system imbalance (particularly those patients receiving plasminogen activators) be placed immediately on melting ice until centrifugation and separation of plasma, and frozen or tested immediately. Two products, Aprotinin and PPACK, have demonstrated their ability to effectively inhibit plasmin in several clinical trials of thrombolytic therapy. These substances can be added to the standard sodium citrate collection tube for coagulation testing.

**Unsuitable Specimens**

Plasma specimens that are hemolyzed or contain even small clots are unacceptable for coagulation studies. Hemolysis may cause activation of clotting and should be avoided. Likewise, specimens with significant icterus or lipemia can produce invalid results in optical measurement methods.

**Specimen Transport**

Although it has been recommended that specimens be placed on ice following phlebotomy, current recommendations are to allow the specimen to remain at room temperature to avoid cold activation of factor VII and alteration of platelets, if transportation to site of testing occurs within about 2 hours. Samples which will not be tested within 2 hours should be centrifuged, the plasma removed and refrigerated if testing will be done within 4 hours or quick frozen at -20°C or below. Samples from patients on unfractionated heparin should be processed within an hour of drawing. Samples for prothrombin time determination are stable for 24 hours at room temperature if during transport and storage the specimen tube remains sealed, preventing loss of carbon dioxide and resulting pH increase.

**Centrifugation and Storage**

All specimens, except those for DNA analysis, should be processed to be platelet free to eliminate the potential for the release of platelet factor 4 and phospholipids. Platelet factor 4 is an inhibitor of heparin and can affect the monitoring of heparin. Platelets in a sample can also neutralize the activity of a weak lupus anticoagulant.

All hemostasis specimens, except those for platelet testing and DNA analysis, should be centrifuged within 1 hour of collection at a
minimum of 1500 x g. Specimens should be centrifuged at an adequate speed and time to ensure the plasma platelet count is less than 10,000/μL. It may be necessary to adjust centrifugation force and time to obtain this count. Many laboratories choose to respin or filter plasma after the initial centrifugation to achieve this plasma platelet count.

After centrifugation, plasma should be stored at 2°-4°C and assayed within four hours, or quick-frozen at -20°C or lower when the specimen is to be held more than four hours. It is important to maintain samples in freezers that are not frost-free (i.e. freezers that have automatic defrost cycles).

Samples for DNA testing are kept as whole blood at room temperature or refrigerated.

**Standardization**

Several publications have been issued to ensure standardization among laboratories with regard to collection and processing. In the United States the majority of laboratory standards are set by Clinical Laboratory Standards Institute (CLSI) formerly known as The National Committee for Clinical Laboratory Standards (NCCLS). All specimen requirements contained within this publication follow the guidelines presented in the following documents. Documents that pertain to coagulation testing include: CLSI Document H21-A3; Collection, Transport and Processing of Blood Specimens For Coagulation Testing and Performance of Coagulation Assays (Second Edition), CLSI Document HN18-A; Procedure for the Handling and Processing of Blood Specimens, CLSI Document 28/29-A; One-Stage Prothrombin Time Test and Activated Partial Thromboplastin Test.

**Conclusion**

Pre-analytical variables can result in erroneous test results and delays in accurate patient diagnosis. When standard protocols are employed, variables can be controlled and in some cases eliminated. It is essential that laboratorians ensure quality analysis by reducing pre-analytical variables.

**References**


ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT)

Physiology

The activated partial thromboplastin time (APTT) is the clotting time in seconds when citrated plasma is added to a contact activator (ellagic acid, Kaolin, celite), phospholipid and calcium ions. The APTT tests for deficient activity of procoagulants in the intrinsic and common pathways including high molecular weight kininogen, prekallikrein, factors XII, XI, IX, VIII, X (FXII, FXI, FIX, FXIII, FX, FV), prothrombin, and fibrinogen. Deficiency or inhibition of any of these factors prolongs the APTT result.

Thrombin is first generated in vivo through activation of tissue factor via the extrinsic pathway. Amplification of thrombin activity is achieved when extrinsic pathway generated thrombin activates FXI with subsequent activation of FIX. FIXa complexes with FVIIIa, calcium, and phospholipid (tenase complex), activating FX. FXa binds FVa, calcium, and phospholipid to form a second complex, prothrombinase, which then activates thrombin, forming additional thrombin. Thrombin cleaves soluble fibrinogen to produce fibrin monomers. These monomers polymerize, forming a fibrin clot which is then stabilized by FXIII.

Alternatively, the “intrinsic” system may be activated by negatively charged surfaces such as heart valve prostheses or vascular stents (in vitro, this activation occurs by contact with glass or particulate matter, i.e. silica, kaolin, ellagic acid, etc.). In the presence of a negatively charged surface, FXII becomes activated. FXIIa, in the presence of high molecular weight kininogen and prekallikrein, activates FXIa, and the pathway proceeds as described above.

Acquired and Congenital Factor Deficiencies

The APTT test was originally utilized to detect congenital single factor deficiencies such as those occurring in hemophilia, and is prolonged in all intrinsic pathway factor deficiencies. The APTT is sensitive to deficiencies of high molecular weight kininogen (HMWK), prekallikrein, FXII, FXI, FIX, and FVIII. The prothrombin time (PT) is unaffected by these deficiencies. Severe deficiencies of FX, FV, prothrombin, or fibrinogen result in prolongation of both the APTT and PT. The APTT is often ordered to establish the cause for patient bleeding or to disclose possible coagulation defects in those about to undergo an invasive procedure.

Acquired multiple factor deficiencies may be seen in vitamin K deficiency, disseminated intravascular coagulation (DIC), and in liver disease. In vitamin K deficiency, FIX, FX, FVII and prothrombin activities diminish, prolonging the APTT and PT results. In the other listed conditions, nearly all factors are deficient due to either consumption or lack of production, respectively.

Unfractionated Heparin Therapy and the APTT

Unfractionated heparin is an effective, fast-acting anticoagulant that has been widely used in the past to slow the propagation of thrombi in the venous and arterial systems. It is being replaced as the drug of choice by others (i.e. low molecular weight heparins, direct thrombin inhibitors, etc.) that have better dose response, longer plasma half lives, less chance of heparin-induced thrombocytopenia (HIT) and osteopenia.

Unfractionated heparin’s therapeutic range in plasma is narrow. Overanticoagulation with heparin may cause hemorrhage, while inadequate dosage permits additional thrombus formation. Heparin enhances plasma antithrombin’s ability to bind and inactivate the serine proteases FXII, FXI, FIX, FX, and thrombin, thus prolonging the APTT. The APTT is the most often used test to monitor unfractionated heparin therapy.

Laboratories that offer the APTT for heparin management must establish the optimal therapeutic range based on APTT results. This should be done by performing the APTT on 30 to 60 heparinized patient specimens at all levels of anticoagulation. Anti-FXa assays should also be performed on these same specimens to establish actual heparin levels at the corresponding APTT value. The therapeutic range for the APTT is the range in seconds that corresponds to levels of 0.3 to 0.7 IU/mL of unfractionated heparin. The optimal therapeutic range is affected by the relative sensitivity of the APTT reagent and the instrument used for measurement. The therapeutic range must be recalculated each time there is a change of APTT reagent or instrumentation.

Factor Inhibitors and the APTT

Factor inhibitors are the most common cause for a prolonged APTT in the absence of heparin therapy. The most common inhibitor is the lupus anticoagulant, present in 1% of the unselected population. Lupus anticoagulant, despite its name, is associated with venous and arterial
thrombosis and not with a bleeding diathesis. It is a member of the antiphospholipid protein complex family of antibodies that binds and neutralizes APTT reagent phospholipid to cause prolongation of the APTT test results. Specific inhibitors such as anti-FVIII will also prolong the APTT, and are associated with bleeding manifestations. These may be alloantibodies that arise in response to factor therapy in hemophiliacs or autoantibodies, such as acquired anti-FVIII antibodies, that appear spontaneously in older patients and with pregnancy. Mixing studies are used to help distinguish specific (such as FVIII) and non-specific (lupus anticoagulant) inhibitors, determine heparin presence, and to detect single or multiple factor deficiencies.

**Assay Principle**

Purified phospholipid and negatively charged particulate activator, such as silica, kaolin or ellagic acid, are incubated with test plasma to activate the contact factors. Calcium chloride is then added, and the mixture is timed until a clot forms. The degree of prolongation is proportional to the extent of deficiency or inhibition of factors.

**Assay Performance Characteristics**

APTT reagents vary in responsiveness to factor deficiencies and the effects of heparin and inhibitors. This variation is magnified by the variety of instrument systems available for testing. Any change in reagent lot number or manufacturer, or change in instrument calibration necessitates recomputation of the APTT reference range and heparin therapeutic range.

**Activated Partial Thromboplastin Time Mixing Studies**

An isolated prolonged APTT result with a normal prothrombin time result implies either the presence of heparin, an inhibitor (either specific factor or lupus anticoagulant), or a factor deficiency of the intrinsic system. Unless the cause for the prolonged APTT is known, as in the case of heparin therapy, mixing studies may be necessary to determine the etiology of the prolonged result.

To perform mixing studies, the patient specimen is mixed with an equal volume of normal platelet free plasma, and another volume mixed with an equal volume of veronal buffered saline. APTTs are performed on each of these mixtures. An aliquot of the normal plasma/patient plasma mixture is also incubated at 37°C for one to two hours and then an APTT performed to further clarify the cause of the prolongation. As a control, normal platelet free plasma and patient plasma are also incubated separately and then mixed after this incubation as a control for possible factor degradation during incubation.

**Mixing Studies Assay Characteristics**

If the result obtained with the immediate normal plasma mixture corrects to within the reference range and the saline mixture result increases dramatically, a factor deficiency or specific factor inhibitor is suspected. With factor deficiency, heparin or a specific factor inhibitor, the 1:1 saline mix shows significant prolongation of the APTT result. If the result obtained with the immediate normal plasma mixture shows only partial or no correction and the result obtained with the saline mixture shows near correction to the reference range or only mild prolongation, then a non-specific inhibitor such as lupus anticoagulant (LA) is suspected. After incubation at 37°C, an APTT result equivalent to that of the original mixture is indicative of a factor deficiency or heparin. If the result of the APTT on the incubated mixture demonstrates further prolongation (with the control remaining equivalent to the original result), a time dependent specific factor inhibitor is indicated. A specific FVIII inhibitor may show initial correction of the original mixture, but will demonstrate prolongation upon incubation.

Factor deficiencies should be further identified by performing specific factor assays. Plasma suspected of containing a lupus anticoagulant (LA) should be further tested with other LA specific tests [i.e. Dilute Russell Viper Venom Time (DRVVT), Platelet Neutralization Procedure (PNP), or the Hexagonal Phospholipid Neutralization Procedure] to confirm the presence of a lupus anticoagulant. Specific factor inhibitors such as FVIII antibodies should be identified and assayed using the Bethesda titer methodology.

If heparin is suspected and clinical confirmation is not available, a thrombin time with the addition of a heparin neutralizer may be performed. Hepzyme® (Dade Behring) neutralizes heparin and corrects the thrombin time into or near the reference range.
Reference Range

The APTT adult reference range varies with the reagent/instrument combination used and is approximately 23.7 – 37.7 seconds.

Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen on dry ice for next day delivery.

Test Request Information

APTT (#300040) and APTT Mixing studies (#300806) may be ordered separately from the Esoterix Service Directory. CPT code: APTT 85730, APTT mixing studies 85730 and 85732 x3.

Reflex Recommendations

An isolated prolonged APTT result implies either the presence of heparin, an inhibitor (either specific factor or lupus anticoagulant), or a single or multiple factor deficiency of the intrinsic system. Unless the cause for the prolonged APTT is known, as in the case of heparin therapy, mixing studies may be necessary. If mixing studies indicate the possibility of single or multiple factor deficiencies, a PT may be performed to differentiate between common pathway and intrinsic pathway deficiencies. Factor assays should then be performed to determine specific factors involved. If a factor inhibitor is suspected specific factor assays and inhibitor titers may be necessary. If heparin is suspected without clinical confirmation, a thrombin time after neutralization of heparin should be performed on the original specimen. A lupus anticoagulant may be confirmed by doing the DRVVT, PNP and/or hexagonal phospholipid neutralization tests as well as antiphospholipid antibody immunassays. A patient with an initial positive antiphospholipid antibody or lupus anticoagulant test should be retested after twelve or more weeks to determine if the antibody is persistent.

References

Physical Characteristics

Activated protein C (APC) is the functional form of the naturally occurring, vitamin K dependent anticoagulant, protein C. Resistance to the anticoagulant function of activated protein C is a common condition that can be inherited or acquired. In at least 90% of cases, a poor response to activated protein C is due to a single point mutation in the factor V (FV) gene that results in a substitution of glutamine for arginine in amino acid number 506 (nucleotide 1691). This mutation is also known as FV Leiden mutation (G1691A or R506Q) and in the heterozygous state, occurs in about 5% of the Caucasian population. The FV Leiden mutation causes the FV protein to be relatively resistant to APC-induced inactivation. The presence of APC resistance, as determined by a clotting based assay, is often used to screen for the FV Leiden mutation.

Physiology

Activated protein C functions as an anticoagulant by inactivating normal coagulation factors Va and VIIIa in the presence of its cofactor protein S. When added to normal plasma, therefore, APC causes prolongation of clotting time due to inactivation of FV and FVIII. Single nucleotide polymorphisms (SNP) in the FV molecule at the APC binding sites (amino acid 306 and 506) have been described that impair activated protein C’s ability to inactivate FVa. FV Leiden (R506Q) is the most common hereditary cause of APC resistance. FV Hong Kong (R306G) and FV Cambridge (R306T) are FV polymorphisms involving the APC binding site, with very low frequencies in the general population. Although plasma from patients with FV Hong Kong do not typically demonstrate resistance to APC, plasma from FV Cambridge individuals do. Another inherited cause of APC resistance is the HR2 haplotype. This haplotype is commonly identified by a polymorphism that replaces histidine with arginine at amino acid 1299 in the B domain of the FV protein, also referred to as A4070G mutation or R2 polymorphism. The R2 polymorphism is linked to at least 6 other polymorphisms, and this collection of SNP’s that is inherited as a group, is referred to as HR2 haplotype.

The presence of acquired APC resistance depends significantly on the assay used to measure it. Assays can be based on the activated partial thromboplastin time (APTT) or the Russell’s viper venom time (RVVT). Individuals with elevated FVIII levels have a greater frequency of APC resistance when evaluated with APTT based assays. This acquired form of APC resistance occurs because elevated FVIII levels shorten the APTT and blunt the anticoagulant effect of APC. Lupus anticoagulants may cause a falsely low APC ratio due to prolongation of the clotting time. Acquired APC resistance has been described in individuals with underlying malignancy especially malignant myeloma and in women on estrogen therapy or during pregnancy. Acquired APC resistance is common in pregnancy, especially the third trimester due to elevations of factors V and VIII as well as decreased levels of protein S.

Incidence of Activated Protein C Resistance

Resistance to APC is to date the most common cause of inherited thrombophilia and is demonstrated in 20%-60% of patients with recurrent venous thrombosis. The FV Leiden genetic mutation is present in 3%-7% of persons of Northern European descent making this defect at least ten times more prevalent than other known genetic abnormalities predisposing to thrombosis. This cause of inherited thrombophilia is virtually absent from Asian, African, and Mediterranean populations. Acquired APC resistance is seen in up to 30% of pregnant women in the third trimester.

Clinical Significance

Patients with APC resistance ratios less than the laboratory’s established cut-off are at an increased risk for venous thrombosis. Deep venous thrombosis (DVT) is the most common clinical manifestation of APC resistance in a manner similar to protein C, protein S, and antithrombin deficiencies. Patients with heterozygous FV Leiden have a 5- to 10-fold increased risk of thrombosis, while patients with homozygous FV Leiden have a 50- to 100-fold increased risk of thrombosis. The risk of thrombosis is increased significantly if other contributing factors such as a genetic deficiency of protein C or protein S, FII G20210A polymorphism, pregnancy, oral contraceptive usage, or surgery is present concomitantly with APC resistance. Although APC resistance carries a life-long risk of thrombosis, many patients having the abnormality remain asymptomatic throughout their lifetime.

Individuals with APC resistance in the absence of FV Leiden are at increased risk of venous thrombosis and this risk appears to be graded, that is, the greater the APC resistance (the lower the ratio), the greater the risk of thrombosis.
**Assay Principle**

The assay for activated protein C resistance can be performed using an APTT-based or RVVT-based assay. The most common assay used is a modified APTT-based assay. Results are reported as the ratio of the APTT with and without the addition of exogenous activated PC. A common modification is performed by most laboratories whereby patient plasma is first mixed with FV deficient plasma and the heparin neutralizer polybrene. This is to correct for any factor deficiencies as may occur in patients on oral anticoagulant therapy and to normalize the APTT in patients on heparin therapy. The APTT is performed on two samples, one with APC added and the other without and a ratio of the two clotting times determined. This modified assay is highly specific and sensitive to the presence of the FV Leiden polymorphism. This assay can generally distinguish heterozygous FV Leiden from homozygous FV Leiden. The homozygous state is more common when the ratio is <1.3.

The APC resistance assay can be performed without the addition of Polybrene and FV deficient plasma although this assay shows interference due to heparin and factor deficiencies. Some investigators recommend this assay be performed in addition to the APC resistance assay with added FV deficient plasma in order to screen for abnormalities that may cause APCR other than FV Leiden. A disadvantage to this assay is that it may not distinguish patients with and without the FV Leiden mutation. This assay as well as the modified assay is not recommended in those with an abnormal baseline APTT as may occur in those on heparin or warfarin therapy. This assay cannot distinguish heterozygous from homozygous and may be affected by elevated FVIII levels.

**Reference Range**

The patient is APC resistance positive (using the added FV deficient plasma assay) if the ratio of the APTT with the addition of purified APC to the activated partial thromboplastin time without APC is below the established laboratory cut-off (e.g. less than 2.4). Unaffected persons have an APC ratio with FV deficient plasma ratio of 2.4 to 4.0. The reference range for APCR without FV deficient plasma is 1.9 to 4.0.

**Assay Performance Characteristics**

The assay is routinely performed using patient plasma diluted with FV deficient plasma and the reagent frequently contains a heparin neutralizer. The addition of FV deficient plasma makes the assay more specific for the mutations in the FV molecule and allows the assay to be performed on plasma from patients on oral anticoagulant or heparin therapy. The assay with added FV deficient plasma may also perform better in those with lupus anticoagulant, especially if an RVVT-based clotting time is used. However, patients with an activated partial thromboplastin time prolongation due to the presence of lupus anticoagulant are not appropriate candidates for APC resistance testing. Lupus anticoagulants may interfere with the assay and cause a falsely low APCR. Testing for the presence of FV Leiden in such individuals should be performed using a molecular assay. The normal range for healthy individuals and heterozygotes overlaps; therefore, a low normal ratio may require genetic confirmation. The APC resistance screen does not reliably distinguish between patients with heterozygous or homozygous resistance to APC, although some investigators believe that APC ratios below 1.3 indicate homozygosity. APC resistance has been shown to interfere with some functional protein S assays. Repeated freeze-thaw cycles of plasma samples may result in lowering of the APC ratio also.

**Specimen Requirements**

Citrate plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109) buffered sodium citrate. Ensure complete fill of the evacuated tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.
Test Request Information

The activated protein C resistance assay (#300716) can be ordered individually from the Esoterix Service Directory. CPT Code 85307.

Reflex Recommendations

Due to its relatively high prevalence, APC resistance should be evaluated in patients with a history of venous thrombosis. When the APC resistance assay is positive (ratio < 2.4 in this laboratory), it is recommended that DNA confirmation of the FV Leiden mutation be performed. Evaluation of the HR2 haplotype may be of value, as it is believed that the risk for developing thrombosis is further increased when both FV Leiden and the HR2 haplotype are present. This combination may cause a lower APCR than the presence of FV Leiden alone. The kindred of individuals with APC resistance or FV Leiden should be screened for the abnormality to assess thrombotic risk in family members. If the FV Leiden genetic assay is negative, the APC resistance may be acquired or due to another mutation as is the case in approximately 5 to 10% of APC resistant patients.

Therapy for Activated Protein C Resistance

Acute thrombotic episodes are managed by heparin and long-term warfarin therapy maintaining an INR of 2 to 3. Prophylactic therapy may be given in high-risk situations and prolonged therapy is recommended for those who suffer recurrent thrombosis.

References

**Physical Characteristics**

Cleavage of the ultra-large von Willebrand factor (ULvWF) multimers is mediated by a protease, which is in the ADAMTS family of metalloproteases. ADAMTS stands for "A Disintegrin-like And Metalloproteinase with ThromboSpondin type 1 repeats." The particular protease responsible for cleavage of ULvWF multimers is ADAMTS-13, so named, as it is the 13th member of the 19 members of the ADAMTS family of proteases discovered to date.

**Physiology**

ADAMTS-13 requires the presence of a divalent cation such as Ca++ to function. ADAMTS-13 cleaves the peptide bond tyrosine 842-methionine 843 in the vWF. When the activity of ADAMTS-13 is diminished, the ULvWF multimers accumulate and remain bound to the vascular surface where they serve to collect platelets. In the presence of ADAMTS-13, the ultra-large vWF multimers are cleaved and the bound platelets are released back into the circulation. This ADAMTS-13 based cleavage assists to prevent the development of microvascular platelet aggregates, also known as hyaline thrombi.

**Etiology of Deficiency**

Low levels of ADAMTS-13 are associated with thrombotic thrombocytopenic purpura (TTP). Levels of ADAMTS-13 can be decreased due to a congenital or acquired deficiency. TTP is estimated to be 1 per 200,000 individuals per year or higher. TTP is not a uniform disease; it has a variety of different etiologies and varies significantly in its clinical presentation between patients. In most adults and about 20% of children, the development of TTP is idiopathic. TTP may occur in the setting of underlying infection, pregnancy, cancer, organ transplantation, collagen vascular disorders and with certain drug therapies (such as clopidogrel and ticlopidine). In the majority of children and in a minority of adult patients, the predominant etiology is infection with Shiga toxin producing E-coli (0157:H7) or less commonly other infectious agents associated with the development of bloody diarrhea. Acute idiopathic TTP accounts for about 90% of all cases in adults. Most individuals suffer a single episode of TTP but it may be recurrent or episodic in up to 20% of patients and in about 10% if the disorder follows a chronic course. Rarely, TTP occurs as a familial disorder due to a homozygous or doubly heterozygous mutation in the ADAMTS-13 protease, leading to a severe protease deficiency.

**Clinical Significance of Deficiency**

ADAMTS-13 deficiency can be congenital or acquired. Severely decreased levels of vWF-protease activity (less than 5% of normal human plasma) is associated with the development of TTP. Deficiency of ADAMTS-13 predisposes a patient to microvascular thrombosis as the ultra-large vWF multimers persist and collect platelets on the vascular surface causing the development of hyaline thrombi. It is generally believed that the thrombotic microangiopathy ensues only when secretion of vWF is enhanced, such as following DDAVP administration or in the third trimester of pregnancy.

**Assay Principle: von Willebrand Protease Activity**

Test plasma is added to a small synthetic fragment of vWF. Cleavage of this synthetic peptide between two modified residues by ADAMTS-13 in the sample releases the fluorescence quenching capabilities. The activity is detected fluorimetrically.
**Assay Principle: von Willebrand Protease Inhibitor (Autoantibody)**

An inhibitor to ADAMTS-13 may be detected by mixing with normal plasma in a Bethesda-type inhibitor assay and assaying in the ADAMTS-13 activity assay.

**Assay Performance Characteristics von Willebrand Protease**

VWF protease is deficient in hereditary TTP, idiopathic acute TTP, intermittent relapsing TTP and ticlopidene and clopidogrel-induced TTP but is generally normal in Hemolytic Uremic Syndrome (HUS) and bone marrow transplant-related TTP. The low VWF protease activity can be due to a constitutional deficiency or more commonly, reflect the presence of a neutralizing antibody (autoantibody).

Severe deficiency of ADAMTS-13, i.e., levels less than 5%, is considered to be specific for TTP. The presence of antibodies to VWF protease would be considered confirmatory for TTP. While ADAMTS-13 levels less than 5% are rather specific for TTP, the sensitivity of this deficiency for TTP remains questionable. In a study of patients with non-idiopathic TTP, ADAMTS-13 was severely decreased in only 47 of 66 patients suggesting a sensitivity of only 71%. Slightly decreased protease activity, in the range of 25 – 50% and even moderately decreased activity in the 10 – 25% range, occurs rather commonly in a variety of disorders including disseminated intravascular coagulation (DIC), heparin – induced thrombocytopenia (HIT) and immune thrombocytopenia purpura (ITP). Levels are also commonly decreased, but not into the severely decreased range, in the newborn period and in the second and third trimesters of pregnancy.

**Assay Performance Characteristics von Willebrand Protease Inhibitor**

Antibodies against VWF protease have been identified in acute idiopathic and intermittent relapsing TTP and occasionally in hereditary TTP. Mild to moderate deficiency of ADAMTS-13 activity has been observed in multiple medical conditions. Levels are generally normal in patients with marrow transplant-associated thrombotic microangiopathy and childhood hemolytic uremic syndrome. Recent plasma exchange therapy may alter ADAMTS-13 activity and inhibitor levels.

This assay result may assist in diagnosis. Its utility for management of patients with acute thrombotic microangiopathies remains to be defined through clinical observations.

**Reference Range**

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<td>von Willebrand Factor Protease Activity</td>
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**Specimen Requirements**

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Aliquot the plasma into two plastic tubes – 1mL each, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimens overnight on dry ice.
Test Request Information

vWF protease activity and vWF protease inhibitor (#300326) can be ordered together from the Esoterix Service Directory. This assay is not performed at Esoterix, Inc. but is a send-out test. CPT Codes: vWF protease activity – 85246; vWF protease inhibitor – 85335.

Reflex Recommendations

If the vWF protease is <6%, evaluation for the vWF protease inhibitor should be performed.

References


Physical Characteristics

α2-antiplasmin, also known as α2-plasmin inhibitor, is a 70,000 Dalton glycoprotein produced in the liver. It is found at low concentrations in the alpha granules of platelets. α2-antiplasmin circulates in two forms in the plasma which differ in their capacity to bind plasminogen. Approximately 70% of circulating α2-antiplasmin is able to bind plasminogen. With a plasma concentration of 7 mg/dL, α2-antiplasmin has a concentration approximately half that of plasminogen. Its plasma half-life is approximately 3 days; however, once bound to plasmin, the complex has a half-life of 12 hours.

Physiology

α2-antiplasmin serves three major functions: 1) It is considered the major inhibitor of plasmin and does so by rapidly forming a 1:1 complex, 2) It interferes with the adsorption of plasminogen to fibrin by noncovalently binding to lysine binding sites on the plasminogen and plasmin molecules; and 3) It is cross-linked with α-chains of fibrin during clotting though the action of factor XIIa making the clot resistant to fibrinolysis. α2-antiplasmin is the primary and most abundant physiologic inactivator of plasmin. Its binding to plasmin decreases the rate of local fibrinolysis and enhances clot retention. α2-antiplasmin is not effective against plasmin located near the fibrin surface of the clot since the fibrin interferes with the binding of α2-antiplasmin to plasmin.

Etiology of Deficiency

Congenital deficiency is inherited in an autosomal dominant manner. Acquired deficiencies of α2-antiplasmin are found in liver disease, nephrotic syndrome, cardiopulmonary bypass, systemic amyloidosis, acute promyelocytic leukemia, disseminated intravascular coagulation, and during L-asparaginase therapy. Very low levels of α2-antiplasmin can be seen during thrombolytic therapy.

Incidence of Deficiency

The inherited deficiency state is rare, while acquired deficiencies are more common.

Clinical Significance of Deficiency

Congenital deficiencies may be associated with severe bleeding disorders in the homozygous individual and symptomatic or mild hemorrhagic manifestations in the heterozygous individual.

Assay Principle

Diluted plasma is incubated with a precise excess of plasmin, resulting in rapid complex formation between plasmin and functional α2-antiplasmin in the sample. Plasmin inhibition is directly proportional to antiplasmin in the sample. The residual plasmin hydrolyzes a chromogenic substrate and liberates the pNA chromophore which is read photometrically at 405 nm. Color intensity is inversely proportional to α2-antiplasmin activity in the sample.

Assay Performance Characteristics

At low levels of α2-antiplasmin, an increased level of α2-macroglobulin may interfere with the assay.

Reference Range

The reference range by chromogenic assay for α2-antiplasmin activity in plasma is 80 - 150%.
Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109) buffered sodium citrate. Ensure complete fill. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Test Request Information

Alpha 2-antiplasmin activity (#300039) can be ordered separately from the Esoterix Service Directory, CPT Code 85410.

Reflex Recommendations

This assay is usually requested as part of a fibrinolytic evaluation which may include fibrinogen, PAI-1, tissue plasminogen activator, and plasminogen. It may also be ordered as part of an evaluation for a bleeding diathesis with a normal APTT and PT.

References

**Physical Characteristics**

Lupus anticoagulants (LA) and “antiphospholipid” antibodies (APLs) represent a heterogeneous family of IgG, IgM or IgA antibodies (immunoglobulins) associated with a variety of clinical conditions, most commonly arterial or venous thrombosis and fetal loss. The “antiphospholipid syndrome” (APS) is a term used to describe the association of these clinical constellations with laboratory identification of a specific group of antibodies, either lupus anticoagulants or anticardiolipin antibodies (IgG and IgM). The term “antiphospholipid” is now realized to be a misnomer, as these antibodies are not directed solely against anionic phospholipids as once believed, but rather phospholipid binding proteins. Many of the proteins which bind phospholipid to form these antibody-inducing complexes are involved in coagulation and include β2 glycoprotein-1, prothrombin, factor XI, activated Protein C (PC), Protein S (PS), high molecular weight kininogen and annexin V. The antiphospholipid protein complex family of antibodies includes, but is not limited to, anticardiolipin, antiphosphatidylserine, anti-β2 glycoprotein 1, anti-annexin antibodies and antiprothrombin antibodies, in addition to lupus anticoagulants.

**Physiology**

Antiphospholipid antibodies are part of a heterogeneous group of antibodies that, when persistent, may be associated with the antiphospholipid syndrome characterized clinically by arterial or venous thrombosis and fetal loss. APLs react with negatively charged phospholipids in the presence of protein cofactors such as (β2-GPI) or prothrombin. APLs, if persistent, are the most common form of acquired thrombophilia.

**Incidence and Etiology**

APLs are detected in 1% -2% of unselected individuals. APLs are present in up to 50% of patients with systemic lupus erythematosus (SLE) and are also seen in other autoimmune diseases. In these circumstances, the antibodies are called secondary and the syndrome, if present, is referred to as secondary APS. APLs may also arise in association with malignancies and immune deficiency syndrome, as well as with certain drugs. Transient APLs are found with many types of infection, especially viral, and appear to have no adverse clinical effects. APLs may also arise spontaneously to cause primary APS.

Certain APLs will, in addition to reacting in enzyme immunoassay systems, prolong the results of phospholipid-dependent clot-based assays such as the activated partial thromboplastin time (APTT), dilute Russell’s viper venom time (DRVVT), platelet neutralization procedure (PNP), kaolin clotting time (KCT), hexagonal phospholipid neutralization, and tissue thromboplastin inhibition test (TTIT). When this occurs, they are called lupus anticoagulants (LA), although they have no anticoagulant properties in vivo.

**Screening for APLs is important in a number of clinical scenarios and should be considered in the following populations:**

1. Patients who suffer venous thrombosis, especially if idiopathic.
2. Patients who suffer an arterial occlusive event such as myocardial infarction or stroke before the age of 50 years.
3. Individuals greater than 50 without typical risk factors for arterial occlusive disease (e.g. hyperlipidemia, hypertension, diabetes mellitus) who suffer MI or stroke.
4. Women who suffer 3 or more first trimester pregnancy losses or a single unexplained fetal loss in the second or third trimester.
5. Women who suffer severe preeclampsia or intrauterine growth retardation without a known provocator.
6. All patients with systemic lupus erythematosus.

Drugs that may trigger APL formation include phenothiazine, various antibiotics, hydralazine, quinine and quinidine, calcium channel blockers, procainamide, and phenytoin. The antibodies induced by drugs are occasionally associated with thrombosis, and should be considered clinically significant if they persist.
ANTIPHOSPHOLIPID ANTIBODIES (cont.)

Clinical Significance

Antiphospholipid antibodies found in association with APS may present as either arterial or venous thrombosis or fetal loss. Arterial thrombotic events associated with APS include myocardial infarction (MI), stroke, and transient ischemic attacks (TIA). Venous-associated findings are most commonly deep vein thrombosis (DVT) and pulmonary embolus (PE). Pregnancy related complications include infertility, intrauterine growth retardation and recurrent fetal loss (spontaneous abortion) or fetal demise, as well as severe preeclampsia. Not all individuals with APLs suffer clinical consequences of these antibodies. Development of APS is more likely when patients test positive in multiple assay systems such as lupus anticoagulant, anticardiolipin antibodies (ACA), anti β2-glycoprotein I and antiphosphatidylserine assays. Literature suggests that LA is more often related to thrombotic complications than ACA alone and that antibodies to, β2-GP I are also more predictive of clinical consequences than presence of ACA antibodies alone.

Anti-annexin antibodies may be of particular importance in a pregnant population and/or a population trying to conceive. Placental villi are coated with annexin A5, a potent anticoagulant protein. This protein functions as an anticoagulant as it shields the negatively charged phospholipids that are necessary to support fibrin clot formation. The presence of antibodies to annexin theoretically may interfere with this natural shield and predispose to vascular complications within the placenta. Annexin 5 is necessary for maintenance of placental integrity.

Laboratory Diagnosis of Lupus Anticoagulant

A diagnosis of LA should be made in accordance with the ISTH criteria. Before a diagnosis of LA is made, 4 criteria should be met, specifically there should be:

1. Prolongation of a phospholipid dependent clotting assay such as the APTT, hexagonal phospholipid neutralization, dRVVT or KCT.
2. Demonstration of the presence of an inhibitor through either mixing studies or factor assays.
3. Demonstration that the inhibitor is phospholipid dependent.
4. Demonstration that no other inhibitor is present such as a specific factor VIII inhibitor.

Due to the heterogenous nature of this family of antibodies, 2 or 3 phospholipid dependent clotting assays should be performed before the presence of a LA is ruled out. Only persistent antibodies are thought to be of clinical significance and for this reason, repeat analysis in twelve or more weeks is recommended.

| Antiphospholipid Antibody Syndrome*#: International Criteria for Classification |
|-------------------------------|-------------------------------|
| **Clinical**                  | **Laboratory**                |
| Venous thrombosis             | ACA positive, IgG or IgM at moderate to high levels |
| Arterial thrombosis           | β2-GP I positive IgG or IgM or both |
| Small vessel thrombosis       | LA positive: based on ISTH criteria |
| Pregnancy morbidity           |                               |
| - Recurrent fetal loss        |                               |
| One loss or morphologically normal fetus after 10 weeks |                               |
| 3 or > spontaneous abortions before 10 weeks |                               |
| - Preeclampsia or placental insufficiency with premature birth |                               |

* Patient classification as antiphospholipid antibody syndrome positive requires at least 1 clinical and 1 laboratory finding with demonstration of persistence over time. A repeat positive test in twelve weeks is required for the diagnosis.

Anticardiolipin Antibody (ACA) Immunoassay Principle

Diluted test plasma or serum is pipetted into a microtiter plate coated with purified cardiolipin antigen. Anticardiolipin antibodies in the test plasma bind to this antigen. Anti-human IgG, IgM or IgA is then added, binding captured antibody if present. A chromogen is added and the amount of color produced is directly proportional to the amount of antibody present as compared to a standard reference curve.

Anticardiolipin Antibody (ACA) Immunoassay Performance Characteristics

Diagnosis of APS requires that ACA IgG or IgM is positive at a moderate to high level and that these results are persistent over at least a 3 month period of time. Serum from seropositive syphilis patients may have elevated ACA results. Confirmatory syphilis testing should be performed to rule this out if clinically appropriate. ACAs may appear transiently during many infections and should be retested at least twelve weeks after the initial positive test result. When IgM ACA titers are extremely elevated, consideration should be given to the presence of a monoclonal immunoglobulin. Rheumatoid factor may interfere with the determination of IgM ACA. ACA testing is the most commonly used solid phase assay for the detection of antiphospholipid antibodies. This presence of antibodies to cardiolipin is relatively sensitive for APS but not very specific for the thrombotic and obstetric complications associated with APS.

Antiphosphatidylserine (APTS) Immunoassay Principle

Diluted test plasma is pipetted into a microtiter plate coated with purified phosphatidylserine. Anti-phosphatidylserine antibodies in the test plasma bind to this antigen. Anti-human IgG or IgM is then added, binding captured antibody if present. A chromogen is added and the amount of color produced is directly proportional to the amount of antibody present as compared to a standard reference curve. β2 glycoprotein I is added to the test system to provide optimal binding of antiphosphatidylserine antibodies present in the plasma.

Antiphosphatidylserine (APTS) Immunoassay Performance Characteristics

Diagnosis of APS should not be made on the basis of one APTS result alone. APTS antibodies may appear transiently during many infections and should be retested after a minimum of six months. Like ACAs, antibodies to phosphatidylserine are sensitive for APS but not specific for the thrombotic and obstetric complications associated with APS.

Anti-β2 Glycoprotein I (β2-GP I) Immunoassay Principle

Diluted test plasma is pipetted into a microtiter plate coated with purified β2 antigen. β2 glycoprotein I antibodies in the test plasma bind to this antigen. Anti-human IgG, IgM or IgA is then added, binding captured antibody if present. A chromogen is added and the amount of color produced is directly proportional to the amount of antibody present as compared to a standard reference curve.

Anti-β2 Glycoprotein I (β2-GP I) Immunoassay Performance Characteristics

Diagnosis of APS should not be made on the basis of one β2-GP I result alone as persistence should be demonstrated. The anti-β2-GP I assay is thought to be a more specific marker of thrombotic risk than is the ACA test. It has been shown that β2-GP I is the target antigen for anticardiolipin antibodies. When elevated results are obtained with the β2-GP I assay in conjunction with elevated ACA results, there is increased association with thrombosis over positive ACA results alone.
**Antiprothrombin Antibody (APT) Immunoassay Principle**

Diluted test plasma is pipetted into a microtiter plate coated with purified prothrombin antigen. Antiprothrombin antibodies in the test plasma bind to this antigen. Anti-human IgG or IgM is then added, binding captured antibody if present. A chromogen is added and the amount of color produced is directly proportional to the amount of antibody present as compared to a standard reference curve.

**Antiprothrombin Antibody (APT) Immunoassay Performance Characteristics**

This assay detects antibodies directed against prothrombin which in rare individuals, more commonly children, may lead to hypoprothrombinemia resulting in a bleeding diathesis in patients having a LA. The presence of antiprothrombin antibodies, however, does not always coincide with decreased prothrombin activity levels.

**Antiannexin Antibody Immunoassay Principle**

Diluted test plasma is pipetted into a microtiter plate coated with purified annexin V antigen. Antiannexin antibodies in the test plasma bind to this antigen. Anti-human IgG or IgM is then added, binding captured antibody if present. A chromogen is added and the amount of color produced is directly proportional to the amount of antibody present as compared to a standard reference curve.

**Antiannexin Antibody Immunoassay Performance Characteristics**

This assay may be useful in the evaluation of women with miscarriages and in anti-phospholipid syndrome. False weak positive reactions may occur in patients with underlying inflammatory or infectious diseases or in those with autoimmune disorders or high concentrations of IgG or IgM as may be associated with malignant myeloma.

**Reference Ranges**

<table>
<thead>
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<th>Anti-Annexin V (Test Code 300233)</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
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<th>IgM</th>
<th>IgA</th>
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<tr>
<td>Normal</td>
<td>&lt;23</td>
<td>&lt;11</td>
<td>&lt;22</td>
</tr>
<tr>
<td>Low Positive</td>
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<td>Moderate Positive</td>
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<td>36-45</td>
</tr>
<tr>
<td>High Positive</td>
<td>&gt;50</td>
<td>&gt;30</td>
<td>&gt;45</td>
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**Anti-β2 GP I Antibodies** (Test Code 300166)

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>&lt;21</td>
<td>&lt;21</td>
<td>&lt;21</td>
</tr>
<tr>
<td>Low Positive</td>
<td>21-50</td>
<td>21-50</td>
<td>21-50</td>
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<tr>
<td>Moderate Positive</td>
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<td>1-100</td>
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<tr>
<td>High Positive</td>
<td>&gt;100</td>
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**Anti-Phosphatidylserine Antibodies** (Test Code 300153)

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<th>IgM</th>
</tr>
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<td>Normal</td>
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<td>&lt;22</td>
</tr>
<tr>
<td>Low Positive</td>
<td>16-30</td>
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<td>31-50</td>
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<td>High Positive</td>
<td>&gt;50</td>
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**Anti-Prothrombin Antibodies** (Test Code 300230)

<table>
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<th></th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;21</td>
<td>&lt;21</td>
</tr>
</tbody>
</table>

**Specimen Requirements**

APL specimens may be either frozen plasma or serum; serum is the specimen of choice.

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Serum specimen: Collect blood in a plain red-top tube and allow to clot for 30 minutes. Centrifuge 10 minutes at 2500g and transfer serum to a plastic freezer tube using plastic transfer pipettes. Label appropriately and freeze immediately at 20°C or lower. Ship the frozen specimen overnight on dry ice.

**Test Request Information**

The ACA IgG/IgM (#300165), ACA IgG/IgM/IgA (#300034), ACA IgA (#300164), APTS IgG/IgM (#300153), β2-GP I IgG/IgM/IgA (#300166) and APT IgG/IgM (#300230), anti-annexin V IgG, IgM RUO (#300233) tests may be ordered separately from the Esoterix Service Directory. APL testing is typically performed in parallel with clot-based lupus anticoagulant testing in order to achieve the greatest probability for detection of APLs in the diagnosis of APS. CPT Codes: APT IgG, IgM 0030T x 2, Anticardiolipin IgG, IgM 86147 x 2, Anticardiolipin IgG, IgM, IgA 86147 x 3, Anticardiolipin IgA 86147, Antiphosphatidylserine Antibody IgG, IgM 86148 x 2, β2 GPI IgG, IgM, IgA 86146 x 3, anti-annexin V 83516 X 2.
ANTIPHOSPHOLIPID ANTIBODIES (cont.)

Reflex Recommendations

The APL family is heterogeneous. If there is clinical suspicion of APLs, ACA or APTS testing should be performed in combination with clot-based lupus anticoagulant testing, as well as β2-GP I and APT tests to ensure the greatest success of detection of APS and/or LA. If the patient’s prothrombin time is elevated in the absence of warfarin therapy, factor II activity should be evaluated. A patient with an initial positive APL should be retested after twelve weeks to determine if the antibody is persistent.

Therapy for Antiphospholipid Antibody

Long-term oral anticoagulant therapy, monitored by PT/INR or in some instances the factor X chromogenic assay, is the most effective treatment in those individuals with recurrent thrombosis due to antiphospholipid antibodies. PT/INR values in some patients with LA may not accurately predict the level of anticoagulation present and these patients may be monitored with a chromogenic factor X assay. Asymptomatic individuals should not be treated in most instances. In pregnancy with previous recurrent fetal loss and persistent APLs, low molecular heparin therapy appears to be beneficial.

References

**Physical Characteristics**

Antithrombin, also known as AT, Antithrombin III, or ATIII, is a 58,000 Dalton glycoprotein produced in the liver. Antithrombin's plasma concentration averages 30 mg/dL by microlatex particle-mediated immunoassay and its plasma biological half-life is 48 - 72 hours.

**Physiology**

Antithrombin inhibits coagulation by irreversibly binding a number of serine proteases including thrombin (IIa), IXa, Xa, XIa, and XIIa at their catalytic sites. Antithrombin-serine protease complexes, such as thrombin-antithrombin, are rapidly cleared from the plasma.

Antithrombin's protease binding reaction is enhanced 1000-fold by the availability of endothelial cell-derived heparan sulfate or exogenously administered heparin. A portion of the heparan sulfate or heparin molecule binds a specific site on the antithrombin molecule, the heparin binding site, causing a conformational change in the antithrombin structure allowing the AT to more effectively bind and inhibit the activated coagulation factor.

Abnormalities in either the heparin binding site or the protease binding site of antithrombin have been reported.

**Acquired or Congenital Deficiency**

Antithrombin deficiency is common in a variety of clinical conditions and situations. According to Kottke-Marchant and Duncan (see references), acquired AT deficiency is due to either impaired synthesis or loss of protein. Impaired synthesis occurs in individuals with liver disease, malnutrition, inflammatory bowel diseases, extensive burns and in premature infants. AT deficiency is demonstrated in almost all cases of disseminated intravascular coagulation and levels may have prognostic value. Antithrombin levels are usually diminished due to consumption or loss in nephrotic syndrome, malignancy, thrombotic microangiopathy, sepsis and acute thrombotic episodes. Antithrombin is decreased during L-asparaginase therapy; it is also commonly decreased following bone marrow transplant, surgery and while on heparin therapy (although levels below 50% are unusual unless the patient has other causes for AT deficiency).

Congenital antithrombin deficiency is typically inherited as one of two types of autosomal dominant traits. Quantitative, or “type I” deficiency, describes structurally normal antithrombin molecules but diminished plasma levels. Type I deficiencies are usually the result of deletion of a major portion of the antithrombin gene or of a small deletion that generates a premature stop codon. Qualitative, “type II,” deficiencies are thought to be as common as type I deficiencies. Type II deficiency describes a functionally abnormal molecule. A point mutation may cause a defective heparin binding site or a defective serine protease binding site. In type II deficiencies, antithrombin antigen assay results are normal, but activity levels are diminished. As AT deficiency is due to many different genetic mutations, genetic analysis is not practical for diagnostic purposes and diagnosis is dependent on evaluation of plasma levels.

**Incidence of Deficiency**

Congenital antithrombin deficiency in the heterozygous state has been detected in two to four of 1000 unselected individuals and is the probable cause of approximately 1% of cases of recurrent venous thrombosis. Prevalence in the general population is 0.2 – 0.4%. Acquired antithrombin deficiency may be observed more frequently than congenital deficiency and occurs in the clinical conditions listed above. Homozygous deficiency is considered incompatible with life except for those individuals in which the abnormality involves the heparin binding site. In this rare population, thrombosis tends to develop in infancy or childhood.

**Clinical Significance of Deficiency**

AT deficiency is associated typically with venous rather than arterial thrombosis. Deep venous thrombosis, pulmonary emboli and thrombosis in unusual sites such as the mesenteric or cerebral veins are the most common manifestations of AT deficiency. The relative risk for
thrombosis is increased 25-50 fold for type I antithrombin deficiency. Nearly 85% of individuals with inherited antithrombin deficiency experience thrombotic episodes by age 55, the initial event commonly occurring between 20 and 30 years of age. Second and third events are likely. In one-third of congenital deficiencies thrombosis is spontaneous; in the remainder it may be traced to trauma, surgery, oral contraceptive usage, or other events. Both the severity and frequency of thrombotic episodes are inversely correlated to antithrombin activity. Antithrombin deficiency is a rare cause of resistance to unfractionated heparin therapy. Type II antithrombin deficiency, in which the defect involves the heparin binding site rather than the protease binding site, carries a lower thrombotic risk. AT deficiency is also associated with recurrent miscarriage, still birth, infants born small for gestational age and pre-eclampsia.

Plasma Antithrombin Activity Chromogenic Substrate Assay Principle

Antithrombin activity is measured using a chromogenic substrate, or amidolytic method. Test plasma is mixed with an excess of heparin and a serine protease, usually Xa, although IIa may be used. The mixture is allowed to incubate at 37°C for several minutes. During incubation the heparin-activated antithrombin irreversibly binds a proportion of the added Xa or IIa. Chromogenic substrate, provided as a second reagent, is then hydrolyzed by residual protease. The degree of hydrolysis, measurable by colored end product intensity, is inversely proportional to the activity of antithrombin in the test plasma.

Chromogenic Substrate Assay Performance Characteristics

The chromogenic substrate test for plasma antithrombin activity detects both quantitative and qualitative antithrombin deficiencies. Oral anticoagulant (warfarin) therapy has been reported to raise the antithrombin level although this is unlikely of clinical significance. The antithrombin level may be diminished for several days after surgery or a thrombotic event, so during this period the assay cannot be used to establish a congenital deficiency. When human thrombin is used in the assay, there may be crossreactivity with heparin cofactor II causing a falsely increased AT level. This does not occur in the presence of bovine thrombin.

Plasma Antithrombin Antigen Latex Immunoassay (LIA) Principle

The test reagent is a suspension of anti-antithrombin antibody-coated microlatex particles. In the absence of antithrombin, the wavelength of monochromatic incident light exceeds the microlatex particle diameter, so it passes through unabsorbed. In the presence of antithrombin, the particles form large aggregates by immune binding. Antithrombin concentration is directly proportional to the rate of light absorption change due to aggregate formation.

Latex Immunoassay Antigen (LIA) Performance Characteristics

The microlatex particle-mediated immunoassay for plasma antithrombin antigen concentration detects most acquired deficiencies and quantitative congenital deficiencies, but does not detect qualitative congenital abnormalities.

Reference Ranges

Plasma antithrombin activity levels by chromogenic substrate assay range from 77 - 123%. Antithrombin antigen levels range from 72-124% by latex immunoassay (LIA). Adult levels are reached by three to twelve months of age and remain steady throughout middle age. Antithrombin levels decrease with advanced age.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin Activity</td>
<td>77% - 123%</td>
</tr>
<tr>
<td>Antithrombin Antigen</td>
<td>72% - 124%</td>
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</tbody>
</table>
Specimen Requirements for the Plasma Antithrombin Activity and Antigen Tests

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). A second spin may be necessary to achieve this. Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Test Request Information

The antithrombin activity plasma (#300030) and the antithrombin antigen plasma (#300033) can be ordered separately from the Esoterix Service Directory. CPT Code: antithrombin activity plasma 85300, antithrombin antigen plasma 85301.

Reflex Recommendations

If the antithrombin activity is below 70%, an antithrombin antigen assay may be performed to distinguish qualitative from quantitative deficiencies. Since therapy is the same for both type I and type II AT deficiency, such testing may not be cost effective. Acquired causes of AT deficiency should be excluded. The antithrombin antigen assay should not be performed alone as a screen as it will not detect qualitative antithrombin deficiencies.

Following initial detection, all presumed congenital antithrombin deficiencies should be confirmed by repeat analyses after several weeks. The kindred of antithrombin deficient individuals should also be screened for antithrombin deficiency both to confirm its hereditary nature and to assess thrombotic risk to family members.

Therapy for Deficiency

Most acute thrombotic episodes are managed by heparin therapy followed by warfarin prophylaxis. Rare individuals with AT deficiency are heparin resistant. In disseminated intravascular coagulation, heparin resistance, and in situations where hemorrhage is a risk such as parturition or surgery, antithrombin concentrates may be useful.

References

BLEEDING TIME

**Physiology**

In the bleeding time test, a precision lancet-type device is used to produce a standard wound on the volar surface of the forearm and the time interval required for the wound to stop bleeding is measured. Physical trauma induced by the lancet disrupts the blood vessel endothelial layer, exposing subendothelial collagen. Assisted by von Willebrand factor (vWF), platelets bind the exposed collagen to form an occlusive plug.

**Etiology**

Prolonged bleeding times may indicate an anomaly in the formation of the platelet plug. Prolonged bleeding time tests have been observed as a result of: 1) alterations in the vessel wall such as in connective tissue disorders; 2) abnormal adhesion between the platelet and subendothelium such as in patients with von Willebrand disease (vWD), Glanzmann’s thrombasthenia (GT), and Bernard Soulier Syndrome (BSS); 3) in platelet function disorders such as platelet storage pool disease and platelet release abnormalities; 4) acquired causes of platelet dysfunction (e.g. myeloproliferative disorders); 5) defects in fibrin formation such as afibrinogenemia; and 6) drug effect in response to those agents known to impair platelet function (e.g. aspirin); 7) decreased hematocrit; or 8) in the presence of renal failure.

**Clinical Significance**

The bleeding time interval reflects the in vivo function of platelets, vasculature, and vWF. Platelet defects, vascular disorders, and vWD may lead to systemic hemorrhage, characterized by bleeding in the skin and mucous membranes. Patients with such defects typically experience bruising, epistaxis, menorrhagia, and gingival bleeding. In patients whose platelet count is greater than 100 x 10^9/L, a prolonged bleeding time may indicate a vascular disorder, vWD, afibrinogenemia, or a qualitative platelet defect such as storage pool disorder, platelet release defect, GT, or BSS. The bleeding time test may be used to monitor the effectiveness of therapy in vWD when measures of vWF activity or antigen are not available and in patients with uremia following DDAVP or estrogen therapy.

**Assay Principle**

The test is offered only onsite as it requires an incision in the patient’s forearm. A sphygmomanometer cuff is placed on the forearm and inflated to a pressure of 40 mmHg, 20 mmHg in newborns and infants, to standardize the venous pressure. The cuff is inflated at least 30 seconds before the incision is made. A 5 mm long, 1 mm deep incision, 3.5 by 1 mm deep for children, or 2.5 by 0.5 mm deep for infants, is made in the volar surface of the forearm utilizing a standardized bleeding time device, and the incision is wicked with filter paper every 30 seconds in a manner not to disturb the forming clot. The incision may be made parallel to or perpendicular to the length of the arm, but the choice must be consistent within an institution to normalize the results. The time interval from incision until bleeding stops is recorded.

**Assay Performance Characteristics**

There is no evidence to suggest that a prolonged bleeding time is predictive of hemorrhagic risk following surgery or other invasive procedures in unselected populations. The predictive value of this test is acceptable in selected populations, such as those with a hemorrhagic tendency, and members of kindreds with vWD, BSS, GT, or other inherited anomalies. A number of variables may prolong the bleeding time and diminish its specificity. Biological variables include platelet count below 100 x 10^9/L, hematocrit below 30%, and uremia. Platelet activity is suppressed by aspirin and other non-steroidal anti-inflammatory drugs such as indocin and sulfipyrazone. Other drugs may prolong bleeding time such as ticlopidine, penicillin, and cephalosporins. It is imperative that the patient history include specific questions on these medications, and that the patient refrain from their use before the bleeding time is performed. Non-steroidal anti-inflammatory drugs may prolong a bleeding time but only as long as the drug is in the circulation, typically about 4 hours. A number of technical variables affect results: venous pressure, depth of incision, temperature of the arm, skin thickness, technologist’s “wicking” technique, and repeated contraction of the arm or excessive movement. As a result of the incision, patients may develop scarring or keloid formation. An abnormal result should be repeated for confirmation.
Reference Range

Normal adult values typically range from 2-9 minutes, although females' times are slightly longer. Ranges may vary between laboratories. Times typically shorten with age. Newborn infants' bleeding time ranges from 1.6-5.2 minutes; the interval is 10-13 minutes in children from 1-12 years of age.

Specimen Requirements

The bleeding time test must be performed on site by a well-trained laboratory professional.

Test Request Information

Bleeding time assay (#300501) can be ordered separately from Esoterix Service Directory. CPT Code: 85002.

Reflex Recommendations

Abnormal bleeding time tests suggest a defect in the formation of the platelet plug. When the bleeding time is prolonged in the presence of a normal platelet count, platelet dysfunction or vWD should be considered and platelet aggregation studies, vWF activity (ristocetin cofactor), or vWF antigen testing may be indicated.

References

CRYOFIBRINOGEN

Physiology and Etiology

Cryofibrinogen refers to plasma fibrinogen that precipitates at a low temperature. Plasma cryoprecipitates are complexes that form between fibrinogen, fibrin, and fibronectin. Cold-induced precipitation from plasma differentiates cryofibrinogenemia from cryoglobulinemia where proteins (mainly immunoglobulins) are precipitated from serum.

Clinical Significance

Cryofibrinogen is not normally present in the healthy individual. Individuals with cryofibrinogenemia are asymptomatic in the majority of cases. In some persons, however, cryofibrinogenemia may produce a clinical picture that is similar to cryoglobulinemia with cold sensitivity that results in purpura, venous or arterial thrombosis and peripheral gangrene. Cryofibrinogenemia should be suspected in patients who have cold sensitivity and peripheral ischemia with skin ulcerations or in individuals without known cause of vascular occlusion.

Cryofibrinogenemia has been reported in association with autoimmune disorders, neonatal infections and neoplastic disorders. Cryofibrinogenemia may also be a primary disorder.

Assay Principle

Cryofibrinogen is measured by dividing plasma into 2 equal portions in glass test tubes. The tubes are covered, and one tube is refrigerated for 12 hours, while the remaining tube is left at room temperature. The formation of heavy precipitate, light strands in the plasma, or gel formation in the refrigerated sample tube indicates the presence of cryofibrinogen. The precipitate will disappear when the refrigerated plasma tube is placed in a 37°C water bath.

Reference Range

Cryofibrinogen is not normally present.

Specimen Requirements

Both plasma and serum samples are required. Citrate plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Serum specimen: Collect blood into a plain red top tube. Allow the specimen to sit at least 30 minutes to clot. Centrifuge the specimen for 10 minutes. Dispense the serum into plastic tube. Label and freeze immediately. Place frozen specimen in container to be shipped overnight.

Cryofibrinogen Test Request Information

Cryofibrinogen (#300203) can be ordered from the Esoterix Service Directory. CPT Code 82585

References

**Physiology**

The fibrinolytic system is initiated following activation of the contact factors in the coagulation cascade. Fibrinolytic system activation leads to the production of plasmin, a proteolytic enzyme capable of degrading fibrin and fibrinogen as well as other plasma proteins. The euglobulin lysis time (ELT) test is a global screening assay that evaluates the fibrinolytic system function by measuring the time it takes for an in vitro clot to dissolve in the absence of plasmin inhibitors.

**Clinical Significance**

Increased fibrinolytic activity is suggested by clot lysis that occurs in less than 2 hours. A shortened ELT result implies excessive fibrinolytic activity that may be primary or could be secondary to pregnancy, hypofibrinogenemia, excessive inflammation, malignancy (especially acute leukemia and prostate cancer), extensive trauma, severe liver disease, obstetric complications (such as hydatidiform mole) or thrombolytic therapy. Clinical bleeding is a possible consequence of excessive fibrinolysis. Excessive fibrinolysis may result from increased levels of tissue plasminogen activator (tPA), increased plasmin activity, decreased levels of plasminogen activator inhibitor-1 (PAI-1) or decreased α2-antiplasmin activity. The effect of fibrinolysis is the production of plasma fibrin(ogen) degradation products (FDP or FSP) and in most instances D-dimer fragments.

A prolonged ELT result implies a defect in the fibrinolytic system such as elevated plasminogen activator inhibitor (PAI-1) levels, elevated levels of α2-antiplasmin, a plasminogen deficiency, or decreased tissue plasminogen activator (tPA) activity. The ELT may also be prolonged if the fibrinogen level exceeds 600 mg/dL. Inadequate fibrinolysis may be accompanied by superficial or deep venous thrombosis, pulmonary embolism, coronary thrombosis, transient ischemic attack, or stroke.

**Assay Principle**

Following blood collection and centrifugation, plasma inhibitors of fibrinolysis are physically removed (the euglobulin fraction is produced) and the reaction of fibrinogen, plasminogen, and plasminogen activators are assayed. The euglobulin fraction of the plasma refers to plasma proteins that precipitate at low pH and decreased ionic strength. The precipitate includes tissue plasminogen activators, plasminogen, plasmin, and fibrinogen. The inhibitors of lysis, such as α2-antiplasmin and α2-macroglobulin, do not precipitate and the precipitation of PAI-1 is variable. In the test system, the euglobulin precipitate is redissolved in buffer, and clotted with CaCl₂. The clots are formed in microwells and turbidity at 340 nm is monitored automatically, using a plate reader to measure the clot lysis. The time required for the intrinsic plasmin to lyse the fibrin clot equates to the euglobulin lysis time. On the turbidimetric assay profile, this equates to the time taken for the turbidity to reach a baseline plateau level. The controls tested with every assay to verify assay performance are a normal plasma control and an abnormal plasma control with shortened euglobulin lysis time. Test samples and controls are tested in duplicate.

**Assay Performance Characteristics**

The diagnostic potential of euglobulin lysis times is limited by the extreme variation in lysis times among healthy individuals. Both hypofibrinogenemia and factor XIII (FXIII) deficiency may result in a shortened lysis time. In the case of hypofibrinogenemia, the shortened time is due to the decreased amount of fibrin to be lysed. In FXIII deficiency, the clot is not stabilized by covalent crosslinking of fibers and can be readily lysed by plasmin.

Traumatic venipuncture, prolonged stasis, incorrect sample preparation and elevated fibrinogen levels may invalidate test results.

**Reference Range**

The normal reference range for the euglobulin lysis time is 180 – 700 minutes.
**Specimen Requirements**

Citrate plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube to within 90% of its capacity. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

**Euglobulin Lysis Time Assay Test Request Information**

The euglobulin lysis time (#300403) may be ordered separately from the Esoterix Service Directory. CPT Code 85360.

**Reflex Recommendations**

Other assays that may be of value when the ELT is short include PAI-1 activity and tPA activity. When the ELT is prolonged, consideration should be given to evaluation of PAI-1 activity, plasminogen and fibrinogen.

**References**


**Physical Characteristics**

**Factor II**

Synonym: prothrombin. Factor II (FII) is a 72,000 Dalton single chain glycoprotein precursor, synthesized in the liver. The synthesis of FII is vitamin K dependent. FII's plasma concentration is 100 μg/mL and its plasma half-life is approximately 60 hours.

**Factor V**

Synonym: proaccelerin. Factor V (FV) is a 330,000 Dalton single chain non-enzymatic cofactor similar in size and structure to factor VIII (FVIII). It is synthesized in hepatocytes, megakaryocytes, and vascular endothelial cells. FV's plasma concentration is 7 μg/mL, and its plasma half-life is 15-36 hours. Twenty-five percent of circulating factor V is carried on platelet surfaces and in platelet alpha granules.

**Factor VII**

Synonym: proconvertin. Factor VII (FVII) is a 48,000 Dalton single chain glycoprotein precursor, synthesized in the liver. The synthesis of FVII is vitamin K dependent. FVII's plasma concentration is 0.5 μg/mL, and its plasma half-life is 4-6 hours.

**Factor X**

Synonym: Stuart-Prower factor. Factor X (FX) is a 54,800 Dalton two-chain glycoprotein serine protease precursor synthesized in the liver. The synthesis of FX is vitamin K dependent. FX's plasma concentration is 10 μg/mL and its plasma half-life is approximately 40 hours.

**Physiology**

Fibrinogen, tissue factor, and FII, FV, FVII, and FX are the coagulation factors of the extrinsic and common coagulation pathway, now called the tissue factor pathway.

The coagulation process begins with exposure of plasma to tissue factor thus triggering FVII activation. FVIIa then forms a complex with the exposed tissue factor and nearby cell membrane phospholipids. This complex activates FX. FXa in the presence of Ca++, FVa and phospholipid (Prothrombinase complex) cleaves prothrombin (FII) to form thrombin (FIIa). Thrombin then cleaves the fibrinogen molecule and fibrin monomer is formed. Polymerization of fibrin monomers follows and a soluble fibrin clot is formed.

The tissue FVIIa complex also activates FIX, forming a complex with FVIIIa, to activate FX. This in vivo pathway is bypassed in the prothrombin time test, thus FVIIa and FIX are not considered to be part of the extrinsic pathway.

The extrinsic pathway's activity is short-lived. Soon after tissue factor and FVIIa join with their substrate, FX, the FVIIa-FXa-TF complex is neutralized by plasma tissue factor pathway inhibitor (TFPI). Subsequent fibrin formation relies upon the intrinsic pathway, which is driven by thrombin's activation of FXI.

**Incidence and Etiology of Abnormalities**

Inherited as autosomal recessive traits, FII, FV, FVII, or FX deficiencies cause hemorrhagic disorders of varying severity. Both sexes are affected in all ethnic groups in all parts of the world. Quantitative and qualitative deficiencies have been described, although quantitative deficiencies predominate. These disorders are rare.

A few cases of combined congenital FII, FVII, FIX and FX (vitamin K-dependent) factor deficiencies have been reported. Combined FV and FVIII deficiencies have been found in over 60 families in and around the Mediterranean basin.

Acquired FII deficiencies are seen with vitamin K deficiency, liver disease, during warfarin therapy and rarely in association with lupus anticoagulant.

Acquired FV deficiency occurs in liver disease, disseminated intravascular coagulation (DIC) syndromes and in association with acquired inhibitors. FV inhibitors generally occur postoperatively, especially after multiple exposures to bovine topical thrombin that contains residual FV.
Acquired FVII deficiency is seen with vitamin K deficiency, liver disease and during warfarin therapy. Extreme elevations in FVII may be due to cold activation of the sample prior to analysis. This may be caused if the sample was placed on ice after phlebotomy or if it was stored in a frost-free freezer. This preanalytical variable will result in erroneous test results not only of FVII testing but of other tissue factor-based testing.

Acquired FX deficiency occurs in vitamin K deficiency, warfarin therapy and in liver disease. This deficiency may also occur in patients with primary amyloidosis and paraproteinemia, multiple myeloma or in the elderly with no apparent disease. Acquired FX inhibitors are rare.

**Clinical Significance of Deficiencies**

Hemorrhagic manifestations of prothrombin (FII) deficiency include easy bruising, hematomas, epistaxis, menorrhagia and bleeding after surgery or trauma. Hemarthroses are rare. A polymorphism in the prothrombin gene, 20210 G to A, present in approximately 2% of the population, has been associated with increased plasma prothrombin levels: a prothrombin level above 115% may increase the risk of thrombosis.

Congenital FV deficiency, also known as parahemophilia, is a variable bleeding disorder. Patients may exhibit surgical or trauma-induced hemorrhage, post partum hemorrhage, menorrhagia, neonatal intracranial hemorrhage, epistaxis, as well as rare deep hematoma or joint bleeding while other individuals with identical plasma levels exhibit little or no bleeding tendency. The severity of FV deficiency has been correlated with the amount of FV stored in the platelets.

Bleeding manifestations of FVII deficiency vary from patients being asymptomatic to those seen in classical hemophilia. Onset is seen in an infant as umbilical stump bleeding or intracranial hemorrhage. Other hemorrhagic manifestations include oral mucosal bleeding, epistaxis, hemarthroses, gastrointestinal bleeding, severe menorrhagia and post partum or post-surgical hemorrhage.

FX deficient individuals may exhibit mild to severe bleeding including hemarthroses, deep hematomas, menorrhagia and post-surgical hemorrhage.

Deficiency of any of these extrinsic pathway factors slows thrombin generation and allows bleeding. When the plasma activity of any of these factors is below 30%, excessive bleeding may follow any traumatic event. When the activity is below 1%, spontaneous bleeding may occur.

**Clot-based Assay Principles**

Extrinsic pathway factors are measured using a modification of the prothrombin time (PT) test. At least three different dilutions are made of the test plasma in imidazole buffer. These dilutions are then mixed 1:1 with reagent plasma immunologically depleted or congenitally deficient in the factor being measured; for example, FVII-depleted plasma is added to the plasma dilutions of a patient suspected of having a FVII deficiency. The factor-depleted plasma is added to ensure that all factors except for the one under investigation are present at greater than 75% of normal levels thus, only the factor under study will affect the outcome of the PT. Next, the PT reagent (thromboplastin), consisting of tissue factor, phospholipid, and calcium chloride, is added and the mixture is timed until clot formation occurs. Results are calculated by comparing the dilutions of the test plasma to that of a curve prepared using a standard reference plasma. The clotting time interval of each dilution is inversely proportional to the activity of the factor being studied.

**Clot-Based Assay Performance Characteristics**

The clotting factors are assayed using multiple dilutions to detect possible inhibitors. The inhibitor may affect either the specific factor being assayed or may affect the test system of other factors being tested. If the inhibitor is directed against the factor being assayed, the percentage activity of that factor will remain relatively constant at all dilutions. If the inhibitor is directed towards a factor not specifically being assayed, the factor level being assayed will increase as dilution of the test plasma.
**Factor VII and X Antigen (Immunoassay) Principle**

Immunooassays are available for FVII and FX but are designated as research use only assays (RUO). Test plasma is pipetted into a microtiter plate well coated with anti-human coagulation factor antibody, either anti-FVII or anti-FX. A second anti-human factor antibody, coupled with an enzyme, binds to the captured factor. A substrate is added and the amount of color produced is directly proportional to the factor concentration.

**FVII and FX Antigen (Immunoassay) Performance Characteristics**

The immunoassay for FVII or FX detects quantitative, but not qualitative deficiencies, and thus should be employed to confirm functional results or to distinguish between a quantitative and qualitative deficiency. The immunoassay returns a normal result in the presence of cross-reacting material (CRM+), i.e., defective molecule with normal antigenic levels but abnormal function.

**FX Chromogenic Assay Principle**

FX is measured by a chromogenic methodology with color production proportional to the FX activity in the sample.

**FX Chromogenic Assay Performance Characteristics**

In most patients, the international normalized ratio (INR) is used to monitor warfarin therapy. Lupus anticoagulants may produce prolonged prothrombin times which result in an INR that does not accurately reflect the level of anticoagulation. The FX chromogenic assay may be used to more accurately monitor warfarin therapy in patients with a lupus anticoagulant. The therapeutic FX chromogenic range correlates to an INR of 2.0 – 3.5.

**Reference Ranges**

<table>
<thead>
<tr>
<th>Factor Assay</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II (prothrombin) Activity</td>
<td>75 - 135%</td>
</tr>
<tr>
<td>Factor V Activity</td>
<td>70 – 150%</td>
</tr>
<tr>
<td>Factor VII Activity</td>
<td>50 - 155%</td>
</tr>
<tr>
<td>Factor VII Antigen - RUO*</td>
<td>50 - 150%</td>
</tr>
<tr>
<td>Factor X Activity</td>
<td>65 - 135%</td>
</tr>
<tr>
<td>Factor X Antigen – RUO*</td>
<td>65 - 150%</td>
</tr>
<tr>
<td>Factor X chromogenic (lupus anticoagulant patient on oral anticoagulant - therapeutic)</td>
<td>20 - 44%</td>
</tr>
<tr>
<td>Factor X chromogenic (no anticoagulant)</td>
<td>75 - 155%</td>
</tr>
</tbody>
</table>

*RUO – Research Use Only*
Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Test Request Information

The extrinsic pathway activity assays factor II #300100, factor V #300101, factor VII #300102, and factor X #300105 may be ordered separately from the Esoterix Service Directory. Factor VII antigen (RUO) #300112 and factor X antigen (RUO) #300206 may be ordered separately from the Esoterix Service Directory. Factor X Chromogenic Assays (#300211 and #300215) can be ordered separately from the Esoterix Service Directory. CPT Codes: factor II activity 85210, factor V activity 85220, factor VII activity 85230, factor VII antigen 85230, factor X activity 85260, factor X antigen 85260, factor X chromogenic 85260.

Reflex Recommendations

A prolonged PT on a specimen with a normal APTT may indicate a rare FVII deficiency or mild deficiencies of FV and/or FX. FVII activity and antigen assays are used for confirmation and to determine whether the deficiency is quantitative (Type I) or qualitative (Type II).

If both the PT and APTT are prolonged, and the patient is receiving anticoagulants, there may be a multiple factor deficiency or a single factor deficiency of FII, FV, or FX. Because FII, FVII, FIX and FX are vitamin K dependent, all four become mildly deficient in oral anticoagulant therapy or in vitamin K deficiency. PT mixing studies are performed to rule out the presence of heparin, lupus anticoagulant, or specific factor inhibitors; then individual assays of FII, FV, or FX are performed.

A FVII antigen assay may be used in conjunction with protein C antigen or protein S antigen to obtain a presumptive diagnosis of protein C or S deficiency while a patient is on stable oral anticoagulant therapy. Because they are vitamin K dependent, protein C and S levels begin to fall a few hours after warfarin therapy is started. The protein C antigen/FVII antigen ratio and the protein S antigen/FVII antigen ratio may be used for preliminary diagnosis. A final diagnosis of protein C or S deficiency should not be made until an individual has been removed from warfarin therapy for a minimum of two weeks and then testing repeated.

Therapy for Extrinsic Pathway Factor Deficiency

Therapy for acute bleeding episodes in congenital deficiencies of FII, FV, FVII or FX, may be accomplished by giving fresh frozen plasma until a plasma level of approximately 25 percent is reached. For acquired deficiencies, the underlying condition must be identified and treated. Vitamin K administration corrects vitamin K deficiency, immunosuppression may be useful in treating acquired single factor inhibitors.
References

**Physical Characteristics**

**Factor VIII**

Synonym: Anti-hemophilic factor (AHF). Factor VIII (FVIII) is a 320,000 dalton glycoprotein cofactor. FVIII circulates in the plasma bound to von Willebrand factor (vWF). FVIII’s plasma concentration is approximately 0.15 μg/mL and its plasma half-life is 9-18 hours. The normal range of FVIII varies with the ABO blood group, with lower mean normal levels in type O blood. It is an acute phase reactant, elevated in times of stress or in response to certain medications.

**Factor IX**

Synonym: Plasma Thromboplastin Component (PTC) or Christmas factor. Factor IX (FIX) is a 72,000 dalton single-chain glycoprotein proenzyme synthesized in the liver. The synthesis of FIX is vitamin K dependent. FIX’s plasma concentration is 3-5 μg/mL and its plasma half-life is approximately 24 hours.

**Factor XI**

Synonym: Plasma Thromboplastin Antecedent (PTA) or Rosenthal factor. FXI is a 160,000 dalton single-chain glycoprotein zymogen synthesized in the liver and megakaryocytes. FXI’s plasma concentration is 4-6 μg/mL and its plasma half-life is approximately 60 hours.

**Factor XII**

Synonym: Hageman factor. FXII is an 80,000 dalton single-chain serine protease glycoprotein zymogen synthesized in the liver. FXII’s plasma concentration is 30 μg/mL and its plasma half-life is approximately 50 hours.

**Prekallikrein**

Synonym: Fletcher factor. Prekallikrein is a single-chain serine protease zymogen gamma globulin synthesized in the liver that circulates in two forms having molecular weights of 85,000 and 88,000 daltons. Prekallikrein’s plasma concentration is 35-45 μg/mL. It circulates in an equimolar complex with high molecular weight kininogen (HMWK), and has a plasma half-life of 24 hours. Kallikrein liberates kinins from kininogens, activates FXII and plasminogen, converts prorenin to renin, destroys C1 components and interacts with leukocytes.

**High molecular weight kininogen**

Synonym: Fitzgerald factor. High molecular weight kininogen (HMWK) is a 110,000 dalton single-chain nonenzymatic cofactor synthesized in the liver which is central to contact activation reactions. It forms a complex with prekallikrein and factor XI. HMWK’s plasma concentration is 70 μg/mL and its plasma half-life is approximately 144 hours.

High molecular weight kininogen proteolysis leads to the production of bradykinin, a mediator of vasodilation, smooth muscle contractions and increased vascular permeability. Other functions of HMWK include inhibition of thrombin-induced platelet aggregation, participant in fibrinolysis as well as having surface binding anti-adhesive properties.

**Physiology**

FVII, FIX, FXI, prekallikrein, and HMWK are the coagulation factors of the intrinsic coagulation pathway. FXII, high molecular weight kininogen, and prekallikrein are also called the “contact” factors. FXI is sometimes included in this designate of “contact” factors because of its interaction with the others listed.

Tissue F-VIIa complex generated by the extrinsic pathway activates FIX. FIXa binds FVIIa, calcium, and phospholipid; this complex, called “tenase,” then activates FX and the coagulation cascade proceeds through the common pathway. Thrombin generated from both the extrinsic and intrinsic pathways activates more FIX (also FV and FVIII), augmenting the formation of the tenase complex.
FXI is also activated by FXIIa formed through activation of FXII by a HMWK-prekallikrein complex on endothelial cells. With production of FXIIa and kallikrein, activation of the kinin, fibrinolytic and complement systems occur. The major inhibitor of FXIIa and kallikrein is C1 inhibitor, others include antithrombin (AT), plasminogen activator inhibitor (PAI) and α2-macroglobulin.

**Intrinsic Pathway Factor Abnormalities: Incidence and Etiology**

**Factor VIII Deficiency**

Hemophilia A or classic hemophilia is an X chromosome-linked hereditary disorder, characterized by defective or deficient FVIII molecules. About 30% of FVIII deficiencies arise from new gene mutations; the remainder are inherited through a carrier mother. Hemophilia A is seen in males of all ethnic groups in all parts of the world. It is the second most common of the inherited bleeding abnormalities, with an estimated incidence of 1 in 5,000 live male births. Bleeding manifestations may rarely present in women who are carriers of hemophilia A. Symptomatic female carriers may have low FVIII levels. von Willebrand disease (vWD) is the most common inherited bleeding disorder with an incidence of 1 in 100. Although vWD is genetically unrelated to hemophilia A, approximately 90% of vWD patients have moderately to severely decreased FVIII levels because the von Willebrand factor is the carrier protein of FVIII in the plasma, and prolongs the FVIII half life in circulation.

Inhibitors to FVIII may develop in hemophilia A patients, especially those with severe hemophilia, due to the production of alloantibodies directed against the FVIII in replacement products. Acquired FVIII deficiency is a rare (1 in 1,000,000) condition caused by the development of autoantibodies to FVIII. These acquired FVIII inhibitors may arise following pregnancy, in elderly individuals, or in patients with autoimmune disorders. They may cause severe bleeding disorders resembling that of hemophilia A.

**Factor VIII Elevation**

FVIII, like vWF, is an acute phase reactant. FVIII levels rise during strenuous exercise, epinephrine or DDAVP administration, estrogen administration (birth control or hormone replacement therapy), during periods of acute stress, following surgery, and in most inflammatory conditions. FVIII levels are elevated in carcinoma, leukemia, liver disease, renal disease, hemolytic anemia, diabetes mellitus, deep vein thrombosis, and myocardial infarction. In otherwise healthy individuals, levels are elevated at birth, increase during pregnancy, and also with age.Persistently elevated FVIII levels are thought to have, in part, a genetic basis. Elevation of FVIII above 150% is seen in 11% of unselected individuals with venous thrombosis. Risk associated with elevated FVIII levels is graded and levels above 150% are associated with a 6x increase in thrombosis risk. Elevation of FVIII may shorten the APTT and diminish sensitivity in testing for factor deficiencies, heparin therapy, or lupus anticoagulant.

**Factor IX Deficiency**

Hereditary FIX deficiency, known as hemophilia B or Christmas disease, is inherited in an X chromosome-linked recessive pattern, similar to that of hemophilia A. It is a heterogeneous disorder with varying FIX activity and FIX antigen levels produced from many different genetic mutations. Hemophilia B occurs in 1 of every 25,000-30,000 live male births in all ethnic groups in all parts of the world with most affected families showing a unique genetic mutation.

Acquired FIX occurs in anti-vitamin K therapy, liver disease and with vitamin K deficiency. Physiologic FIX deficiency is found in the newborn but generally normalizes by 6 months of age.

Inhibitors to FIX develop in approximately 3% of hemophilia B patients, generally those with a severe deficiency, due to the production of alloantibodies in response to replacement therapy. Rarely, spontaneous autoimmune inhibitors of FIX arise to cause acquired hemophilia B. These acquired inhibitors are most often seen in patients with an underlying autoimmune disorder.

**Factor XI Deficiency**

Hereditary FXI deficiency, known as Hemophilia C or Rosenthal’s syndrome, is inherited in an autosomal recessive pattern. Heterozygotes have levels between 25% and 60% and homozygotes have levels of less than 15%. Over half of reported cases have occurred in Jewish individuals. The Ashkenazi Jewish population in Israel has a heterozygote frequency of 1:8.

Acquired inhibitors are extremely rare in FXI deficient patients. Spontaneous autoantibodies to FXI are more common, generally in patients with an underlying autoimmune disorder such as rheumatoid arthritis or systemic lupus erythematosus (SLE) or with drugs such as chlorpromazine.
Factor XII, Prekallikrein and High Molecular Weight Kininogen Factor Deficiencies

Deficiencies of these contact factors have no hemorrhagic consequence, however they are necessary for normal APTT clot formation, so deficiency of any of the three produces markedly prolonged APTT results. Hereditary deficiencies of these factors are inherited through autosomal recessive patterns.

The prevalence of FXII deficient individuals in the general population is estimated to be 1.5 to 3.0%. Homozygotes have levels of less than 1% and heterozygotes have levels between 17-83%. Of these three contact factors, a mild FXII deficiency is the most common cause of prolongation of the APTT in the non-bleeding patient without a lupus anticoagulant. Congenital FXII deficiency has been associated with an increased incidence of venous thrombosis, possibly not reflecting true FXII deficiency but rather spurious FXII deficiency due to a lupus anticoagulant.

Hereditary deficiencies of prekallikrein and high molecular weight kininogen are extremely rare. The presence of HMWK regulates the activation of kallikrein and subsequently FXIIa. Therefore, HMWK deficiency interferes with optimal contact factor activation resulting in decreased activation of FXI as well as decreased fibrinolytic and kinin system activation.

Acquired alterations of these three factors, both increased and decreased, have been seen in many disorders including septicemia, coronary artery disease, pharmacologic thrombolysis and angioedema. Decreases of these factors may be seen in liver disease, renal disease, hyperlipoproteinemia and in respiratory distress syndrome.

Clinical Significance of Deficiency

Patients with classical hemophilia (hemophilia A) have a myriad of bleeding manifestations including deep muscle and joint hemorrhage, hematomas, easy bruising, post-traumatic bleeding, post-surgical and laceration bleeding, oozing after tooth extraction, intracranial hemorrhage, gastrointestinal and renal bleeding, and retroperitoneal bleeding. Chronic musculoskeletal hemorrhages and recurrent hemarthroses leads to chronic muscle injury and fibrosis of the joint tissue. Female carriers of hemophilia A may have an increased bleeding tendency especially following trauma or surgery. Clinical manifestations of hemophilia B are the same as in hemophilia A. Markedly low FIX levels may be seen in female carriers of hemophilia B. Symptoms of hemophilia A and B are classified as mild (5%-25% activity), moderate (1%-5% activity), and severe (<1% activity) on the basis of the factor assay results. In moderate and severe hemophilia, bleeding may be spontaneous.

Hemorrhagic manifestations of FXI deficiency include prolonged bleeding following surgery, dental extraction or childbirth, epistaxis, menorrhagia or easy bruising. The frequency and severity of bleeding in FXI deficient individuals does not always correlate with factor assay levels.

Although the APTT is prolonged in deficiencies of FXII, prekallikrein and high molecular weight kininogen there is generally no clinical evidence of bleeding unless other contributing factors are present. These deficiencies are generally diagnosed when evaluating a prolonged APTT with no other explanation (i.e., other screening tests and clinical history is negative for a bleeding disorder).

Clot-based Assay Principle

Intrinsic pathway factors are measured using a modification of the APTT. At least three different dilutions are made of the test plasma in imidazole buffer. These dilutions are then mixed with an equal volume reagent plasma immunologically depleted of the factor being measured; for example, FVIII-depleted plasma is added to the plasma dilutions of a patient suspected of having a FVIII deficiency. The factor-depleted plasma is added to ensure that all factors except for the one under investigation are present at greater than 75% of normal levels thus, only the factor being studied will affect the outcome of the APTT. Next, the APTT reagent, consisting of phospholipid and a particulate activator (silica, ellagic acid, kaolin, or other particulate substance) is added and the mixture is allowed to incubate for a prescribed interval of time to activate the contact factors. Calcium chloride is then added and the mixture is timed until clot formation occurs. Results are calculated by comparing the dilutions of the test plasma to that of a curve prepared using a standard reference plasma. The clotting time interval of each dilution is inversely proportional to the activity of the factor being studied.
**Clot-based Assay Performance Characteristics**

The clotting factors are assayed using multiple dilutions to detect possible inhibitors. Specific factor inhibitors may affect either the specific factor being assayed or may affect the test system of other factors being tested. If the inhibitor is directed against the factor being assayed, the percentage activity of that factor will remain relatively constant at all dilutions. If the inhibitor is directed at a factor not specifically being assayed, the factor level being assayed will increase as dilution of inhibitor increases.

**Factor VIII Chromogenic Assay Principle**

FVIII can be measured by a chromogenic methodology using an amidolytic substrate specific for FIX. Since FVIII is a cofactor for FIX, color production is proportional to the FVIII activity in the sample.

**Factor VIII Chromogenic Assay Performance Characteristics**

Polybrene has been added to the reagent test system to minimize heparin effect up to 1.0 IU/mL. This assay may be useful in differentiating between a specific factor VIII inhibitor and decreased factor VIII levels that may be due to a nonspecific inhibitor such as a lupus anticoagulant.

**Factor IX Immunoassay Principle**

Diluted test plasma is pipetted into a microtiter plate well coated with anti-human coagulation FIX antibody. The FIX in the test plasma binds to this antibody. A second antibody, coupled with an enzyme, is then added binding the captured factor. A substrate is added and the amount of color produced is directly proportional to the factor concentration.

**Factor IX Immunoassay Performance Characteristics**

The immunoassay for FIX detects and measures quantitative not qualitative deficiencies, and thus should be employed to confirm functional results or to distinguish between a quantitative and qualitative deficiency. The immunoassay gives a normal result in the presence of cross-reacting material (CRM+), i.e., defective molecule with normal antigen levels but abnormal function.

**Reference Range**

<table>
<thead>
<tr>
<th>Factor Assay</th>
<th>Reference Range</th>
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<tr>
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</tr>
<tr>
<td>Factor VIII Chromogenic</td>
<td>50 - 170%</td>
</tr>
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<td>Factor IX activity</td>
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<td>60 - 150%</td>
</tr>
<tr>
<td>Factor XII activity</td>
<td>50 - 150%</td>
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<tr>
<td>Prekallikrein (Fletcher factor)</td>
<td>65 - 135%</td>
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<tr>
<td>High molecular weight kininogen</td>
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<td>(Fitzgerald factor)</td>
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</table>

COAGULATION HANDBOOK
Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then centrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Test Request Information

Factor VIII activity assay (#300103), factor VIII chromogenic (#300214), factor IX activity (#300104), factor IX antigen (#300205), factor XI activity (#300106), and factor XII activity (#300107), Prekallikrein (#300121) and high molecular weight kininogen (#300123) may be ordered separately from the Esoterix Service Directory. CPT Code: factor VIII activity 85240, factor VIII chromogenic 85240, factor IX activity 85250, factor IX antigen 85250, factor XI activity 85270, factor XII activity 85280, prekallikrein (Fletcher factor) 85292, high molecular weight kininogen 85293. For information on test orders for inhibitors to the above factors, please refer to the Esoterix Service Directory.

Reflex Recommendations

A prolonged APTT on a specimen with a normal PT may indicate an intrinsic factor pathway deficiency or inhibitor. APTT mixing studies are performed to distinguish a deficiency from the presence of heparin, a lupus anticoagulant, or a specific factor inhibitor. If mixing studies result in correction (indicating a deficiency) and the patient has hemorrhagic symptoms, evaluation for deficiencies of FVIII, FIX, or FXI should be performed. If bleeding is not present and FVIII, FIX and FXI results are normal, evaluation for deficiencies of FXII, prekallikrein or high molecular weight kininogen can be performed.

Some patients with a mild bleeding disorder may have a normal APTT. This may be due to the sensitivity of the APTT reagent to a specific factor. In these instances, factor assays may be performed to rule out a mild specific factor deficiency.

Intrinsic pathway factor assays may be performed to monitor therapy or the development of an inhibitor in hemophilia A, hemophilia B, and hemophilia C. The time, frequency, and dosage of therapeutic product should be recorded prior to specimen collection.

Therapy

Patients with hemophilia require prompt correction of their factor deficiencies to attain hemostatic levels in acute bleeding or in potential bleeding episodes. Treatment given preventatively or at first onset of symptoms limits not only the amount of bleeding but the extent of tissue damage that may occur if bleeding is left unchecked. In general, 30-50 percent of normal factor levels are needed for most bleeding episodes; 50-100 percent for treatment or prevention of life threatening or post surgical hemorrhage.

FVIII deficient patients may be given DDAVP (desmopressin) in association with Amicar (inhibitor of clot lysis by the fibrinolytic system) for mild defects, or FVIII concentrates (anti-hemophilic factor, AHF) for moderate and severe deficiencies. The plasma half-life of therapeutic FVIII is approximately 12 hours. Extended periods of prophylactic therapy to prevent recurrent bleeding may be needed. Complications of AHF therapy include infection by the Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV) or parvovirus leading to chronic active hepatitis, cirrhosis, hepatocellular carcinoma and liver failure. With the development and administration of recombinant FVIII protein products (i.e., Recombinate®, Kogenate® and Refacto®), viral transmission does not occur. Another complication of therapy, especially in severe hemophiliacs, is the development of FVIII inhibitors. These inhibitors occur in up to 30% of severe hemophilia A patients independent of type of therapy.
For FIX deficient patients, administration of FIX concentrates (i.e., Mononine®, Alphanine SD® and Benefix®) are used. These preparations are free of HIV and hepatitis viruses. Prior to these, a prothrombin complex concentrate (PCC) was used. This product was not viral-free and contained activated factors VIIa and Xa, making it thrombogenic and sometimes requiring heparin therapy during administration. Development of FIX inhibitors occurs in 3% of severe cases. The half-life of therapeutic FIX concentrate is approximately 24 hours.

For FXI deficient patients, administration of fresh frozen plasma or FXI concentrate may be used. The half-life of therapeutic FXI is 45-55 hours. An acquired FXI inhibitor may develop in transfused patients but incidence of this is rare.

Other therapeutic advances, such as gene therapy, are continually being made for the treatment of hemophilia patients leading to a possible cure for these diseases.

References

FACTOR V MUTATION (LEIDEN) AND HR2 HAPLOTYPE

Factor V Leiden Mutation Physical Characteristics

In at least 90% of cases, activated Protein C resistance (APCR) is due to a single point mutation in the factor V (FV) gene that results in substitution of glutamine (Gln) for arginine (Arg) in the 506th amino acid position of the FV molecule (R506Q). This corresponds to nucleotide position G1691A. This mutation, also known as FV Leiden mutation, was first described by Dahlback in 1993.

HR2 Haplotype Physical Characteristics

The HR2 haplotype was first described in 1996, by Lunghi, et al. as a polymorphism that replaces histidine with arginine at amino acid 1299 (His 1299 Arg) in the B domain of the FV protein. It was called R2 polymorphism because of its susceptibility to the Rsa1 restriction enzyme. This R2 polymorphism is a single nucleotide polymorphism (SNP) at nucleotide 4070 and is also referred to as A4070G mutation because adenine is replaced by guanine. The R2 polymorphism is linked to at least 6 other polymorphisms, and this collection of SNPs that is inherited as a group, is referred to as the HR2 haplotype. Haplotype is a term used to describe closely linked genetic markers that tend to be inherited together. Some of the polymorphisms in the HR2 haplotype are clinically silent. Evaluation of the HR2 haplotype is generally performed by detection of the R2 polymorphism (A4070G) only, due to observed linkage disequilibrium with the other polymorphic sites.

FV Leiden Mutation Physiology

Activated Protein C (APC) normally inhibits clot formation by proteolytically inactivating normal coagulation factors Va and Vlla in the presence of Protein S. The FV Leiden point mutation makes FV resistant to the proteolytic action of APC, thereby promoting increased thrombin generation and thrombosis.

HR2 Haplotype Physiology

The physiologic basis of the potential prothrombotic potential of the HR2 haplotype has been recently elucidated. Plasma and platelet FV normally exists as two isoforms, FV1 and FV2. These isoforms differ in the level of glycosylation of the C2 domain which is that portion of the FV molecule involved in phospholipid membrane interaction. The FV1 isoform increases thrombin generation due to decreased cofactor activity in the inhibition of FVIIIa. Individuals with the HR2 haplotype have increased levels of FV1 isoform.

FV Leiden Mutation Incidence

APCR is demonstrated in 20%-60% of patients with recurrent venous thrombosis. The FV Leiden mutation is present in 3%-7% of persons of Northern European descent, making this defect at least ten times as prevalent as other known genetic abnormalities predisposing to thrombosis. This cause of inherited thrombophilia is virtually absent in Asian, African, and Mediterranean populations.

HR2 Haplotype Incidence

The HR2 haplotype is common in the general population with an overall frequency of 6 to 11%. Unlike FV Leiden, which is seen predominantly in Caucasians, HR2 has a broader distribution with higher allelic frequencies in diverse populations including African Americans, Hispanics and Asians.
FV Mutation Clinical Significance

Patients with activated Protein C resistance ratios of less than the laboratory established cut-off are at increased risk for venous thrombosis. Deep venous thrombosis (DVT) is the most common clinical manifestation of APC resistance in a manner similar to Protein C, Protein S, and antithrombin deficiencies. The risk of thrombosis is significantly increased if other contributing risk factors such as a genetic deficiency of Protein C or Protein S, pregnancy, oral contraceptive usage, or surgery is present concomitantly with APC resistance. Patients with heterozygous FV Leiden have a 5- to 10-fold increased risk of thrombosis while patients with homozygous FV Leiden have a 50- to 100-fold increased risk of thrombosis. Although FV Leiden carries a life-long risk of thrombosis, many heterozygous patients remain asymptomatic.

HR2 Haplotype Clinical Significance

There is an association between the R2 polymorphism and decreased levels of FV. In addition to low FV levels, studies have reported an increased resistance to APC in those individuals with HR2 haplotype. Furthermore, recent studies have demonstrated an association between the HR2 polymorphism and increased risk of venous thrombosis, which occurs both in the presence and absence of FV Leiden. In the absence of the FV Leiden mutation, the HR2 haplotype is associated with a twofold-increased risk of venous thrombosis. However, these 3 main parameters, namely, decreased FV levels, increased resistance to APC, and increased risk of venous thrombosis, are not consistently associated with each other and some investigators have published a non-correlation. The risk of developing venous thrombosis, associated with HR2 haplotype has also been shown to be significantly increased only in patients with acquired risk factors such as surgery, immobilization, malignancy and pregnancy.

FV Leiden and HR2 Haplotype Dual Inheritance Clinical Significance

Because both mutations have high allelic frequency, predicted coinheritance of both mutations is 1 in 400 in the general population and 3 in 100 in a thrombophilic group. Coinheritance of FV Leiden and HR2 haplotype may explain in part the thrombotic tendency in some but not all individuals with FV Leiden. Resistance to APC is exaggerated in individuals who are heterozygous for both the HR2 and FV Leiden polymorphisms and is in fact similar to the level of APC resistance seen in individuals homozygous for FV Leiden. Inheritance of the HR2 polymorphism with FV Leiden further increases thrombotic risk threefold over heterozygous FV Leiden alone.

FV Leiden Mutation and HR2 Haplotype Assay Principle

These assays use polymerase chain reaction (PCR) technology. The patient’s DNA is isolated from white blood cells and the gene sequence that contains the specific mutation is then analyzed.

Assay Performance Characteristics

The FV Leiden assay indicates resistance to activated Protein C caused by the Arg-Gln substitution (R506Q) and is unable to detect resistance to activated Protein C due to any other abnormality. The HR2 haplotype assay detects only the R2 polymorphism (A4070G). Recently, evidence has suggested that alterations to the FV gene other than FV Leiden may contribute to poor response to activated Protein C.

Reference Ranges

In persons without thrombotic risk due to these factors, the assay for either mutation is negative (normal). The FV Mutation (Leiden) as well as the HR2 haplotype assays can detect persons that are heterozygous or homozygous for these mutations.
Specimen Requirements

EDTA or ACD whole blood maintained at room temperature (do not freeze). Collect blood into a lavender (EDTA) or yellow (ACD) stopper tube, ensure complete fill of the tube, and invert gently six times immediately after filling. The specimen should be kept at room temperature and should not be centrifuged. Label and place specimen in container to be shipped overnight within 5 days of collection.

Test Request Information

FV Leiden (#120719) and HR2 haplotype (#300734) can be ordered separately from the Esoterix Service Directory. CPT Codes for FV Leiden: 83891, 83894, 83900, 83912.

CPT Codes for HR2 Haplotype: 83891, 83892, 83894, 83898, 83912. Both assays are also included in the ThrombAssureSM Profile (#129260), ThrombAssureSM Plus Profile (#129250) and the ThrombAssureSM Enhanced Profile (#300931). These profiles contain other thrombotic risk testing, refer to the Esoterix Service Directory for complete descriptions.

Reflex Recommendations

The kindred of individuals with FV Leiden should be screened for the abnormality to assess thrombotic risk to family members. To detect the cause of thrombosis, APCR is often assayed with other molecular markers of thrombosis such as prothrombin gene mutation, lupus anticoagulant, antithrombin, Protein C, and Protein S.

References

Physical Characteristics

Plasma factor XIII (FXIII), also known as fibrin stabilizing factor, is composed of two protein units forming a tetrameric molecule of two A-subunits and two B-subunits. It has a molecular weight of 320,000 Daltons and circulates as a zymogen. There is a cellular form of FXIII found in macrophages and platelets.

Physiology

The A subunit of FXIII is biologically active as it contains the active site, activation peptide and calcium binding site. The B subunit of FXIII is integral to the regulation of FXIII activation and appears to stabilize the A subunit. Plasma FXIII is activated through the action of thrombin in the presence of calcium. When FXIII is activated, it catalyzes the development of covalent bonds between fibrin molecules, crosslinking these molecules forming a stable fibrin clot.

Clinical Significance of Deficiency

Severe FXIII deficiency is associated with a bleeding diathesis. Severe deficiency occurs with a homozygous FXIII deficiency. Patients with severe hereditary FXIII deficiency can have a life-long bleeding tendency and affected women, in addition, suffer recurrent spontaneous abortions. Delayed wound healing occurs in about 15% of affected individuals. Bleeding from the umbilical stump immediately following birth, while a unique site of bleeding, is common in homozygous FXIII deficiency. Intracranial bleeding commonly occurs as a spontaneous event or in association with mild trauma and is a major cause of morbidity in this population. FXIII replacement therapy in the form of fresh frozen plasma, cryoprecipitate or FXIII concentrate (Fibrogammin P) appears to diminish bleeding in these patients and has been shown to allow pregnancies to continue until term. Even a small amount of FXIII (>2%) appears to protect against bleeding and pregnancy loss.

Heterozygous FXIII deficient patients do not suffer a bleeding diathesis, but women may suffer a greater frequency of spontaneous abortions. FXIII levels are decreased in the newborn period and acquired FXIII deficiency occurs in patients with disseminated intravascular coagulation, severe liver disease, and acute leukemia. The development of autoantibodies (IgG) to FXIII, although rare, may result in a severe bleeding diathesis; usually manifest as extensive ecchymoses. The development of autoimmune FXIII inhibitors is more common in those on isoniazid but may occur spontaneously most commonly in an elderly population. Laboratory evaluation reveals a low FXIII level and 1:1 normal plasma mix fails to show correction of the FXIII activity into the expected range. The mortality associated with FXIII antibodies is high, despite therapy.

Assay Principle

FXIII present in the patient sample is converted to FXIIIa by thrombin. The action of thrombin also converts fibrinogen to fibrin within the sample. Fibrin formed by the action of thrombin is prevented from forming a clot by an aggregation-inhibiting peptide reagent in the test system. The FXIIIa produced, links a specific peptide substrate with a glycine ethyl ester, releasing ammonia. A second enzyme system measures the ammonia production which is proportional to FXIII activity.

Assay and Inhibitor Screen

To screen for the presence of a FXIII inhibitor, a FXIII level is first determined. Inhibitor screen and titer are not performed unless the FXIII level is less than 60%. To screen for an inhibitor, test plasma is mixed with normal plasma at varying dilutions and the activity assay repeated. Failure to normalize activity suggests the presence of an inhibitor. The titer is reported as the last dilution that the inhibitor effect is noted.
FACTOR XIII ACTIVITY (cont.)

**Performance Characteristics**

Fibrinogen levels above 600 mg/dL or below 80 mg/dL may result in falsely low FXIII activity levels. The lower limit of detection is in the range of 10 – 12%. A urea clot solubility assay is sometimes used to screen for FXIII deficiency. This solubility assay, however, is typically abnormal only when FXIII levels are below 2% and, therefore, may be normal in the presence of a clinically significant FXIII deficiency.

**Reference Range**

The reference range for FXIII activity is 60% to 150%.

**Specimen Requirements**

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete filling of the tube. Invert gently six times immediately after drawing. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then centrifuge for an additional ten minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma into two plastic tubes, cap, label, and freeze immediately at -20° or below. Ship the frozen specimen overnight on dry ice.

**Test Request Information**

Factor XIII activity RUO (#300108) factor XIII inhibitor screen RUO (#300308) and factor XIII inhibitor titer RUO (#300309) may be ordered separately from the Esoterix Service Directory. CPT Codes: 85290; 85290 X 2, 85335; 85290 X 3, 85335.

**References**

FACTOR INHIBITOR (BETHESDA TITER) ASSAYS

Physical Characteristics

Factor inhibitors (also called circulating anticoagulants or inactivators) are endogenously produced antibodies, commonly specific for their respective coagulation factors, that interfere with coagulation in vivo or in vitro.

Physiology, Incidence, and Etiology

Specific factor inhibitors can be classified as neutralizing or non-neutralizing. Neutralizing inhibitors interact with the functional component of the coagulant protein impairing its function and may cause clinical bleeding. Non-neutralizing antibodies react with the protein somewhere other than the functional epitopes and may be clinically silent or may lead to increased clearance of the factor.

Factor VIII (FVIII) inhibitors may arise as IgG4 (non-complement fixing) alloantibodies or autoantibodies. FVIII alloimmunization occurs in 15%-20% of hemophilia A patients in response to treatment with FVIII concentrates. Alloimmunization occurs primarily in severe hemophilia A when the patient's plasma FVIII concentration is less than 1%. Alloimmunization is idiosyncratic, affecting some, but not all severe hemophilia A patients. Autoimmune FVIII inhibitors occur in one per one million individuals, primarily in pregnancy and the postpartum period, in patients over 55 years old, and in people with primary autoimmune disorders. Porcine FVIII inhibitors may arise in hemophilia A patients treated with porcine factor VIII (Hyate C®) therapy.

Factor IX (FIX) inhibitors arise as alloantibodies in 3%-5% of hemophilia B (Christmas disease) patients in response to FIX concentrate, prothrombin complex concentrate, or fresh frozen plasma therapy. Inhibitors to FXI deficient patients are extremely rare. Inhibitors to factor V (FV) have been shown to develop after exposure to bovine topical thrombin "fibrin glue" and may also arise spontaneously. Inhibitors of all other coagulation factors are extremely rare as deficiencies of these factors are rare. Autoimmune anti-factor X (FX) inhibitors have been described.

Clinical Consequences of Factor Inhibitors

Alloimmune factor inhibitors are suspected when hemophilia patients' symptoms increase in severity, or bleeding continues despite infusion of ever increasing dosages of factor concentrates. The normal plasma half-life of FVIII, for example, is 12 hours; however, Hemophilia A patients with inhibitors may have undetectable FVIII levels a few minutes after factor VIII concentrate infusion. These hemophilia A patients may require specialized therapy to halt acute bleeding.

Clinically significant factor inhibitors form covalent complexes with their target coagulant factor molecules, rendering the factors non-functional. For alloimmune FVIII inhibitor, this is typically a first order reaction with complete in vitro inactivation of available FVIII. Autoimmune FVIII inhibitors and some alloimmune inhibitors exhibit in vitro second order kinetics, achieving equilibrium with FVIII leaving some residual FVIII unbound in the plasma. In vivo, however, autoimmune FVIII inhibitors cause “acquired hemophilia” with symptoms as severe as the more common congenital severe hemophilia. The mortality rate for acquired hemophilia is up to 22%.

Factor Inhibitor (Bethesda Titer) Assay Principle

Factors VIII, IX, XI, and XII Inhibitors

(The factor VIII inhibitor assay is used as an example of testing principle.)

Serial dilutions are made of patient plasma with veronal buffered saline, then mixed with normal plasma containing close to 100% factor VIII activity. The serial dilutions are then incubated for 2 hours. An activated partial thromboplastin time (APTT)-based FVIII assay using factor VIII-depleted plasma substrate is performed on each of these incubated mixtures. Results are compared to those of incubated normal plasma. One Bethesda unit is defined as the amount of factor VIII inhibitor that neutralizes 0.5 IU of factor VIII in this system. The number of serial dilutions tested is based on the anticipated level of the inhibitor.

FIX, FXI, and FXII inhibitor assays are performed in the same manner as the factor VIII inhibitor assay, substituting the respective FIX, FXI, and FXII depleted substrate plasmas in place of the FVIII depleted substrate plasma.
FACTOR INHIBITOR (BETHESDA TITER) ASSAYS (cont.)

Porcine Factor VIII Inhibitors

The porcine FVIII inhibitor assay is performed using the FVIII inhibitor test protocol above, except that porcine FVIII (Hyate: C®) concentrate is used in place of normal plasma. The porcine FVIII (Hyate: C®) concentrate is mixed with human FVIII depleted substrate prior to mixing with the patient’s serial dilutions.

Factors II, V, VII, and X Inhibitors

Assays for these factor inhibitors are performed in a manner similar to the FVIII inhibitor assay, except that the testing method used is a prothrombin time-based system, using thromboplastin reagent, rather than an APTT testing system.

Factor Inhibitor (Bethesda Titer) Assay Performance Characteristics

It is difficult to obtain an accurate Bethesda titer in patients whose inhibitors demonstrate second order kinetics. Reported results in this circumstance represent our best approximation. If there is residual coagulation factor, it could falsely lower the results. Lupus anticoagulant activity must be ruled out prior to assaying for specific factor inhibitors.

Reference Ranges

<table>
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<tr>
<th>Assay</th>
<th>Reference Range</th>
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<tbody>
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<td>Factor II inhibitor</td>
<td>&lt;0.8 Bethesda units</td>
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<tr>
<td>Factor V inhibitor</td>
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<td>Factor VII inhibitor</td>
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<td>Factor VIII inhibitor</td>
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<td>Factor VIII inhibitor (porcine)</td>
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<td>&lt;0.8 Bethesda units</td>
</tr>
<tr>
<td>Factor XII inhibitor</td>
<td>&lt;0.8 Bethesda units</td>
</tr>
</tbody>
</table>

Factor inhibitors are not normally present in plasma. When present, factor inhibitors are measured by Bethesda titer units (BU). Titors of less than 5 BUs are classified as low responders, titers greater than 10 BUs as high responders. Responder status influences the approach for clinical treatment.

Specimen Requirements

Two citrated plasma specimens: Collect blood into two blue-stopper collection tubes containing 3.2% (0.109M) buffered sodium citrate. Ensure the blood reaches the fill line. Invert gently six times immediately after filling. Centrifuge the capped tubes at 2000g for ten minutes. Transfer the plasma to two plastic centrifuge tubes using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to two additional plastic tubes, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimens overnight on dry ice.
Test Request Information

All factor inhibitor assays are ordered individually as required and can be ordered separately from the Esoterix Service Directory.

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<th>Assays</th>
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Reflex Recommendations

Factor inhibitor assays are ordered in response to clinical impression, and are performed periodically on individuals who have inhibitors and are receiving therapy. During therapy, periodic assays are essential, both to manage the dosage and to establish whether a patient is a low or high responder. Low responders maintain titers consistently under 5 BUs while high responders may generate extremely high titers and rapid anamnestic responses to therapy. Assays must also be performed repeatedly during immune tolerance therapy.

Therapy for Factor Inhibitors

Acute Bleeding Episodes

To resolve acute or life-threatening hemorrhage, low responders may be given increased factor concentrate (VIII or IX) dosages to neutralize the inhibitor and achieve therapeutic factor levels. Fibrinolyis inhibitors such as Amicar or tranexamic acid may be given simultaneously. High responders, or patients demonstrated to be unresponsive to therapy, are given porcine factor VIII concentrate (Hyate C®) or recombinant FVIIa (Novoseven®). Patients who develop porcine FVIII inhibitors may be given FVIII bypassing preparations such as prothrombin complex concentrate (PCC, Proplex®), activated PCC (FEIBA®), or recombinant FVIIa. Low dose heparin infusions may be given with activated preparations such as Proplex® and FEIBA® but not Novoseven® to reduce the risk of thrombosis or DIC.
References


**Physical Characteristics**

Fibrinogen, also known as factor I, is a large glycoprotein synthesized in the liver with a molecular weight of approximately 340,000 Daltons. Fibrinogen, the most abundant hemostatic protein at approximately 300 mg/dL, circulates in plasma but is also found in platelet granules. It has a half-life of approximately 4 days.

**Physiology**

Thrombin converts plasma fibrinogen to fibrin through a series of enzymatic steps involving removal of polar peptides. These peptides, known as fibrinopeptide A and B, are considered markers of fibrinogen conversion to fibrin specifically by thrombin. Fibrinopeptides A and B constitute approximately 3% of the total fibrinogen molecule. The remaining portion of the molecule, fibrin monomers spontaneously polymerize due to electrostatic interactions and are stabilized by factor XIII through cross-linkage.

Fibrinogen, present in the plasma in a much greater quantity than von Willebrand factor, competes for the platelet glycoprotein IIb/IIIa binding site during the platelet activation sequence of clot formation. Due to fibrinogen's concentration in the plasma, the majority of glycoprotein IIb/IIIa sites are bound to fibrinogen.

**Etiology of Deficiency**

The congenital absence of fibrinogen, termed afibrinogenemia, is inherited as an autosomal recessive trait and is likely a subset of dysfibrinogenemia. Hypofibrinogenemia, with both recessive and dominant modes of inheritance, is characterized by low fibrinogen levels, typically in the range of 10 to 50 mg/dL, for both functional and antigenic levels. Using strict definitions, however, hypofibrinogenemia is defined as immunologic fibrinogen levels less than 150 mg/dL. The presence of structurally abnormal or qualitatively defective fibrinogen molecules is termed dysfibrinogenemia. In patients with dysfibrinogenemia, functional fibrinogen levels are lower than antigenic levels resulting in a low functional to immunologic ratio, usually in the range of 1:2. Dysfibrinogenemia may manifest as defects of fibrin polymerization, fibrinopeptide release, or abnormal lysis of the fibrin clot. Congenital dysfibrinogenemia is inherited in an autosomal dominant manner. There are a large variety of dysfibrinogenemias, typically named after the city in which they were first discovered (i.e. Chapel Hill II, Naples, Detroit, Baltimore I, etc.).

Acquired hypofibrinogenemia may be seen in a variety of disease states, most commonly liver disease, but has been described in disseminated intravascular coagulation (DIC), renal disease, bone marrow transplantation and during L-asparaginase therapy.

**Clinical Significance**

Because it is an acute phase reactant protein, following any kind of stress, trauma, infection or inflammation, fibrinogen levels may increase and, therefore, may vary significantly in a given individual. Levels also increase with age, pregnancy and oral contraceptive usage. Even smoking may produce a transient elevation in fibrinogen levels. Elevated fibrinogen contributes to increased plasma viscosity, and is a well-established independent risk factor for cardiovascular and cerebrovascular disease. Its relationship to increased risk of ischemic heart disease has been demonstrated in a number of studies including the Northwick Park Heart Study, the Göteborg Study, the Leigh Study, the Framingham Study, and the Caerphilly Study.

Patients diagnosed with afibrinogenemia have a severe, lifelong bleeding diathesis. These individuals present with umbilical cord hemorrhage, deep muscle and joint bleeding, menorrhagia and other mucous membrane hemorrhage. Spontaneous abortions are also common in this population. Patients with hypofibrinogenemia usually have only a mild bleeding tendency even with prolonged PT and APTT results. Surgery or trauma may initiate severe bleeding in these individuals. They may also present with menorrhagia, recurrent abortions and placental abruptio. Patients with the diagnosis of hereditary dysfibrinogenemia may be asymptomatic (55%), have a chronic history of bleeding following surgery or trauma (25%), poor wound healing or may even have a tendency toward thrombosis (20%). Acquired dysfibrinogenemia shows little if any increase in thrombotic risk. (In dysfibrinogenemia, immunological levels of fibrinogen may be low and functional levels even lower.)
Activity Assay Principle

Fibrinogen activity may be measured using a variety of methods including Clauss, Ellis-Stransky or Ratnoff-Menzie, as well as a derived fibrinogen using the curve generated from the prothrombin time. In the Clauss method, the most widely used, plasma is diluted with buffer and thrombin is then added. The clotting endpoint is compared to a standard reference curve and fibrinogen concentration is determined.

Activity Assay Performance Characteristics

The evaluation of fibrinogen must take into account that fibrinogen is an acute phase reactant and as such can be markedly elevated in patients with inflammation, trauma, and infection. Plasma clot-based assays may show artificially low values due to substances that interfere with the rate of clot formation such as heparin and fibrin(ogen) degradation products (FDP). Reagent test systems vary in their sensitivity to interfering substances and are not typically influenced unless greater than 0.6 - 1.0 U/mL heparin and/or greater than 100 ug/mL fibrin(ogen) degradation products are present. Lipemia or hemolysis may interfere with methods that utilize optical density changes in clot end point detection.

Antigen Assay Principle

Fibrinogen present in the test plasma forms immune complexes with rabbit anti-human-fibrinogen antibodies. These complexes scatter a beam of light passed through the sample by the nephelometer. The intensity of scattered light is proportional to the concentration of the fibrinogen in the sample.

Antigen Assay Performance Characteristics

Artifactual increases may occur in patients with significant levels of circulating fibrin degradation products (e.g. samples from patients undergoing fibrinolytic therapy, or in DIC) as the antiserum to human fibrinogen also detects fibrinogen degradation products. Very lipemic samples, or frozen samples which are turbid after thawing, must be clarified by centrifugation prior to analysis to avoid interference with the assay.

Reference Range

<table>
<thead>
<tr>
<th>Fibrinogen Assay</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen Activity</td>
<td>160 – 420 mg/dL</td>
</tr>
<tr>
<td>Fibrinogen Antigen</td>
<td>180 – 400 mg/dL</td>
</tr>
</tbody>
</table>

Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count < 10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.
**Test Request Information**

Fibrinogen activity (#300200) and fibrinogen antigen (#300199) can be ordered separately from the Esoterix Service Directory. CPT Code Fibrinogen Activity 85384, Fibrinogen Antigen 85385.

**References**


Physical Characteristics

Fibrinogen degradation products, breakdown products of fibrinogen or fibrin, have multiple sizes and thus varying molecular weights. Specifically, the X fragment has a molecular weight of 250,000 Daltons while the D fragment has a molecular weight of 100,000 Daltons and Y fragment has a molecular weight of 150,000 Daltons. The D-dimer fragment has a half-life in circulation of 3 to 6 hours.

Fibrinogen Degradation Products Physiology

Fibrinogen degradation products (FDP), also known as fibrinogen split products (FSP), are the result of plasmin digestion of both crosslinked and non-crosslinked fibrin or fibrinogen. Fragments X and Y are considered the early split products from fibrinogen and from non-crosslinked fibrin. FDPs are normally cleared rapidly from circulation. When present in large quantities FDPs can act as anticoagulants through several mechanisms including; forming soluble complexes with fibrin monomer, prohibiting the fibrin monomer from polymerizing, and binding platelet surfaces and interfering with normal platelet function.

D-dimer Physiology

D-dimer is a terminal degradation product of cross-linked fibrin molecules. This degradation product is therefore unique to fibrin clot formation. D-dimer fragments have unique epitopes that are detected in the D-dimer assay.

Clinical Significance

The presence of FDP in plasma can provide important information for the diagnosis of hemostatic disorders. Increased FDPs are seen in primary fibrinolysis as well as during fibrin clot breakdown. Elevated FDP levels are a hallmark finding in disseminated intravascular coagulation (DIC). In fact, greater than 95% of cases of DIC have increased levels of FDP. However, elevated FDP levels are not specific for DIC and increased FDP levels also are seen in primary fibrinolysis, severe liver disease, including alcoholic cirrhosis, eclampsia, during acute thrombotic episodes, following surgery or trauma, and after lytic therapy.

Most healthy individuals have low levels of circulating D-dimers. D-dimer levels increase with age and with gestation during pregnancy. Levels may increase with venous or arterial thrombosis and increased levels post-myocardial infarction are predictive for a recurrent event. D-dimer levels are increased in those with atherosclerotic vascular disease and levels may correlate with disease severity. Levels also increase in patients with visceral tumors and, in some instances, levels correlate with tumor stage. D-dimer levels are increased with systemic inflammatory conditions and severe infections. Elevated levels also have been seen in patients with disseminated intravascular coagulation and those with sickle cell disease as long as six months following a painful crisis. Elevated D-dimer levels are specific for fibrinolysis. Levels of D-dimer may be normal in cases of primary fibrinogenolysis.

Increased D-dimer levels above a laboratory’s specific predetermined cutoff level are seen in patients with venous thrombosis including pulmonary embolism (PE), and deep vein thrombosis (DVT). The ELISA method of D-dimer and other sensitive, automated quantitative assays have sensitivity for DVT of 90% or greater and a high negative predictive value of 90% or greater by most studies. This suggests that the finding of negative D-dimer levels by a sensitive quantitative method has a high negative predictive value and provides exclusion of the venous thrombosis diagnosis unless clinical suspicion is high. Elevated D-dimer levels following thrombosis and a recommended course of anticoagulant therapy has been shown to predict an increased risk for recurrence. While elevated D-dimer levels are not specific for venous thrombosis, low D-dimer levels may be used to rule out thrombosis. Studies have suggested that a D-dimer level of less than 500 ng/mL fibrinogen equivalent units using an ELISA technique can rule out venous thrombosis, although each assay should be evaluated to determine the cut-off levels for diagnostic application. In patients on anticoagulant therapy, D-dimer levels typically do not rise to the same degree as in patients not anticoagulated leading to the potential of a false negative D-dimer in an anticoagulant patient with venous thrombosis. Importantly, D-dimer has been used in conjunction with clinical probability for the diagnosis of DVT and PE. In studies of patients with clinically suspected PE, the use of D-dimer levels was beneficial in patients with indeterminate ventilation/perfusion scans in deciding whether or not to perform pulmonary arteriography.
Fibrin(ogen) Degradation Products Assay Principle

The FDP assay is performed using latex particles coated with monoclonal antibodies against FDP. When FDPs are present, macroscopic agglutination occurs. Plasma FDP levels may be semi-quantitated by using dilutions of the patients plasma and comparing the agglutination pattern to a positive control. In the semi-quantitative method, the patient plasma is diluted 1:2 and 1:8. When agglutination is not present in the 1:2 dilution or the 1:8 dilution, the semi-quantitative result is less than 5 mg/mL FDP. When agglutination is present in the 1:2 dilution but not in the 1:8 dilution, the semi-quantitative result is equal to or greater than 5 mg/mL and less than 20 mg/mL FDP. When agglutination is present in the 1:2 dilution and in the 1:8 dilution, the semi-quantitative result is equal to or greater than 20 mg/mL FDP.

D-dimer Quantitative Manual ELISA Assay Principle

This is not the standard D-dimer assay performed. The quantitative assay is based on a manual enzyme immunoassay. The patient D-dimer is bound by D-dimer monoclonal antibody covalently bound to the microtiter well. The wells are washed and a detector antibody conjugated to horseradish peroxidase is added. Color production is directly proportional to the concentration of D-dimer in the patient sample. This method has a lower limit of quantitation than immunoturbidometric methods of D-dimer testing. This manual ELISA is offered only for clinical trials.

D-dimer Quantitative Immunoturbidometric (Latex Immunoassay [LIA]) Assay Principle

Antibody-coated latex particles bind D-dimer fragments in patient samples forming aggregates. Monochromatic light passed through the sample is absorbed in a manner proportionate to the degree of aggregation and, therefore, D-dimer level in the test plasma.

Fibrin(ogen) Degradation Products Assay Performance Characteristics

The assays for FDP do not distinguish fibrinogen degradation products from those of fibrin. The assay has a lower detection limit of 2.5 mg/mL and patients with marked elevations in FDP will require dilution to obtain accurate results. Patients with dysfibrinogenemia may demonstrate false positive FDP titers as a result of incomplete clotting. Manual latex slide agglutination requires visual interpretation and as such can be insensitive and subjective.

Fibrin(ogen) Degradation Products Reference Range

Normal levels of FDP are 0-5 mg/mL.

D-dimer Assay Performance Characteristics

The D-dimer assay is specific for fibrin degradation products formed by the enzymatic action of plasmin. The specificity of the enzyme immunoassay D-dimer for venous thrombosis varies between manufacturers and with cut-off. The range of specificity is 30-75%. The ELISA method does not show interference by hyperbilirubinemia or rheumatoid factor. Manual latex slide agglutination requires visual interpretation and as such can be insensitive and subjective. Manual latex slide agglutination assays are semi-quantitative and, in general, should not be used to exclude venous thrombosis.

D-dimer Reference Range

The reference range for D-dimer manual ELISA quantitative is <251 ng/mL D-dimer units and for the immunoturbidometric assay is less than 256 ng/mL D-dimer units. Results can also be expressed in fibrinogen equivalent units (FEU), where one D-dimer unit equals 2 FEU.
Fibrinogen Degradation Products Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.105 – 0.109) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Fibrinogen Degradation Products and D-dimer Test Request Information

D-dimer LIA Quantitative (#300415) and FDP (#300201), can be ordered separately from the Esoterix Service Directory. CPT Codes D-dimer LIA Quantitative 85379 and FDP 85362.

References

**Physiology**

The unfractionated form of heparin (UFH) is a heterogeneous negatively charged sulfated mucopolysaccharide with a molecular weight between 5,000 and 30,000 Daltons. Conversely, low molecular weight heparin (LMWH) is a subfraction of UFH with molecules that have a molecular weight of <10,000 Daltons. These two forms of heparin differ not only in molecular weight but also in their effectiveness in catalyzing antithrombin's inactivation of thrombin and factor Xa (FXa). Unfractionated heparin catalyzes inactivation of Xa and thrombin in a 1:1 ratio, but low molecular weight heparin favors Xa activation 2:1 to 4:1.

Danaparoid sodium is an antithrombotic agent that exerts its anticoagulant properties through the inhibition of FXa and factor Ila (FIIa). The product contains primarily heparan sulfate, 84%, as well as dermatan sulfate and chondroitin sulfate. This drug is no longer available in the U.S.

Fondaparinux is a pentasaccharide that binds antithrombin and potentiates its FXa inhibitor effect. Fondaparinux does not inhibit thrombin. This relatively new anticoagulant agent has an immediate anticoagulant effect when administered intravenously. This drug may be used in individuals with heparin-associated thrombocytopenia.

**Clinical Significance**

Heparin is the most commonly administered antithrombotic agent in the acute setting and frequently is chosen for the treatment and prevention of thrombotic disorders because of its immediate onset of action following intravenous administration. The anticoagulant effect of unfractionated heparin (UFH) is variable among patients due to varied concentrations of acute phase reactants and variations in clearance. For this reason, UFH must be monitored in the laboratory when administered in therapeutic doses. LMWH is rapidly absorbed from the subcutaneous tissue and has rapid onset of action. It has greater predictability of dose response than UFH, and it may be less likely to trigger heparin-induced thrombocytopenia. UFH, LMWH and fondaparinux can be monitored using a heparin anti-FXa assay. LMWH and fondaparinux cannot be monitored using an APTT assay. Heparin Anti-FXa levels should be drawn 6 hours after initiation of UFH therapy or change in dose whereas with LMWH therapy levels should be drawn 6 hours after administration when given once daily and 3-4 hours when administered twice daily.

Fondaparinux has reliable bioavailability and is dosed based on patient's weight. Routine monitoring is not necessary when administered in therapeutic of prophylactic doses. This drug is not associated with the development of antibody induced thrombocytopenia and thrombosis. There is no known antidote to reverse the drug's effect. Fondaparinux levels should be measured 3 hours post administration.

**Anti-FXa Unfractionated Heparin Assay Principle**

The Heparin Anti-FXa assay measures the effect of unfractionated heparin on Xa activity in the plasma using an amidolytic method that employs a synthetic chromogenic substrate. Patient plasmas or unfractionated heparin standards are incubated in the presence of antithrombin and an excess of FXa resulting in an antithrombin-heparin-Xa complex. The Xa remaining in solution reacts with the chromogenic substrate to form a color end product. The color intensity is inversely proportional to the heparin concentration in the sample.

**Anti-FXa LMWH Assay Principle**

The assay for LMWH heparin is performed in the same manner as the Heparin Anti Xa for UFH with the exception that LMWH standards are used. Since the anticoagulant activity of LMWH and danaparoid sodium is predominantly anti-FXa dependent, the effect of the drug on the prothrombin time and activated partial thromboplastin time assays is minimal. To date, LMWH is monitored using the anti-FXa activity to assess its biological activity.
HEPARIN ANTI-FXa (cont.)

**Heparin Anti-FXa Fondaparinux Assay Principle**

Although routine monitoring is not necessary, this agent can be evaluated using the anti-FXa activity assay to assess its biological activity. The assay for fondaparinux is performed in the same manner as the Heparin Anti Xa for UFH with the exception that fondaparinux standards are used. Since the anticoagulant activity of fondaparinux and danaparoid sodium is predominantly anti-FXa dependent, the effect of the drug on the prothrombin time and activated partial thromboplastin time assays is minimal. To date, fondaparinux is monitored using the anti-FXa activity to assess its biological activity.

**Heparin Solution Quantitation Assay Principle**

This assay uses the same principle as the Heparin anti-FXa test to assay therapeutic heparin solutions. The solution is diluted prior to testing. The test procedure may be used for both LMWH and UFH solutions.

**Anti-FXa Assay Performance Characteristics**

Because standard heparin is not well absorbed by the gastrointestinal tract, standard heparin is given by subcutaneous injection, or intravenously by continuous or intermittent infusion. UFH levels should be obtained 6 hours following administration or change in dose. LMWH is given subcutaneously; therefore, the peak and trough times will provide important clinical data as to the patient’s therapeutic range. Levels below 0.05 IU/mL are reported as negative as this is the lower limit of detection for LMWH and the lower limit of detection is 0.1 for UFH.

Although typically monitored by the activated partial thromboplastin time (APTT), unfractionated heparin can be monitored using the Heparin anti-FXa assay as well. In fact, certain interferences with the APTT including the presence of lupus anticoagulant, elevated FVIII levels, and elevated fibrinogen levels may make the Heparin anti-FXa assay more appropriate.

**Anti-FXa Reference Ranges**

The reference range depends on the drug being monitored and whether it is administered at prophylactic or therapeutic doses. For LMWH, reference range depends on whether the drug is administered once a daily or twice per day.

<table>
<thead>
<tr>
<th>Assays</th>
<th>Therapeutic Range</th>
<th>Prophylactic Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin Anti-FXa (unfractionated)</td>
<td>0.3 – 0.7 IU/mL</td>
<td>0.1 – 0.2 IU/mL</td>
</tr>
<tr>
<td>Heparin Anti-FXa LMWH</td>
<td></td>
<td>0.05 – 0.5 IU/mL</td>
</tr>
<tr>
<td>Twice/day dosing</td>
<td>0.6 – 1.1 IU/mL</td>
<td></td>
</tr>
<tr>
<td>Once/day dosing</td>
<td>1.0 – 2.0 IU/mL</td>
<td></td>
</tr>
<tr>
<td>Fondaparinux</td>
<td></td>
<td>0.39 – 0.50 mg/mL</td>
</tr>
</tbody>
</table>

**Anti-FXa Assay Specimen Requirements**

Citrate plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Process immediately or within one hour after collection of specimen. Centrifuge the capped tube at 2000g for ten minutes. Specimen must be platelet free (platelet count <10 x 10^9/L). Plasma must be separated
from the red cells within one hour of phlebotomy to avoid falsely low heparin levels. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

**Heparin Solution Quantitation Assay Specimen Requirements**

Five mL of the heparin solution (heparin vial, syringe or IV bag) to be evaluated should be sent to the laboratory with an expected value in U/mL. This will assist the technologist in approximating the necessary dilutions to perform the assay within the limits of linearity.

**Anti-FXa Reflex Recommendations**

Unfractionated heparin levels also can be evaluated using the APTT. The therapeutic level is an expression in seconds correlating to a heparin plasma level of 0.3 IU/mL to 0.7 IU/mL. This APTT value is highly dependent on the sensitivity of the partial thromboplastin reagent used, and it is recommended that each laboratory establish a heparin therapeutic range to ensure adequate anticoagulation. The APTT is not a sensitive assay for measuring low molecular weight heparin or fondaparinux.

**References**

HEPARIN COFACTOR II

Physical Characteristics

Heparin cofactor II (HC II) is a glycoprotein that belongs to the serine protease inhibitor family. HC II, also known as heparin cofactor A or dermatan sulfate cofactor, has a molecular weight of approximately 65,000 Daltons and has a plasma concentration of 9 mg/dL. HC II is synthesized by the liver and has a plasma half-life of approximately 60 hours.

Physiology

HC II specifically inhibits thrombin, in contrast to antithrombin which inhibits thrombin, factor Xa, and other serine proteases. The inhibition of thrombin by HC II is approximately 10 times slower than antithrombin-mediated inhibition. The antithrombotic activity of HC II is greatly enhanced (over 1000-fold) in the presence of heparin and dermatan sulfate. The inhibition of thrombin occurs through the formation of equimolar complexes between the reactive site of the inhibitor and the active site of thrombin. The physiologic function of the molecule is unclear, but its role may be to serve as an antithrombotic agent in the presence of dermatan sulfate in the vasculature as well as the inhibition of fibrin-bound thrombin. Decreased HC II activity may be associated with placental dysfunction.

Etiology of Abnormalities

Acquired deficiencies of HC II are reported in patients with acute and chronic liver disease and disseminated intravascular coagulation. Decreased levels have also been reported in patients with heparin-induced thrombocytopenia and may be transiently decreased postoperatively. Conversely, increased levels of HC II may be observed in individuals with renal disorders with proteinuria, during the third trimester of pregnancy, and with oral contraceptive usage. Inherited deficiency of HC II has been reported in rare instances and is inherited as an autosomal dominant trait. Both type 1 and type 2 deficiencies have been reported.

Clinical Significance

A clear relationship between increased risk of thrombosis and HC II deficiency has not been established since deficiency of HC II is observed in both healthy individuals and those with thrombotic episodes. In limited studies, it has been observed that heterozygosity for HC II is not a likely risk factor for thrombosis without other concomitant risk factors. Limited studies demonstrated that 36% of individuals with HC II deficiency had thrombotic episodes.

Assay Principle

Patient plasma is incubated with excess thrombin in the presence of dermatan sulfate. The residual thrombin activity reacts with the chromogenic substrate to form a color end product. The color intensity is inversely proportional to HC II levels.

Assay Performance Characteristics

The HC II assay is not affected by heparin levels up to 1U/mL.

Reference Range

In healthy adults, HC II reference range in plasma is 65%-145%. At birth, plasma levels of HC II are approximately 50% of adult levels and approach adult levels at six months of age. However, levels may remain about 10% below adult normals until 16 years of age.
## Specimen Requirements

Citrate plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

## Test Request Information

Heparin cofactor II (#300133) can be ordered separately from the Esoterix Service Directory. This assay is approved for research use only. CPT Code: 85130.

## Reflex Recommendations

HC II assays may be useful in patients with recurrent thrombosis with no other identifiable congenital or acquired risk factors.

## References

HEPARIN-INDUCED PLATELET ANTIBODY

Synonyms: Heparin-Induced Antibody, Heparin-Induced Thrombocytopenia Antibody (HITA), Heparin-Induced Platelet Antibody (HIPA), Heparin-Associated Thrombocytopenia Antibody (HAT)

Etiology

An adverse effect of heparin therapy is the development of antibodies. This may result in heparin-induced thrombocytopenia (HIT), also known as heparin associated thrombocytopenia (HAT), a clinical finding characterized by a decrease in the platelet count following administration of heparin. HIT is observed in one to five percent of patients receiving standard unfractionated heparin and is seen more commonly in patients receiving bovine preparations than porcine preparations. HIT may be observed with any route of administration, any dosage regimen, or any heparin type. The development of antibodies appears to be more common in surgical rather than medical patients that receive heparin. It is uncommon following exposure to LMWH. Two forms of HIT are seen. The most common form appears as mild thrombocytopenia one to five days after initiation/exposure to heparin in a patient who has not previously received the drug and is thought to be caused by non-immune mechanisms (Type I). Type I HIT is generally a self-limiting disorder and platelet counts return to normal with or without the cessation of heparin therapy. The second form (Type II) of HIT is an immune-mediated response to heparin and can lead to life-threatening complications. Heparin-induced platelet antibodies are detected with Type II HIT.

Clinical Significance

Immune-mediated HIT (Type II) is typically associated with decreased platelet count and may be associated with severe thrombosis. The onset of thrombocytopenia is typically 5-10 days following exposure to heparin. While moderate thrombocytopenia is the most common manifestation, platelet count in those with HIT can be in the normal range depending on platelet count prior to development of HIT. A 50% decrease in platelet count is considered significant. Thrombocytopenia is rarely severe. If HIT antibody is not associated with a significant drop in platelet counts, thrombosis is unlikely. Patients who previously have been exposed and sensitized to heparin may develop thrombocytopenia within hours to three days of re-exposure if heparin has been administered in the last 100 days.

The antibodies responsible for immune-mediated HIT are directed against the heparin-PF4 complex, a neoepitope, that forms following heparin administration. The severe thrombosis reported in HIT can be venous or arterial with severe and fatal consequences. Only a minority of patients with heparin antibodies will develop clinical manifestations such as thrombosis.

Heparin-Induced Thrombocytopenia – Clinical Rules for Diagnosis

The clinical course of the development of thrombocytopenia in relation to heparin exposure is critical in determining if the patient has HIT. Drs. Warkentin and Greinacher have developed ten clinical “rules” for diagnosing HIT which are listed below.

Disclaimer: The following ten clinical “rules” have been formulated primarily for didactic purposes, and are not intended necessarily to imply any standard of care in relation to their clinical application.

Rule 1: A thrombocytopenic patient whose decrease in platelet count began between days 5 and 10 of heparin treatment (inclusive) should be considered to have HIT unless proved otherwise (first day of heparin use is considered “day 0”).

Rule 2: A rapid fall in the platelet count soon after starting heparin is unlikely to represent HIT unless the patient has received heparin in the recent past, usually within the past 100 days.

Rule 3: A platelet count decrease of more than 50% from the postoperative peak between days 5 and 14 after surgery associated with heparin treatment can indicate HIT even if the platelet count remains higher than 150 X 10^9/L.

Rule 4: Petechiae and other signs of spontaneous bleeding are not clinical features of HIT, even in patients with very severe thrombocytopenia.

Rule 5: HIT is associated with a high frequency of thrombosis despite discontinuation of heparin with or without substitution by coumarin: the initial rate of thrombosis is about 5-10% per day over the first 1-2 days; the 30-day cumulative risk is about 50%.
Rule 6: Localization of thrombosis in patients with HIT is strongly influenced by independent acute and chronic clinical factors, such as the postoperative state, atherosclerosis, or the location of intravascular catheters in central veins or arteries.

Rule 7: In patients receiving heparin, the more unusual or severe a subsequent thrombotic event, the more likely the thrombosis is caused by HIT.

Rule 8: Venous limb gangrene is characterized by (1) in vivo thrombin generation associated with acute HIT; (2) active deep vein thrombosis in the limb(s) affected by venous gangrene; and (3) a supratherapeutic international normalized ratio (INR) during coumarin anticoagulation. This syndrome can be prevented by delaying coumarin use in acute HIT until therapeutic anticoagulation is achieved with an agent that reduces thrombin generation (e.g., danaparoid) or that inhibits thrombin directly (e.g., lepirudin).

Rule 9: Erythematous or necrotizing skin lesions at heparin injection sites should be considered dermal manifestations of the HIT syndrome, irrespective of the platelet count, unless proved otherwise. Patients who develop thrombocytopenia in association with heparin-induced skin lesions are at increased risk for venous and, especially, arterial thrombosis.

Rule 10: Any inflammatory, cardiopulmonary, or other unexpected acute event that begins 5-30 min after an intravenous heparin bolus should be considered acute HIT unless proved otherwise. The post-bolus platelet count should be measured promptly, and compared with pre-bolus levels, because the platelet count decrease is abrupt and often transient.

**Assay Principle - PF4/Heparin Antigen Assay**

HIT antibody is measured by enzyme immunoassay using platelet factor 4 complexed to heparin or to another linear polyanionic compound such as polyvinyl sulfonate (PVS) coated microwells in stoichiometric proportions. The patient serum sample is added to a microwell, and, if present, heparin-induced platelet antibody binds to the complex coating the well. After addition of an enzyme-linked conjugate antibody and substrate, production of a chromophore is a direct function of the concentration of heparin-induced platelet antibody in the test sample.

**Assay Principle - Normalized OD (Optical Density)**

The OD cut-point between positive and negative for the heparin-induced platelet antibody assay varies with each assay run. For this reason the patient results are reported as patient OD ÷ cut-point OD and the ratio reported. Clinical HIT is more likely if the patient OD is high and this would correlate to a high OD ratio. The results indicate the highest dilution at which the patient is positive for heparin-induced antibodies, compared to a normal serum sample. Titer correlates well with OD.

**Assay Performance Characteristics – Antigen Assay**

Antigen assays tend to be more sensitive for the detection of HIT antibodies and are less sensitive for clinical HIT. According to Warkentin, only a subset of PF4/heparin-reactive antibodies can activate platelets and cause clinical HIT.

Clinical HIT is more likely if the OD of the antigen assay is high. For example, an OD of >1.5 is more likely to be associated with clinical HIT than an OD of 0.6. As OD will increase over time in patients developing HIT antibodies, repeat HIT antigen testing is recommended when an initial antigen result is borderline.

Microbial contamination, lipemia, icterus, or hemolysis may interfere with valid test results and should be eliminated or the patient sample recollected. The presence of immune complexes or immunoglobulin aggregates in the patient sample may cause increased non-specific binding and produce false-positive results. The presence of excess heparin in the sample (greater than 1 U/mL) can interfere with the assay and cause false negative results.

The heparin-induced antibody test is intended as a screening test and should not be relied upon solely to establish the diagnosis of heparin-induced thrombocytopenia.
HEPARIN-INDUCED PLATELET ANTIBODY (cont.)

Assay Principle – Platelet Activation Assay – Serotonin Release

Platelets prepared from healthy subjects, previously demonstrating responsiveness to HIT-IgG, are incubated with [14C]-serotonin. Test serum or plasma and the appropriate type of heparin (porcine or bovine if known) is added at a low and high concentration. Following an incubation and centrifugation, the concentration of serotonin is measured in the supernatant.

Assay Performance – Platelet Activation Assay – Serotonin Release

The serotonin release assay has greater specificity for clinical HIT than antigen assays. The performance of this assay is highly dependent on the appropriate responsiveness of donor platelets. A small percentage of the normal population contains HLA antibodies or immune complexes that may cause platelet activation to occur at both low and high heparin concentrations. This would be considered an indeterminate result.

Reference Range – Antigen Assay

Heparin-induced platelet antibodies are usually not detected in healthy individuals.

Reference Range – Serotonin Release Assay

Patient’s serum does not release serotonin in the presence of heparin.

Specimen Requirements

Serum blood specimen: Collect blood into a plain red top tube. Allow the specimen to sit at least 30 minutes to clot. Centrifuge the specimen for 10 minutes. Dispense the serum into plastic tube. Label and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice. 3.2% citrated plasma collected in a blue-top tube and processed as for serum may also be used.

Test Request Information

Heparin Antibody Profile (includes both Heparin Induced Antibody and Serotonin Release Assay #300935), Heparin-Induced Platelet Antibody Test (#300522), Heparin-Induced Platelet Antibody Titer (#300528), or Serotonin Release (C-14) Assay (#300082) can be ordered separately from the Esoterix Service Directory. CPT Codes Heparin Antibody Profile – 86022 x 3, Heparin Induced Platelet Antibody Test – 86022, Heparin Induced Platelet Antibody Titer – 86022 x 3, Serotonin Release Assay – 86022 x 2.

Reflex Recommendations

The PF4/heparin antigen test is intended to be used as a screening test. The results of this assay should not be relied on solely to establish the diagnosis of heparin-induced thrombocytopenia, as the diagnosis must be made in conjunction with the clinical picture. If the antigen test is positive, consideration should be given to evaluation of the more specific activation assay by serotonin release.

Therapy for Heparin-Induced Platelet Antibody
When heparin-induced antibodies are detected, the administration of heparin should be discontinued and an alternate form of anticoagulation be considered when necessary. Heparin-coated catheters should be sought and removed. Once heparin administration has ceased, the platelet count should return to normal within a few days, if indeed the thrombocytopenia was due to heparin antibodies. If the suspicion for clinical HIT is high in the presence of a positive laboratory assay, consideration should be given to initiation of anticoagulant therapy other than UFH, LMWH and warfarin therapy.

References

**Physical Characteristics**

C-reactive protein is a 105 kDa structure produced in the liver, composed of 5 identical non-glycosylated subunits, similar in structure to serum amyloid P. This protein was named by Tillet and Francis in 1930 due to its ability to bind and therefore precipitate polysaccharides from the “C” fraction in their experimental system.

**Physiology**

In healthy individuals C-reactive protein is present at very low levels. Levels can increase several hundred-fold within 24 to 48 hours of an acute inflammatory stimulus due to increased hepatic synthesis, mostly in response to pro-inflammatory cytokines, predominantly interleukin-6. This inflammation–dependent increase in levels is also called acute phase response. There are several acute phase proteins whose levels are non-specifically increased as a result of inflammation, including fibrinogen and factor VIII.

CRP binds to polysaccharides and in doing so, serves as an opsonin for bacteria and immune complexes. This protein can also activate the complement cascade and monocytes, inducing expression of tissue factor.

**Clinical Significance**

CRP is a marker of systemic inflammation. As an indicator of acute phase response, levels are generally greater than 8 mg/L. It has been demonstrated that levels, previously considered in the normal range, that is 0.5 – 3.0 mg/L, may be predictive of cardiovascular disease. This is in agreement with the concept that the development of cardiovascular disease is associated with a low-level inflammatory component. Levels of C-reactive protein and other inflammation-sensitive proteins including fibrinogen and factor VIII predict future cardiovascular events. Of these 3 proteins, C-reactive protein appears to be the most sensitive. In the absence of an acute inflammatory stimulus, C-reactive protein levels are relatively stable with time. C-reactive protein is considered an independent, prospective cardiovascular risk factor. Risk appears to be graded over a range of 0.5 mg/L to 3.0 mg/L.

**Assay Principle**

In the presence of CRP from the test sample, agglutination of polystyrene particles coated with monoclonal antibody to CRP occurs, which is measured using nephelometric analysis. CRP concentration is determined based on a calibration curve generated from dilutions of a known standard. The descriptor “high sensitivity” (hs) refers to the lower limits of detection of the assay and thus, higher sensitivity. This is determined by both the methodology and the CRP standard used. Typical CRP assays detect levels as low as 3.0 mg/L whereas the hsCRP assay detects levels as low as 0.1 mg/L.

**Performance Characteristics**

Turbidity and particles in the sample may interfere with the assay. Highly lipemic samples which cannot be cleared by centrifugation should be rejected. Heat-inactivation of the samples should be avoided since this can lead to diminished CRP values. Therefore, samples should be thawed rapidly at 37°C. Levels in plasma are on average slightly lower than serum samples due to the dilutional effect of sodium citrate in the blue stoppered evacuated tube.
**Reference Range**

hsCRP results over 8 mg/L may indicate an acute inflammatory condition and cannot be used to establish risk of cardiovascular disease. Measurement of hsCRP in the determination of cardiovascular risk should not be made until two weeks after an acute infection. In the determination of cardiovascular risk, the American Heart Association recommends that two hsCRP values, taken at least two weeks apart, be averaged and the averaged value used to determine the level of risk.

When used as a marker for cardiovascular disease, hsCRP results <1.0 mg/L may indicate a low risk of cardiovascular disease in the absence of other risk factors. Values from 1.0 – 3.0 mg/L imply moderate risk, and values >3.0 mg/L imply high risk of disease. Other risk factors such as elevated cholesterol increase the relative risk for cardiovascular disease. When used to assess risk of cardiovascular disease, measurements should be compared to previous values.

**Specimen Requirements**

Citrate plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure the blood reaches the fill line. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice. EDTA plasma also can be used.

Serum specimen: Collect blood using standard venipuncture techniques. Allow sample to clot for 30 minutes and then centrifuge for 10 minutes at 2500g. The sample must not contain any particles or traces of fibrin. The serum must be removed from the coagulated cells within 60 minutes after collection and stored at +2 to +8°C for no more than 8 days. The serum can also be stored at -25°C or below for up to 3 months if they are frozen within 24 hours after collection and repeated freeze-thaw cycles are avoided.

**Test Request Information**

The hsCRP assay (#300312) can be ordered separately from the Esoterix Service Directory. CPT Code 86141.

**References**

Homocysteine and Methylene tetrahydrofolate Reductase (MTHFR C677T and A1298C) Polymorphisms

**Physical Characteristics**

Homocysteine is a naturally occurring, sulfur containing amino acid precursor formed during the metabolism of methionine, an essential amino acid derived from the diet.

**Physiology**

Two pathways metabolize homocysteine: remethylation and transsulfuration. According to Key and McGlennen, remethylation regulates primarily fasting homocysteine levels while the transsulfuration pathway is involved when plasma homocysteine levels are higher as in the post prandial state or following methionine loading. The remethylation pathway requires the presence of methionine synthase, a vitamin B12-dependent enzyme, as well as a methyl group that is donated to the reaction by 5-methyltetrahydrofolate. The completion of this pathway also requires dietary folate and the methylenetetrahydrofolate reductase enzyme (MTHFR), a riboflavin (vitamin B2)-dependent enzyme. The second pathway, present only in the liver and kidney, involves degradation of homocysteine into cysteine and inorganic sulfates. This transsulfuration process is vitamin B6 (pyridoxine)-dependent, initially catalyzed by cystathionine β-synthase. Hyperhomocysteinemia is a result of congenital or acquired defects in either of these metabolic pathways.

**Homocysteine Etiology**

Genetic factors resulting in increased levels of homocysteine include cystathionine β-synthase deficiency, methionine synthase deficiency and deficiency of N5, N10-methylenetetrahydrofolate reductase enzyme (MTHFR). Homozygous deficiency of any of these enzymes leads to markedly increased plasma homocysteine levels in the range of 100 – 400 μM/L and homocystinuria. These inherited disorders typically present with mental retardation and other abnormalities such as ectopia lentis. These individuals manifest a marked predisposition to cardiovascular disease and venous thrombosis. Up to 50% suffer a thrombotic episode by 30 years of age. A variant of the MTHFR enzyme results in reduced activity at 37ºC and enhanced thermolability of the enzyme at 42ºC. This polymorphism is caused by a point mutation, a cytosine to thymine substitution at base 677 (C677T). This is the most common genetic cause of hyperhomocysteinemia but elevations of homocysteine occur only in those that are also deficient in folic acid. Homozygosity occurs in 5% to 9% of the population, and heterozygosity can be detected in another 30% to 41%. A second variant of the MTHFR gene, an adenine to cytosine substitution at base 1298 (A1298C), also results in decreased MTHFR activity but has not been associated with increased homocysteine levels if it is the only MTHFR polymorphism present. Compound heterozygosity of the C677T and A1298C mutations, however, has been shown to increase homocysteine levels.

Environmental factors leading to increased homocysteine levels include dietary deficiencies of vitamin B6, B12, and folate, chronic alcohol or substance abuse, excessive coffee consumption, and smoking. Hyperhomocysteinemia has been found in association with several prescription drugs also, namely, carbamazepine, Phenobarbital, phenytoin, primidone, valproic acid, cyclosporine, metformin, methotrexate, nitrous oxide and theophylline. Elevations are also seen in patients with impaired renal function, post-organ transplantation, hypothyroidism, malignant diseases and those with hypertension.

**Clinical Significance**

Hyperhomocysteinemia is a risk factor for both cardiovascular disease (CVD) and venous thromboembolic disease (VTE). The risk for vascular disease appears to be graded such that the higher the homocysteine level, the greater the risk. A meta-analysis published in 1995 reported that a 5μmol/L increase in homocysteine is associated with an odds ratio for CVD of 1.6. Hyperhomocysteinemia may be a more significant risk factor for recurrent rather than first VTE.
It is not known if hyperhomocysteinemia is a cause of CVD and VTE or rather an effect of either of these entities. It has been demonstrated that homocysteine levels may increase for up to 6 months after an acute arterial thrombotic effect. Whether this occurs following venous thrombosis is not studied.

Severe hyperhomocysteinemia usually is seen only with a homozygous enzyme deficiency such as CBS deficiency. Moderate hyperhomocysteinemia can occur in patients with B12 folate deficiency, especially if associated with homozygous MTHFR C677T polymorphism. This may also occur with renal insufficiency, certain drug therapies and underlying clinical disorders such as hypothyroidism, rheumatoid arthritis and inflammatory bowel disease. Lifestyle influences such as smoking, physical inactivity and caffeine are associated with milder elevations of homocysteine.

**Assay Principle**

Homocysteine can be measured easily by an automated fluorescence polarization immunoassay (FPIA) methodology. Bound homocysteine is reduced to free homocysteine (HCY), converted to S-adenosyl-L-homocysteine that is then tagged with a fluorescent antibody. The intensity of polarized fluorescent light emitted is indirectly proportional to the amount of homocysteine in the test sample as compared to a standard curve.

**Reference Range**

The reference range for serum or plasma homocysteine is 4.0 – 14 μmol/L. In general, normal homocysteine levels tend to increase with age and are higher in men than in women. Although there is no standardized scheme to grade elevation of homocysteinemia, the following scheme may be used.

<table>
<thead>
<tr>
<th>Test</th>
<th>Reference Range</th>
</tr>
</thead>
</table>
| Homocysteine | 4.0 – 14.0 μmol/L
| MTHFR C677T | Normal |
| MTHFR A1298C | Normal |

**MTHFR C677T Polymorphism Assay Principle**

Genetic testing is performed using micro-array technology involving polymerase chain reaction (PCR) and restriction fragment length polymorphism technology (RFLP) or by fluorescent resonance energy transfer (FRET) technology on hemolysate or on whole blood collected in EDTA, ACD or sodium citrate anticoagulant.

**MTHFR A1298C Polymorphism Assay Principle**

Genetic testing is performed using micro-array technology involving polymerase chain reaction (PCR) and restriction fragment length polymorphism technology (RFLP) or by fluorescent resonance energy transfer (FRET) technology on hemolysate or on whole blood collected in EDTA, ACD or sodium citrate anticoagulant.
HOMOCYTEINE (cont.)

MTHFR C677T and A1298C Polymorphisms Assay Performance Characteristics

As genetic assays, the MTHFR C677T and the A1298C tests can determine if an individual is normal (does not have a polymorphism of the gene at nucleotides 677 and 1298), heterozygous or homozygous for the specific MTHFR polymorphism being tested. These assays will not detect other genetic abnormalities that may cause hyperhomocysteinemia.

Homocysteine Specimen Requirements

Patients should be fasting overnight to obtain a true homocysteine value; eating will increase methionine levels thereby increasing homocysteine levels. Methionine loading using a 0.1 g of L-methionine per Kg body weight may be administered and homocysteine levels measured 3 – 6 hours following this oral ingestion. Methionine loading increases the sensitivity and specificity of detecting hyperhomocysteinemia. It has been reported that 25 – 45% of patients will demonstrate hyperhomocysteinemia post-methionine loading despite normal basal levels. Samples should be processed rapidly. Homocysteine in whole blood is very unstable at room temperature and artificial elevations occur as red blood cells metabolize S-adenosylmethionine and release homocysteine into the sample.

Serum sample: Collect blood into a SST red top tube. Allow the specimen to clot. Centrifuge the capped specimen at 2000 x g for 10 minutes. Transfer the serum to a plastic tube, cap, label, and freeze immediately. Ship the frozen specimen overnight on dry ice at -20°C or lower. (Note: Concentrations of homocysteine in serum have been observed to be up to 10% higher then in plasma.)

Plasma sample: Collect blood into a blue top tube containing 3.2% (0.109M) buffered sodium citrate or a purple top (EDTA) tube. Ensure complete fill of the evacuated tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000 x g for ten minutes. Transfer the plasma into a plastic centrifuge tube using a plastic transfer pipette, re-centrifuge for an additional ten minutes. Transfer the plasma to a second plastic tube, cap, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

MTHFR C677T and A1298C Polymorphisms Assay Specimen Requirements

Collect blood into a lavender (EDTA) or yellow (ACD) stoppered tube. Ensure complete fill of the evacuated tube. Invert gently six times immediately after filling. The specimen should be kept at room temperature and should not be centrifuged. Label tube and place specimen in container to be shipped overnight at room temperature within 3 days after collection.

Test Request Information

Homocysteine (#300727), MTHFR C677T polymorphism (#120733) and MTHFR A1298C polymorphism (#120740) may be ordered separately from the Esoterix Service Directory. CPT Codes: Homocysteine-83090, MTHFR C677T-83891, 83892,83894,83898, 83912; MTHFR A1298C-83891, 83892, 83894, 83898, 838912.

Reflex Recommendations

Homocysteine, serum methylmalonic acid, along with serum levels of vitamin B12 and folate are tests used to diagnose and distinguish the clinical entities of vitamin B12 and folate deficiency. Homocysteine levels should be repeated approximately one month following initiation of therapy to determine the effectiveness. The kindred of individuals with hyperhomocysteinemia or MTHFR mutations should be screened for the abnormality to assess thrombotic risk to family members.
Therapy of Hyperhomocysteinemia

Mild to moderate hyperhomocysteinemia can generally be reduced through dietary modification and vitamin supplementation providing adequate amounts of folic acid, vitamin B12 and vitamin B6.

References

Physical Characteristics

Lupus anticoagulants are immunoglobulins of the antiphospholipid antibody (APLA) family. APLAs are IgG, IgM, or IgA antibodies directed against negatively charged phospholipid protein complexes. The proteins that bind phospholipid to form these antibody complexes include β-2 glycoprotein I, prothrombin, (activated) protein C, protein S, factor XI (FXI), high molecular weight kininogen and annexin V.

Lupus anticoagulants are APLAs that may prolong phospholipid-dependent clot-based assays such as the activated partial thromboplastin time (APTT), dilute Russell’s viper venom test (DRVVT), tissue thromboplastin inhibition test (TTIT), and kaolin clotting time test (KCT). They may be neutralized by the phospholipid contained in the reagent of the hexagonal-phase phospholipid or the platelet neutralization procedure (PNP) assays. At least 60% of all APLA prolong the clot-based lupus anticoagulant tests; the remainder are detected with anti-phospholipid immunoassays.

Physiology

Lupus anticoagulants represent a heterogeneous family of antibodies directed against phospholipid-protein complexes that are associated with a variety of clinical conditions, most commonly thrombosis, thrombocytopenia and fetal loss. The biochemical relationship between the antibodies’ in vivo response to target antigen combinations and thrombosis remains to be established.

Incidence

Antiphospholipid antibodies are detected in 1-2% of unselected individuals. APLAs are present in up to 50% of individuals with systemic lupus erythematosus and are also seen in polyarteritis, Sjogren's syndrome, rheumatoid arthritis, and other autoimmune diseases. Transient development of lupus anticoagulants is common and frequently associated with viral infections and certain drug therapies.

Etiology

APLAs may arise in individuals with syphilis, where they may cause biological false positive serological test results (as these individuals do not have antiphospholipid antibodies but rather antibodies to cardiolipin), in bacterial infections, in HIV and other viral infections. These APLA seldom persist, and appear to have no clinical consequences.

In a certain percentage of patients, APLAs persist, and these antibodies have the potential for associated complications. To determine persistence, assays should be repeatedly positive at least twelve weeks apart. At least 30% of persistent APLA are associated with a thrombotic condition. When associated with thrombosis, thrombocytopenia or recurrent fetal loss, the presence of persistent APLA is called antiphospholipid syndrome (APS). In those patients with underlying autoimmune disorders, this is referred to as secondary APS and when they arise spontaneously, it is called primary APS.

Drugs that may trigger APLA formation include phenothiazine, various antibiotics, hydralazine, quinine and quinidine, calcium channel blockers, procainamide, and phenytoin. Antiphospholipid antibodies induced by drugs are occasionally associated with thrombosis, and should be considered clinically significant if they persist.

Clinical Significance

Thirty percent of persistent lupus anticoagulants are associated with APS, a syndrome that may encompass both arterial and venous thrombosis. The arterial symptoms of APS include most commonly, coronary thrombosis, stroke, and transient ischemic attacks. Manifestations of venous thrombosis include superficial and deep vein thrombosis, pulmonary embolism and recurrent fetal loss. Recent studies suggest that APLA is associated with a higher incidence of severe pre-eclampsia, placental abruption, intrauterine growth retardation, and possibly infertility. Thrombocytopenia often accompanies APS but is seldom associated with bleeding.
Assay Principles

Dilute Russell’s Viper Venom (DRVVT)
Russell’s viper venom activates the coagulation system by directly activating FX. Xa forms a complex with Va to activate II (prothrombin). FIIa (thrombin) cleaves fibrinogen, which polymerizes to form a detectable clot. In the DRVVT, the term dilute refers to the reduced concentration of phospholipid used in the reagent, to increase sensitivity for APLA. Lupus anticoagulants typically cause prolongation of the dilute Russell’s viper venom time. The DRVVT may be advantageous over the APTT as a screen for APLA in some instances, as this reaction does not involve FVIII. FVIII can elevate as an acute phase reactant and shorten the APTT, diminishing the degree of prolongation seen with some lupus anticoagulants. DRVVT Confirm is a DRVVT assay which utilizes an increased phospholipid concentration. The DRVVT assay is performed in the presence of dilute phospholipid and concentration increased to show phospholipid dependence. A ratio of screen/confirm is reported.

Platelet Neutralization procedure (PNP)
The PNP reagent is concentrated phospholipid extracted from platelets that neutralizes most lupus anticoagulants. If the addition of PNP reagent corrects a prolonged DRVVT or APTT result by greater than 5-7 seconds, the presence of lupus anticoagulant is implied.

Activated Partial Thromboplastin Time Test (APTT)
The activated partial thromboplastin time test is described in a separate entry, however the APTT is the most popular of the clot-based test procedures for lupus anticoagulant detection because it is used for numerous applications in all acute care hemostasis laboratories. Purified phospholipid of animal, vegetable, or human origin and negatively charged particulate activators are incubated with test plasma to activate the contact factors and consequently the intrinsic system. Calcium chloride is added and the mixture is timed until a clot forms. The intrinsic mechanism requires the presence of all coagulation factors except FVII and XIII. The APTT is frequently prolonged in the presence of a lupus anticoagulant.

Hexagonal Phase Phospholipid
Hexagonal phase phospholipid is a concentrated phospholipid reagent that can neutralize lupus anticoagulants. This reagent includes a sensitive APTT reagent, polybrene to neutralize heparin and normal plasma to correct for any factor deficiencies. It is named for its unique phospholipid configuration which may be more specific for lupus anticoagulant. The addition of the reagent neutralizes lupus anticoagulant, correcting the previously prolonged APTT result by greater than 8-10 seconds, confirming the presence of lupus anticoagulant.

Tissue Thromboplastin Inhibition Test (TTIT)
Tissue thromboplastin, the reagent used in the prothrombin time test, activates the extrinsic coagulation system by activating FVII. Vlla then activates FX, Xa forms a complex with FVa to activate FII (prothrombin). Ila (thrombin) cleaves fibrinogen, which polymerizes to form a detectable clot. In the TTIT, the diluted thromboplastin (and hence diluted phospholipid) is partially neutralized by lupus anticoagulant, causing prolongation of clot formation.

Kaolin Clotting Time (KCT)
In the KCT test, kaolin, a negatively charged particulate activator, is incubated with test plasma to activate the contact factors and the intrinsic system. Calcium chloride is added and the mixture is timed until a clot forms. The intrinsic mechanism requires the presence of all coagulation factors except FVII and XIII. In the KCT, unlike TTIT, DRVVT, and APTT, no reagent phospholipid is added, so plasma phospholipids are required for the reaction to proceed. For this reason the KCT is sensitive to the presence of lupus anticoagulants that if present, will generally prolong the KCT. The unknown sample is mixed with an equal volume of normal pooled plasma prior to testing to correct for any factor deficiencies. The patient result in seconds is divided by a normal pooled plasma result and the dividend multiplied by 100 to produce “KCT units”.

COAGULATION HANDBOOK
CLOT-BASED LUPUS ANTICOAGULANT TESTS (cont.)

Assay Performance Characteristics

All clot-based tests are potentially affected by factor deficiencies. The KCT and APTT tests may be prolonged in any except FVII or XIII deficiency. The TTIT may be prolonged by deficiencies of FX, VII, V, II, and fibrinogen, and the DRVVT by deficiencies of FX, V, II, and fibrinogen. Factor deficiencies must be ruled out using APTT and prothrombin time mixing studies.

All clot-based tests are affected by the presence of therapeutic plasma heparin unless the reagent system contains a heparin neutralizer. When heparin is suspected, the test plasma may be neutralized by adsorption with polybrene or an enzyme to degrade the heparin prior to testing.

The presence of excess platelets in a plasma sample causes all clot-based tests to lose sensitivity for the presence of lupus anticoagulants due to the release of platelet phospholipid. Platelet free plasma, plasma with a platelet count <10 x 10⁹/L, is required for all lupus anticoagulant testing.

Specific inhibitors to coagulation factors cause prolongation of some or all of the clot-based tests for lupus anticoagulant. FVIII inhibitors may give false positive results in a variety of lupus anticoagulant tests, such as the hexagonal phase phospholipid neutralization test. FV inhibitors or the presence of heparin may yield a false positive PNP assay. Specific factor inhibitors are identified using the APTT and prothrombin time mixing studies and measured with a Bethesda titer.

Patients with positive lupus anticoagulant or APLA results should be retested at least twelve weeks because the transient APLAs that may appear subsequent to many infections and other conditions have no clinical significance and should be ruled out.

Laboratory Diagnosis of Lupus Anticoagulant

The laboratory diagnosis of LA should be based on the 1995 ISTH published criteria. In order to diagnose a LA, the following four criteria should be present: the sample should demonstrate 1) prolongation of a phospholipid-dependent clotting assay, 2) demonstration of an inhibitor through mixing studies or factor assays, 3) demonstration that the inhibitor is phospholipid dependent and 4) verification that another coagulopathy is not present. When samples contain anticoagulant agents such as heparin, warfarin or direct thrombin inhibitors, strict criteria can often not be met. In order to rule out the presence of a LA, two to three phospholipid-based clotting assays should be performed.

Reference Ranges

<table>
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<tr>
<th>Assay</th>
<th>Reference Range</th>
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<tbody>
<tr>
<td>APTT</td>
<td>23.7 – 37.7 seconds</td>
</tr>
<tr>
<td>APTT 1:1 Normal Pool Mix</td>
<td>23.7 – 37.7 seconds</td>
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<tr>
<td>APTT 1:1 Saline Mix</td>
<td>24.0 – 150.0 seconds</td>
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<tr>
<td>Dilute Russell's Viper Venom Test (DRVVT)</td>
<td>0.6 – 1.2 ratio</td>
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<tr>
<td>Hexagonal Phospholipid Neutralization</td>
<td>&lt;10.0 seconds</td>
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<tr>
<td>Kaolin Clotting Time (KCT)</td>
<td>50 – 130 units</td>
</tr>
<tr>
<td>Platelet Neutralization Procedure (PNP)</td>
<td>0.0 – 3.0 seconds</td>
</tr>
<tr>
<td>Tissue Thromboplastin Inhibition Test (TTIT)</td>
<td>0.5 – 1.2 ratio</td>
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</tbody>
</table>
Specimen Requirements

Frozen citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the collection tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. Specimens for lupus anticoagulant testing must be platelet free (platelet count <10x10^9/L), as the phospholipids from activated platelet membranes may partially neutralize the lupus anticoagulant. Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Test Request Information

The lupus anticoagulant and anticardiolipin confirmatory profile (#300916) includes anticardiolipin antibody IgG (300161), IgM (#300162), activated partial thromboplastin time (#300040), APTT mixing studies (#300806), dilute Russell’s viper venom test (#300057), prothrombin time (#300080), thrombin time (#300807), the platelet neutralization procedure (#300805) and hexagonal phospholipid neutralization (#300144). These assays can each be ordered separately from the Esoterix Service Directory. CPT codes are as follows: anticardiolipin antibody (86147X2), activated partial thromboplastin time (85730), APTT mixing studies (85730, 85732 x3), dilute Russell’s viper venom test (85613), prothrombin time (85610), thrombin time (85670), platelet neutralization procedure (85597) and hexagonal phospholipid neutralization (85597)

<table>
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<th>Assays</th>
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<tr>
<td>APTT Mixing Studies</td>
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<td>Dilute Russell’s Viper Venom Test</td>
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<tr>
<td>Tissue Thromboplastin Inhibition Test</td>
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Reflex Recommendations

Patients with positive lupus anticoagulant results may be confirmed by the use of enzyme immunoassay for APLAs. Concordance between these clot based and immunoassay abnormalities in patients with APLA is about 60%. Patients with positive lupus anticoagulant or APLA results should be retested after at least twelve weeks because the transient APLAs that may appear subsequent to many infections have no clinical significance and should be ruled out. When investigating thrombosis, consider evaluating the panel of assays related to thrombosis (Venous Thrombosis Profile).

Therapy for Lupus Anticoagulant

Long-term oral anticoagulant therapy, maintaining an INR of 2-3, is the most frequently administered treatment for APS although heparin and aspirin may be used during pregnancy or in addition to warfarin therapy. Some patients with lupus anticoagulants show prolongation of the prothrombin time. This prolongation appears to be, in part, reagent dependent. In these patients, therapeutic monitoring of oral anticoagulant therapy using the prothrombin time and the international normalized ratio (INR) can be compromised. An alternative to the INR in these patients is a chromogenic factor X level. Using this assay, an INR of 2 – 3 reportedly correlates to a chromogenic factor X activity level of 20 to 40% while an INR of 2.5 to 3.5 correlates to a 12 – 35% factor X activity.
CLOT-BASED LUPUS ANTICOAGULANT TESTS (cont.)

References


PIVKA-II

**Etiology**

Factors II, VII, IX, X, protein C, and protein S are vitamin K-dependent coagulation factors. When liver production of these proteins is induced in vitamin K’s absence or antagonism, such as during warfarin therapy, dietary deficiency of vitamin K, or in some types of liver dysfunction (cirrhosis, hepatocellular carcinoma), they are referred to as PIVKA (Proteins Induced by Vitamin K Absence or antagonism). PIVKAs are immunologically similar to the naturally occurring factors but are dysfunctional since they lack the carboxyglutamyl residues required for calcium and phospholipid binding during clotting. The proteins are also termed non- or des-carboxylated proteins. PIVKA-II is prothrombin (factor II) produced in vitamin K’s absence or antagonism and lacks normal coagulant activity.

**Clinical Significance**

Impairment of the carboxylation process that occurs in vitamin K absence/deficiency or in the presence of a vitamin K antagonist such as warfarin produces the protein PIVKA-II. Patients with hepatocellular carcinoma and rarely other liver diseases develop an acquired vitamin K deficiency leading to production of PIVKA proteins. Plasma PIVKA-II levels increase in hepatocellular carcinoma and are related to tumor size. Increased levels are rarely observed in chronic hepatitis and liver cirrhosis. Vitamin K deficiency may also occur as a result of long-term, broad-spectrum antibiotics use, in malnutrition and in gastrointestinal diseases such as diverticulitis. PIVKA-II may aid in diagnosis or in determining severity of the above disorders. Normal PIVKA-II levels confirm adequate vitamin K status.

**Assay Principle**

PIVKA-II is measured by enzyme immunoassay. The patient sample containing PIVKA-II is incubated with mouse monoclonal antibodies against PIVKA-II. A horseradish peroxidase conjugate is added, binding the free antigenic determinants of the PIVKA-II molecule. The color formation is directly proportional to the concentration of PIVKA-II in the test specimen.

**Assay Performance Characteristics**

This assay does not measure the PIVKA proteins of factor VII, IX, X, protein C or protein S.

**Reference Range**

The plasma reference range for PIVKA-II is < 3.6 ng/mL.

**Specimen Requirements**

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.
PIVKA-II (cont.)

Test Request Information

PIVKA-II (#300726) can be ordered separately from the Esoterix Service Directory. CPT Code 83520.

References


**Physical Characteristics**

Plasminogen, a 92,000 Dalton single chain glycoprotein, circulates in the plasma in two forms; native glu-plasminogen and the much more active form lys-plasminogen. Plasminogen is synthesized in the liver as a zymogen. Plasminogen has no proteolytic activity itself and has a half-life of approximately 2 days. Plasminogen is converted to the active form, plasmin, by cleavage of a single peptide bond by the action of either tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA).

**Physiology**

During initial hemostatic plug formation, the generation of thrombin stimulates the release of tissue plasminogen activator (t-PA) from the endothelial cells. t-PA is also released from endothelial tissue during exercise, stasis, stress or desmopressin (DDAVP) administration. t-PA binds tightly with fibrin and the fibrin serves as a cofactor for the conversion of plasminogen to plasmin. The interaction of tPA and plasminogen is enhanced significantly in the presence of annexin II. Lipoprotein (a) competes with binding of plasminogen to annexin II. Elevated homocysteine levels diminish the interaction of tPA and annexin II.

A secondary activator of plasminogen, urokinase (u-PA), produced largely in the kidneys, is also released from the endothelial cells through the action of kallikrein of the contact system, but remains functional largely in tissue. This system is vital to cell mobility within tissue.

An exogenous source of plasminogen activation is streptokinase (SK), an extracellular protein produced by β-hemolytic streptococci. SK has been used as a therapeutic agent to assist in pathologic clot dissolution but due to its high antigenicity in humans, even in its purest commercially prepared form, its use should be closely monitored. Commercially prepared concentrates of u-PA and t-PA are also available for this purpose and do not have the antigenicity of SK.

Plasmin is quickly inactivated by α-2-antiplasmin and to a lesser extent by α-2-macroglobulin. Plasminogen activators such as tPA are rapidly inhibited by plasminogen activator inhibitor-1 (PAI-1). PAI-1 found in platelets, endothelial cells and in plasma, is an acute phase reactant that may reach high levels following stress or trauma.

**Clinical Significance**

Hereditary abnormalities of plasminogen synthesis are rare and include type I, characterized by decreased activity and antigen, and type II deficiency, characterized by a dysfunctional protein as a result of a variety of defects. Type II deficiency is demonstrated by normal plasminogen antigen levels but decreased activity. The majority of reported patients are heterozygous plasminogen deficient. Heterozygous type II plasminogen deficiency occurs in 2 – 4% of Asian populations. Homozygous plasminogen deficiency is associated with abnormal fibrin deposition in a variety of tissues resulting in ligneous conjunctivitis or cervicitis and may represent a risk factor for thrombosis. There is no consistent clinical evidence of an association between homozygous plasminogen deficiency and risk of thrombosis, however. It is not certain if heterozygous deficiency poses thrombosis risks. Some studies have demonstrated a slightly higher incidence of heterozygous plasminogen deficiency in patients with a history of thrombosis, than in the general population.

Decreased levels may be acquired and seen in disseminated intravascular coagulation (DIC) and severe liver disease. Reduced plasminogen levels also occur in patients undergoing thrombolytic therapy, e.g. when receiving exogenous urokinase (u-PA), streptokinase (SK), or tissue plasminogen activator (t-PA). During thrombolytic therapy, it may be of interest to monitor plasminogen levels to make certain levels are sufficient to allow fibrinolytic effect of the thrombolytic agent.

Newborns have about 50% of adult levels and even lower concentrations occur in premature infants. Adult levels are achieved by 6 months of age.
PLASMINOGEN (cont.)

**Activity Assay Principle**

Plasminogen in the test sample is activated to form a plasminogen-streptokinase complex by the addition of excess streptokinase. This complex reacts with a chromogenic substrate resulting in color production. The amount of color formation correlates to the plasminogen activity in the sample.

**Antigen Assay Principle**

Plasminogen in the test plasma is combined with antibody to plasmin. The immune complexes that form, scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of plasminogen antigen in the sample.

**Assay Performance Characteristics**

Because some plasminogen defects are qualitative, the plasminogen activity assay is the preferred screening test. The plasminogen antigen assay may be used with the activity assay to distinguish quantitative (type I) from qualitative (type II) abnormalities.

**Reference Range**

<table>
<thead>
<tr>
<th>Assays</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasminogen Activity</td>
<td>70 – 130%</td>
</tr>
<tr>
<td>Plasminogen Antigen</td>
<td>7.5 – 15.5 mg/dL</td>
</tr>
</tbody>
</table>

**Specimen Requirements**

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

**Test Request Information**

Plasminogen activity (#300400) and plasminogen antigen (#300408) may be ordered separately from the Esoterix Service Directory. CPT Code: Plasminogen activity 85420, plasminogen antigen 85421.

**Reflex Recommendations**

Thrombosis and hemorrhage may occur as a result of inadequate or increased fibrinolysis, respectively. In either event, the plasminogen assay may be accompanied by assays of tissue plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1), and α-2-antiplasmin. The global euglobulin lysis test may also be useful in establishing fibrinolytic pathway abnormalities.
References


PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1)

Physical Characteristics

The plasminogen activator inhibitors (PAIs) are members of the serine protease family. PAI-1 is a single chain glycoprotein serpin with a molecular weight of 52,000 Daltons, a plasma concentration of approximately 5 μg/mL and an estimated half-life of 8-10 minutes. PAI is synthesized in the liver and endothelial cells. Adipocytes are also known to produce PAI-1. PAI-1 exists in 2 different forms in plasma: an active form and a latent form. Most of platelet PAI-1 is present in the latent form. The mechanism of the conversion of latent to active PAI-1 is not known. PAI-1 is found in platelets, endothelial cells and in plasma. PAI-2 is a glycoprotein serpin with a molecular weight of 70,000 Daltons. It is produced in placental tissue and in leukocytes.

Physiology

During initial hemostatic plug formation, the generation of thrombin stimulates the release of tissue plasminogen activator (t-PA) from the endothelial cells. t-PA binds tightly with fibrin and the fibrin serves as a cofactor for the conversion of plasminogen to plasmin. t-PA is also released from endothelial tissue during exercise, stasis, stress or desmopressin (DDAVP) administration. A secondary activator of plasminogen, urokinase (u-PA), produced largely in the kidneys, is also released from the endothelial cells through the action of kallikrein of the contact system, but remains on the surface of the endothelial tissue due to its binding with urokinase plasminogen activator receptor (uPAR). Plasminogen activators are rapidly inhibited by plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). PAI-1 is the major regulator of the plasminogen activation system.

Clinical Significance of PAI-1

As PAI-1 is an acute phase reactant and also demonstrates diurnal variation, interpretation of results is difficult. PAI-1 levels are highest in the morning. Promotion of venous and arterial thrombosis may occur through elevations of PAI-1 levels. Deep vein thrombosis (DVT), myocardial infarction and coronary artery disease (CAD) are associated with increased PAI-1 levels. Elevated PAI-1 levels may help predict risk of re-infarction in survivors of myocardial infarction, particularly in young individuals. PAI-1 activity increases as insulin levels increase. Up-regulation of PAI-1 is seen in obesity, atherosclerosis and malignancy. Elevations have also been reported with diabetes mellitus, hypertension, angina pectoris, alcoholic cirrhosis, pancreatitis, infections with sepsis, and with malignancy.

Plasma PAI-1 levels are partly dependent on genetic factors. Increased plasma PAI-1 levels may be caused by genetic polymorphisms of the PAI-1 gene. The most common of these is the 4G/4G deletion/insertion promoter polymorphism. Those with a 4G/4G genotype have an average 25% greater PAI-1 activity levels than those with a 5G/5G genotype while the PAI-1 levels in the 4G/5G individual are intermediate. The 4G/5G polymorphism influences transcription of the PAI-1 protein. Individuals with this genetic mutation may be at increased risk for thrombosis especially if other risk factors are present.

Deficiency of PAI-1, an extremely rare and difficult diagnosis to make, has been associated with a lifelong bleeding diathesis. Bleeding generally occurs only after surgery or trauma. Hereditary PAI-1 deficiency can be caused by a frame-shift mutation in Exon 4 of the PAI-1 gene. Congenital PAI-1 deficiency has been described as both type 1 (quantitative deficiency) and type 2 (qualitative deficiency). Type 1 deficiency is associated with decreases in both PAI-1 activity and antigen while type 2 deficiency is associated with a normal PAI-1 antigen and low activity. Congenital PAI-1 deficiency has also been described with normal platelet PAI-1 levels. Acquired PAI-1 deficiency due to the development of an acquired inhibitor has been described but appears to be rare.

Laboratory Diagnosis of PAI-1 Deficiency

- Congenital deficiency of PAI-1 is a rare disorder associated with a lifelong bleeding diathesis due to hyperfibrinolysis. Screening tests such as PT, APTT, thrombin time and fibrinogen are normal in these individuals. In order to make the diagnosis of PAI-1 deficiency, the evaluation of specific assays is necessary. Since normal individuals may have near zero PAI-1 activity levels, the diagnosis of PAI-1 deficiency can be difficult.
• PAI-1 is present in both plasma and platelets. Individuals with PAI-1 deficiency may have decreased plasma PAI-1 but normal platelet PAI-1 levels or they may be deficient in both plasma and platelet stores.

• If PAI-1 deficiency is suspected and PAI-1 activity is low, a PAI-1 antigen be performed on the same sample. If both the PAI-1 activity and PAI-1 antigen are low, a serum sample should be drawn and submitted for PAI-1 activity and antigen. If both plasma and serum levels are low in the appropriate clinical setting, this would be consistent with a PAI-1 deficiency. Some PAI-1 deficient patients, however, have normal platelet PAI-1 stores and therefore normal serum PAI-1 levels.

• The diagnosis of PAI-1 deficiency can be supported by an abnormally short euglobulin lysis time.

• The use of a tourniquet test may also assist in the diagnosis of PAI-1 deficiency. The diagnosis is supported by the lack of an appreciable rise in PAI-1 antigen and activity after application of a tourniquet for a period of approximately 10 minutes.

• If congenital PAI-1 deficiency is suspected in this individual with a low PAI-1 activity, PAI-1 antigen should be performed on the same sample followed by other assays as described above, if indicated.

**Clinical Significance of PAI-2**

Increased levels of PAI-2 are found in pregnancy, especially during the third trimester. PAI-2 remains elevated for several days following birth before returning to normal. Normal male and non-pregnant female samples contain only trace amounts of PAI-2.

No correlation has been found between increased PAI-2 levels and increased thrombotic risk. Small increases of PAI-2 in pregnancy have been associated with intrauterine growth retardation, thus making it a possible marker for decreased placental function. At the time of this publication, there is no reliable kit available to measure PAI-2 activity.

**Plasminogen Activator Inhibitor-1 Activity Assay Principle**

PAI-1 present in the test sample complexes with active t-PA bound to the surface of a microtiter plate test well. The bound PAI-1 is then quantitated using horseradish peroxidase (HRP)-conjugated monoclonal anti-PAI-1. HRP sensitive substrate is then added and color development is proportional to the activity of PAI-1 in the sample.

**Plasminogen Activator Inhibitor-1 Antigen Assay Principle**

The PAI-1 antigen assay is based on the sandwich enzyme immunoassay procedure. Monoclonal antibodies to PAI-1 immobilized on microtiter plate test well surfaces bind PAI-1 in the sample. A horseradish peroxidase (HRP) conjugate is added that reacts with the bound PAI-1. A chromophore is then added and color development occurs proportional to the PAI-1 antigen in the sample. The PAI-1 antigen assay measures all forms of PAI-1 that are present in the sample (i.e., active, inactive and that which is complexed with t-PA or u-PA).

**Plasminogen Activator Inhibitor-1 Antigen and Activity Assay Performance Characteristics**

Traumatic venipuncture, prolonged stasis during venipuncture, or inadequate centrifugation may cause erroneous results. Samples that contain excessive numbers of platelets post-centrifugation that are frozen before testing may lead to increased PAI-1 antigen levels. These variables may cause the release of PAI from platelets, which is an inactive form of PAI-1. This may cause a discrepancy between PAI-1 antigen and activity. PAI-1 is an acute phase reactant and increases rapidly following inflammation, trauma, or surgery.

PAI-1 demonstrates diurnal variation and collection of samples should be done at the same time each day. PAI-1 levels are highest in the morning and can reach concentrations greater than twice that observed in the afternoon.
## Reference Ranges

<table>
<thead>
<tr>
<th>Plasminogen Activator Inhibitor Assays</th>
<th>Reference Range</th>
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<tbody>
<tr>
<td>Plasminogen activator inhibitor-1 activity</td>
<td>&lt; 31.1 IU/mL</td>
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<tr>
<td>Plasminogen activator inhibitor-1 antigen</td>
<td>4.0 – 43.0 ng/mL</td>
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</table>

## Specimen Requirements

The patient should be in a resting state and the specimen for PAI-1 activity or antigen collected in the morning to avoid diurnal variation of results.

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then re-centrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20° or lower. Ship the frozen specimen overnight on dry ice.

When pre- and post-occlusion samples are required, the following specimen collection guidelines are recommended.

### Pre-Occlusion

Venipuncture should be non-traumatic and performed without a tourniquet. Collect the sample as indicated above.

### Post-Occlusion

Place a blood pressure cuff on the patient’s arm not used for the collection of the pre-occlusion sample. Inflate the cuff to a point midway between systolic and diastolic pressure. Maintain this pressure for a standard length of time (typically 10 minutes). Perform venipuncture immediately prior to releasing the blood pressure cuff. Collect the sample as outlined above.

## Test Request Information

PAI-1 activity (#300126), PAI-1 antigen (#300128) and PAI-1 4G/5G (#129100) may be ordered separately from the Esoterix Service Directory. CPT Codes: plasminogen activator inhibitor-1 (PAI-1) activity and antigen 85415; 4G/5G 83891, 83892, 83894, 83898, 83912.

## References

**Plasminogen Activator Inhibitor (4G/5G Insertion/Deletion) Polymorphism (PAI-1 4G/5G)**

### Physical Characteristics

Several polymorphisms of the PAI-1 gene have been identified. The 4G/5G insertion/deletion polymorphism is a single guanosine base pair variation (4G or 5G) mutation that occurs 675 base pairs from the start of the promoter region.

### Physiology and Clinical Significance

The 4G/5G insertion/deletion polymorphism affects the regulation of the PAI-1 gene by altering the binding of proteins that regulate its transcription. Individuals with the 4G/5G genotype have PAI-1 levels intermediate between those with the 4G/4G and 5G/5G genotypes. PAI-1 levels are about 25% greater in the 4G/4G genotype than levels seen in those with the 5G/5G genotype. The highest PAI-1 levels tend to be in individuals with the 4G/4G genotype. Levels of PAI-1 are not only under the influence of this polymorphism but are also determined by both environmental and other genetic factors.

A number of studies have evaluated the relationship between this polymorphism and the risk of venous thromboembolism. The Physicians Health Study, which enrolled almost 15 thousand men and followed them for over 8 years, found no correlation between the PAI 4G/5G polymorphism and venous thromboembolic risk. In other studies, risk in those with the 4G/4G genotype was increased only in the presence of other well-documented thrombophilic risk factors. The 4G/4G genotype has also been associated with an increased risk of recurrent fetal loss. Elevated circulating PAI-1 levels have been associated with an increased risk of vascular disease including both venous thromboembolic disease and atherosclerotic vascular disease; whether it is an independent risk factor is less well substantiated. It is difficult to use PAI-1 activity levels as a marker of vascular disease for a number of reasons: 1) Plasma PAI-1 exists in active, latent and bound forms and there is a dynamic transition between these forms, 2) PAI-1 shows circadian variation with higher levels in the morning hours and 3) levels elevate as an acute phase response.

### Assay Principle

The PAI-1 4G/5G polymorphism is detected using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) technologies.

### Assay Performance Characteristics

Genetic testing by PCR provides exceptionally high sensitivity and specificity. Inaccurate results using PCR are limited to rare polymorphisms in primer binding sites and to misidentification of specimens by collectors or laboratory personnel. This assay detects only the PAI-1 4G/5G polymorphism and does not measure genetic abnormalities elsewhere in the genome.

### Reference Range

<table>
<thead>
<tr>
<th>Genotype is reported as:</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>4G/4G</td>
</tr>
<tr>
<td></td>
<td>4G/5G</td>
</tr>
<tr>
<td></td>
<td>5G/5G</td>
</tr>
</tbody>
</table>
Specimen Requirements

Collect blood into a lavender (EDTA) or yellow (ACD) stoppered tube. Ensure complete fill of the evacuated tube. Invert gently six times immediately after filling. The specimen should be kept at room temperature and should not be centrifuged. Label tube and place specimen in container to be shipped overnight at room temperature within 3 days after collection.

Test Request Information

PAI 4G/5G insertion/deletion polymorphism (#129100) can be ordered separately from the Esoterix Service Directory. CPT Codes: 83891, 83892, 83894, 83898, 83912.

Reflex Recommendations

This assay is typically requested as part of a thrombophilia workup or evaluation for the risk of atherosclerotic vascular disease. Consideration should be given to the evaluation of PAI-1 activity and antigen.

References

Platelet Aggregation

Physiology

When a blood vessel is damaged, the subendothelial matrix becomes exposed. Platelets adhere to this matrix, undergo a shape change, aggregate to other platelets, synthesize prostaglandins, and release a variety of substances stored in the platelet granules. Prostaglandin synthesis and released products cause further platelet recruitment and aggregation. The coagulation cascade is then initiated by not only exposure of plasma products to the subendothelium but also by procoagulants released from the platelet granules and thrombin generated. The platelet plug is ultimately incorporated into a fibrin clot.

Normal platelet adhesion to subendothelial tissue requires intact platelet membranes, functional platelet alpha glycoprotein receptor GPIb/V/IX and plasma von Willebrand factor (vWF). Normal platelet aggregation requires intact platelet membranes, functional platelet glycoprotein receptor GP IIb/IIIa, normal plasma fibrinogen, and normal secretion of proteins from the platelet α–granules and dense bodies. The α–granules are the most abundant granules in platelets and contain many plasma proteins, such as fibrinogen, vWF, thrombospondin, factor V (FV), vitronectin, platelet factor 4 and β-thromboglobulin. The dense bodies have high concentrations of calcium, serotonin, ADP, and ATP.

Upon activation of normal beta platelets, platelet phospholipase mobilizes membrane phospholipids to arachidonic acid. The arachidonic acid is then metabolized by the enzymes cyclo-oxygenase and thromboxane synthetase to form thromboxane A2. Thromboxane A2 causes platelet aggregation and storage granule release (secretion) of ATP. Cyclo-oxygenase is obligatory for the conversion of arachidonic acid to thromboxane A2.

Clinical Significance

Results from platelet aggregation assays are clinically useful in the detection and diagnosis of acquired or congenital qualitative platelet defects. The platelet’s ability or inability to respond to particular aggregating agents (agonists) is the basis for differentiating platelet dysfunctions. Clinical symptoms of impaired platelet function usually manifest as a defect in primary hemostasis; e.g., epistaxis, petechiae, purpura, menorrhagia and gingival bleeding. One study suggests that an exaggerated response to certain agonists (such as ADP and epinephrine) may lead to an increased thrombotic tendency. This has been called “sticky platelet syndrome” and its clinical relevance is controversial (see below).

Assay Principle

Platelets are known to aggregate under a variety of conditions and in the presence of a number of different agonists; hence, “platelet aggregation” is the most informative in vitro test of platelet function presently available. The aggregation phenomenon can be induced in vitro by adding aggregating agents (agonists) to platelet rich plasma (PRP) or whole blood. Platelet aggregation response depends on the presence of calcium, the number of platelets present, fibrinogen, other plasma factors, as well as the agonist. The amount of platelet activity will vary with the different agonists and their concentration as well as with the platelet count of the sample being tested.

Multiple agonists such as ADP, epinephrine, collagen, arachidonic acid, and ristocetin, at different concentrations, are used to stimulate the platelets. The individual’s response to these agonists is compared to established normal and abnormal response patterns. Aggregation curves may be interpreted by direct comparison to that of a recently drawn normal donor, which provides quality control for the assay.

Aggregation testing is done at 37°C. Samples are continually mixed by the use of magnetic stir bars as platelets will not aggregate unless they come into contact with other platelets. Patient platelet poor plasma (PPP) is used as the reference blank, providing 100% light transmission through the sample. Patient’s platelet rich plasma (PRP) is used to establish an arbitrary baseline of 0% light transmission. Testing is standardized by diluting patient’s PRP with their own PPP so that it contains an ideal number of platelets (approximately 300,000) for testing. When an agonist is added to PRP, the platelets’ initial response to the agonist is termed primary aggregation which may be reversible. The platelets then undergo a shape change, releasing ATP/ADP from the dense bodies producing a secondary aggregation response. This step is irreversible. The platelets then aggregate to one another, producing clumps of platelets in the plasma, thereby clearing the plasma, producing increased light transmittance through the test sample. The optical aggregation output is proportional to the continuously measured difference in light transmission between the test sample and the reference blank. If the response of the platelets is strong enough to the concentration of agonist added, it will cause the platelets to form large aggregates and the amount of light transmittance through the sample will approach 100%.
von Willebrand's Disease (vWD) is characterized by a deficiency or dysfunction of the plasma cofactor von Willebrand Factor (vWF) required for platelet adhesion to subendothelial tissue. Platelet adhesion also requires the glycoprotein Ib (GP Ib) receptor on the surface of the platelet. The antibiotic ristocetin induces platelet agglutination in the presence of vWF and the GP Ib receptor. Failure to agglutinate in response to ristocetin may be indicative of vWD or absence of GP Ib receptor on the surface of the platelet (Bernard-Soulier Syndrome). Generally, vWF activity must be less than <40% for this effect to be seen in platelet aggregation response. Hyper-responsiveness to low dose ristocetin may be seen in Type 2B and platelet-type vWD and may be confirmed by testing at final concentrations of ristocetin below 0.6 mg/mL. In platelet-type vWD the defect is in platelet GP Ib receptor, whereas in Type 2B vWD, the defect is in the vWF molecule.

Thrombocytopenia is present in nearly all Bernard-Soulier Syndrome (BSS) patients but is variable in its severity. The platelets of the BSS patient are typically very large as seen on the peripheral smear. Failure of the BSS platelets to aggregate in response to ristocetin cannot be corrected by addition of normal plasma (correction will occur in vWD platelets upon addition of normal plasma). The thrombocytopenia and the increased size of BSS platelets may make platelet aggregation studies technically difficult, hindering the process of evaluation.

Glanzmann's thrombasthenia, an abnormality of the platelet GP IIb-IIIa receptor, is characterized by absent aggregation in response to all agonists with the exception of ristocetin. Absent platelet aggregation response is the hallmark of this disorder. Symptoms occur only in patients that are homozygous for Glanzmann's thrombasthenia; the heterozygous condition appears to be asymptomatic.

Storage pool defects, classified by the type of granular deficiency present (either α-granules, dense bodies or a combination of these) are difficult to diagnose unless platelet release responses are measured. The platelets may or may not respond to the different agonists dependent on the amount and type of granules present. Electron microscopy of the platelets is a useful aid in the diagnosis of storage pool deficiencies.

Gray platelet syndrome, a deficiency in the α-granule contents (platelet factor 4, platelet vWF, thrombospondin), is characterized by abnormal collagen aggregation. Platelets appear as larger-than-normal, pale, ghostlike, oval forms on peripheral blood smears. Hemorrhagic manifestations are usually mild in the gray platelet syndrome.

It has been suggested that platelet hyper-responsiveness, coined “sticky platelet syndrome” is a cause of hypercoagulability. Although theoretically plausible, this phenomenon is contended by many researchers. It has been recommended that evaluation for sticky platelets be performed using epinephrine and ADP as agonists at several low concentrations. A diagnosis of sticky platelets is made according to the Chittoor criteria, specifically if the aggregation responses of the patient's platelets is greater than normal and is within the range determined in a pivotal study defining this syndrome.

Normally, individuals do not demonstrate spontaneous aggregation of platelets. An individual's platelet aggregation response to agonists such as arachidonic acid, ADP, collagen, epinephrine and ristocetin is compared to the responses of normal and abnormal donors to these same agonists for interpretation.

A complete drug and pertinent dietary history for 8-10 days prior to analysis of platelet aggregation must be obtained from the patient. Many medications, both prescription and over-the-counter, may have a profound affect on platelet aggregation. Aspirin or aspirin-containing medications destroy cyclo-oxygenase for the lifespan of all platelets in the circulation at time of ingestion, inhibiting thromboxane A2 synthesis. This blocks the release mechanism and results in minimal or lack of response to other agonists upon testing. Other medications too numerous to name affect platelet aggregation. Red wine and several herbal preparations such as St. John's Wort will also diminish platelet aggregation response.
Specimen Requirements

Platelet aggregation studies are performed only on locally collected samples and must be completed within four hours of collection. Call Esoterix for special drawing instructions.

Test Request Information

Esoterix offers platelet aggregation studies including spontaneous aggregation and response to agonists including ADP, Arachidonic Acid, Collagen, Epinephrine, and Ristocetin. Test codes #300043 1.5 uM ADP, #300053-7.0 uM ADP, #300062-Collagen, #300063-Arachidonic Acid, #300064-Spontaneous, #300065-Epinephrine, #300066-00.6 mg Ristocetin, #300077 1.0 mg Ristocetin. Platelet Agg. Profile #300068: contains all agonists except Ristocetin. Sticky Platelet Profile #300170. CPT Code: 85576 for all assays (multiply by number of agonists used).

References

PLATELET ANTIGENS AND ANTIBODIES

Physical Characteristics

The platelet-specific antigens are peptides located on the platelet glycoproteins GPIa/IIa, GPIb/IX, and GPIIb/IIIa. The platelet-specific antigens that are seen most in alloimmune thrombocytopenia and in autoimmune thrombocytopenic purpura are listed in the table under reference ranges. The names in parentheses refer to the new, systematic nomenclature.

In addition to the platelet-specific antigens, HLA class I antigens are often implicated in alloimmunization of patients who receive multiple platelet transfusions from random donors. Most antibodies to platelet antigens are IgG or IgM isotype and are called platelet-associated immunoglobulin (PAIg).

Platelet Antibody Origins and Physiology

Autoimmune: Acute and Chronic Autoimmune Thrombocytopenic Purpura (Idiopathic Thrombocytopenic Purpura, ITP)

No one has identified mechanisms that cause autoimmune platelet antibody formation. Acute development of platelet autoantibodies occurs following viral infection, primarily in childhood. Chronic platelet autoantibodies develop in young adults, predominantly women, and no triggering event is found. In both instances, PAIgs coat the patient’s platelets. Splenic macrophages soon remove the coated platelets from the circulation; indeed, coated platelets survive for less than one day in the peripheral blood. In ITP the platelet count often drops to less than 10,000/mL (reference range 150,000 - 400,000/mL).

Autoimmune PAIg formation may also occur in autoimmune disorders such as lupus erythematosus, rheumatoid arthritis, lymphoproliferative disorders, and HIV infection, or secondary to drug administration.

Alloimmunization: Neonatal Alloimmune Thrombocytopenia (NATP) and Post-transfusion Purpura (PTP)

Alloimmune PAIg antibodies arise when there is an antigenic challenge to individuals who lack specific platelet antigens. About 80% of PAIg antibodies are anti HPA-1a (anti-PLA1). The rest are anti-HPA-3a (Baka), anti-HPA-4 (Penb) and anti-HPA-1b (PLA2).

In NATP, maternal PAIg antibodies form in response to fetal platelet antigens, cross the placental barrier, and coat fetal platelets causing thrombocytopenia in a manner similar to Rh disease of the newborn. Unlike Rh disease of the newborn, NATP may occur with the first pregnancy. In post-transfusion purpura (PTP), donor platelets induce a PAIg antibody response. The antibody subsequently destroys both donor platelets and recipient platelets. The mechanism for the recipient platelet destruction is not understood.

Alloimmunization in Multi-transfused Patients

Patients who have received multiple transfusions of random donor platelets often become refractory to further transfusions. Refractoriness is usually caused by antibodies that react with HLA class I antigens.

Platelet Antibody Incidence

The incidence of NATP is 1 in 5000 deliveries. ITP is the most common cause of thrombocytopenia, with acute ITP appearing in patients under 20 years of age and chronic ITP most common in those over 20. PTP is a rare phenomenon.

Platelet Antibody Clinical Significance

All cases of immune thrombocytopenia yield low platelet counts with an increased risk for bleeding. Platelet counts less than 10,000/mL are associated with spontaneous systemic bleeding, and the possibility for intracranial hemorrhage. Patients with platelet counts between 10,000 and 50,000/mL may experience bleeding after a hemostatic challenge event such as surgery, dental extraction, or physical trauma.
**Acute and Chronic ITP**

Acute ITP occurs in children after six months of age and seldom after the age of 20. Acute ITP patients sometimes develop platelet counts below 10,000/mL with severe, often life-threatening symptoms, but the disorder lasts 2-3 weeks. About 10% of acute ITP cases become chronic.

Chronic ITP, sometimes called “idiopathic” thrombocytopenic purpura, arises in adults, predominantly women of childbearing age and persists for years, causing first mild, then increasingly severe bleeding episodes.

Chronic ITP women who become pregnant may pass PAIgG antibodies across the placental barrier, passively immunizing the fetus. Low neonatal platelet counts increase the risk of intracranial hemorrhage during birth, making special birth techniques, such as cesarean section, necessary. The newborn’s platelet count rises to normal after a few weeks.

**NATP and PTP**

In NATP, maternal PAIg antibody arises in response to fetal platelet antigens absent from maternal platelets. Similar to ITP, low neonatal platelet counts increase the risk of intracranial hemorrhage during birth, making special birth techniques, such as cesarean section, necessary. In some instances, the fetus is treated in utero. The count rises to normal after a few weeks. Symptoms may become manifest up to 12 hours after delivery.

PTP causes severe thrombocytopenia 7-10 days following any blood product transfusion to a patient with previous transfusion or pregnancy. The antibody, triggered by the transfused foreign antigen, cross-reacts with the patient’s own antigen-negative platelets, causing a severe, often life-threatening thrombocytopenia.

**Alloimmunization in Multi-Transfused Patients**

Patients with HLA class I alloantibodies become refractory to additional platelet concentrate transfusions and may subsequently require HLA-matched platelets to prevent hemorrhage.

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**Assay Principles**

**Platelet Antigen Test (HPA-1a, HPA-1b)**

This is a molecular test to genotype the HPA-1 DNA sequences. The wild type platelet glycoprotein HPA-1a (PLA1) is evident in about 98% of the population. A single nucleotide polymorphism changes the antigenic determinant from HPA-1a (PLA1) to HPA-1b (PLA2). Using polymerase chain reaction, the site for HPA-1 is amplified. The amplification products undergo restriction fragment length polymorphism with subsequent application to an agarose gel to which electrophoresis is engaged separating the fragments by molecular weight.

**Direct Platelet Antibody Test**

The direct platelet antibody test employs solid-phase red cell agglutination. The test is designed to detect PAIg antibodies on the patient platelets. Patient rich plasma (PRP) is added to microtiter plates coated with a specific platelet-binding agent. The wells are washed and a suspension of red cells coated with rabbit anti-human IgG is added. Centrifugation brings the indicator red cells in contact with antibodies bound to the immobilized platelets. In the case of positive tests, the migration of the indicator red cells to the bottom of the wells is impeded as anti-IgG bridges are formed between the indicator red cells and the platelet-bound antibodies. As a consequence of such bridging, the indicator red cells will cover the immobilized platelets in a confluent monolayer. In contrast, in the absence of platelet antigen-antibody interactions, i.e. negative tests, the indicator red cells will not be impeded during their migration, and will pellet to the bottom of the wells as tightly packed, well-defined cell buttons.

**Platelet Specific Antibody Test**

The platelet specific antibody employs solid-phase enzyme immunoassay. Microtiter plate wells are initially coated with platelet glycoprotein IIb/IIIa from group O donors with known platelet antigen phenotypes, including the platelet-specific antigens HPA-1a, HPA-1b, HPA-3a, HPA-3b and HPA-4a. Patient serum is incubated in the wells, the wells are washed, and an anti-human IgG/M/A enzyme conjugate is added to detect antibody binding. Wells that develop color indicate the presence of antibodies to their respective platelet-specific antigen.
Platelet Antibody Screen Test

The platelet antibody screen test employs solid-phase enzyme immunoassay. The microtiter plate well is initially coated with HLA class I antigens or with platelet glycoproteins Ia/IIa, Ib/IX, IIb/IIIa, and IV, respectively, from group O donors. Patient serum is incubated in all wells, the wells are washed, and an anti-human IgG/M/A enzyme conjugate is added to detect antibody binding. Wells that develop color indicate the presence of antibodies to their respective HLA class I antigen or to platelet glycoproteins Ia/IIa, Ib/IX, IIb/IIIa, or IV.

Expected Results

Platelet Antigens

The platelet antigen HPA-1a (PLA1) is present in 98% of unselected individuals. Platelets from rare individuals lack HPA-1a or other high incidence platelet antigens such as HPA-1b, HPA-3a, HPA-3b and HPA-4a. These people risk developing anti-HPA-1a or a PAIg antibody to HPA-1b, HPA-3a and HPA-4a upon challenge with antigen positive platelets. These platelet antigens may be introduced during transfusion therapy or pregnancy.

PAIg Bound to Platelets

Normal platelets carry fewer than 200 molecules of PAIg on their surfaces. Platelets coated with increased PAIg are evidence of immune thrombocytopenia that is primary (idiopathic) or secondary to leukemia, systemic lupus erythematosus, or other autoimmune disorders. The direct platelet antibody test detects PAIg from platelet eluates, and is reported as positive or negative for PAIg.

PAIg in Serum

PAIg is normally absent from serum. When PAIg is detected in patient serum or on platelets, it indicates the possibility of an immune thrombocytopenia. In disease, PAIg may be detected in a serum platelet antibody screen and may be identified using platelet specific antibody tests.

Platelet Immunoassay Performance Characteristics

The direct platelet antibody test is used in support of the diagnosis of acute or chronic ITP because it confirms coating of platelets with PAIg. Unfortunately, the high concentration of platelet-stored IgG may cause false positives unless the platelets remain intact and inactivated throughout collection, storage, shipping, and test preparation. A negative direct platelet antibody result does not rule out ITP, however. The test should be performed in combination with the serum platelet antibody screen. Like the direct platelet antibody test, the platelet specific HPA-1a antigen test depends upon the presence of intact platelets throughout the collection, storage, shipping, and test preparation steps.

The serum PAIg antibody tests are negative in ITP in cases where all antibody is adsorbed to platelets, thus they should be performed in combination with the Direct Platelet Antibody Test. PAIg antibody results are increased in several non-immune causes of platelet destruction such as thrombotic thrombocytopenic purpura, and may be elevated in some infections, such as HIV.
Reference Ranges

<table>
<thead>
<tr>
<th>Antigen System</th>
<th>Glycoprotein Location</th>
<th>Representative Antigens</th>
<th>Antigen Frequency</th>
<th>Comment</th>
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</thead>
<tbody>
<tr>
<td>HPA-1 antigen</td>
<td>GP IIb/IIla</td>
<td>HPA-1a (PL^A)</td>
<td>98%</td>
<td>80% of NAIT and PTP involve this</td>
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<td></td>
<td></td>
<td>HPA-1b (PL^B)</td>
<td>2%</td>
<td>Associated with increased risk of cardiovascular disease</td>
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<tr>
<td>HPA-2</td>
<td>GP Ib</td>
<td>HPA-2a (Ko^a)</td>
<td>99%</td>
<td>Occasionally implicated in NAIT and PTP</td>
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<tr>
<td></td>
<td></td>
<td>HPA-2b (Ko^b)</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>HPA-3</td>
<td>GP IIb/IIla</td>
<td>HPA-3a (Bak^a)</td>
<td>90%</td>
<td>Occasionally implicated in NAIT and PTP</td>
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<td></td>
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<td>HPA-3b (Bak^b)</td>
<td>10%</td>
<td>Occasionally implicated in NAIT and PTP</td>
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<tr>
<td>HPA-4</td>
<td>GP IIb/IIla</td>
<td>HPA-4a (Pen^a)</td>
<td>&gt;99%</td>
<td>Occasionally implicated in NAIT and PTP</td>
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<tr>
<td></td>
<td></td>
<td>HPA-4b (Pen^b)</td>
<td>&lt;0.1%</td>
<td></td>
</tr>
<tr>
<td>HPA-5</td>
<td>GP Ia/Ila</td>
<td>HPA-5a (Br^a)</td>
<td>99%</td>
<td>Occasionally implicated in NAIT and PTP</td>
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<td></td>
<td></td>
<td>HPA-5b (Br^b)</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Isoantigen Nak^a</td>
<td>GP IV</td>
<td>C036</td>
<td></td>
<td>8% of Asian and African Americans lack GP IV and may become immunized through transfusion or pregnancy</td>
</tr>
</tbody>
</table>

Specimen Requirements

Platelet Antigen and Direct Platelet Antibody Tests: Whole Blood

The platelet specific HPA-1a antigen and direct platelet antibody tests require 10 mL of room temperature uncentrifuged whole blood collected in EDTA or acid citrate dextrose (ACD). Collect 2 tubes of 5 mL each and ship at ambient temperature. More sample may be necessary when patients are severely thrombocytopenic. Assure that shipment arrives within 48 hours of collection.

Platelet Antibody Screen and Platelet Specific Antibody Tests: Serum

The platelet antibody screen and platelet specific antibody tests both requires 1 tube of 1 mL each frozen serum. Collect two plain red-top tubes and allow to clot for 30 minutes. Centrifuge 10 minutes and transfer serum from each to two plastic freezer tubes using plastic transfer pipettes. Label appropriately and freeze immediately at -20°C or lower. Ship frozen specimens overnight on dry ice. Specimens must remain frozen during shipment.

Test Request Information

The platelet specific HPA-1a antigen, (#300531), direct platelet antibody (#300523), platelet antibody screen (#300520), and platelet specific antibody (#300524) can be ordered separately from the Esoterix Service Directory. CPT codes: Direct Platelet Antibody 86022, Platelet HPA-1a Antigen Test: 83891, 83892, 83894, 83898, 83912. Platelet Specific Antibody Test: 86022, Platelet Antibody Screen Test: 86022
PLATELET ANTIGENS AND ANTIBODIES (cont.)

Reflex Recommendations

ITP
The direct platelet antibody test is performed in combination with the serum platelet specific antibody test or the serum platelet antibody screen to determine the presence of an antibody and to identify the antibody if it is present. The test or test battery is run at intervals to determine the relative titer of antibody over time.

NATP
The platelet specific HPA-1a antigen test may be used to screen women of childbearing age for the antigen's absence if family history suggests the risk of alloimmunization.

Women who are at risk for alloimmunization should be screened intermittently during pregnancy for the present of antibodies. The serum may be tested at intervals during pregnancy using the serum platelet antibody screen, or the serum platelet specific antibody test, to determine the continued presence of maternal antibodies.

PTP
Patients who are suspected of having PTP are tested with the serum platelet antibody screen. If the test is positive for a GP IIb/IIIa epitope antibody, the serum platelet specific antibody test is used to identify the antibody and monitor its continued presence.

Alloimmunization in Multi-Transfused Patients
Patients receiving multiple transfusions of random donor platelets who are refractory to subsequent platelet therapy may be identified and monitored with the serum platelet antibody screen.

Therapy

Acute ITP
If the platelet count is <20,000/mL, or if there is evidence of bleeding, physicians often prescribe oral prednisone or other immunosuppressive drugs. The platelet counts should begin to return to normal after 2-3 weeks.

Chronic ITP
If the platelet count is <20,000/mL, or if there is evidence of bleeding, physicians often prescribe oral prednisone or other immunosuppressive drugs. Splenectomy may be attempted in patients who fail to respond to steroids. In pregnancy, the mother is given prednisone to maintain platelet counts >50,000/mL. Neonatal platelet counts are taken from the cord or scalp vein at the time of labor. To prevent intracranial hemorrhage, an atraumatic delivery method such as cesarean section should be considered.

NATP
Intrauterine diagnosis is recommended if NATP is suspected, and if it is confirmed, early cesarean section is recommended. The newborn may receive washed maternal platelets or antigen negative platelets.

PTP
Immune suppression or plasmapheresis may halt the immune destruction of patient platelets. Patients should not receive further platelet concentrates.

Multi-transfused Patients Refractory to Platelet Therapy
Platelet refractoriness may be prevented by using single donor apheresis platelets, leukocyte-poor blood products, UV-irradiated donor platelets, and cross-match compatible platelet concentrates.
References

9. GTI PAT® package insert 150 N. Patrick Boulevard, Brookfield, WI 53045
PLATELET FACTOR 4

Physical Characteristics

Platelet factor 4 (PF4), is a 30,000 Dalton high-affinity heparin-binding protein, produced in megakaryocytes and stored in platelet alpha granules. It is secreted by stimulated platelets and its plasma biological half-life is <5 minutes. It constitutes 5% of the protein found in circulating platelets. It is a member of the chemokine family of proteins.

Physiology

PF4 is a platelet-specific protein released from platelet α-granules. PF4 induces the release of histamine from basophils and induces the differentiation of monocytes into macrophages. Another property of PF4 is to neutralize the anticoagulant effect of heparin. Heparin therapy induces a significant increase in plasma PF4 as heparin induces endothelial cell release of PF4. Initially PF4 levels are very high but these levels decrease during heparinization as PF4 is consumed. Heparin neutralization occurs by the binding of PF4 to heparin at sites that are different from heparin's antithrombin binding site. Platelet activation leads to in vivo release of α-granule contents, including beta thromboglobulin and PF4. Platelets can be activated in vivo through a variety of stimuli, such as exposure to subendothelial materials, thrombin, atherosclerotic material and tissue factor, to name a few.

Etiology

Increased levels of PF4 are observed in a variety of clinical states that are associated with activation of platelets. These include inflammatory or infectious diseases, disseminated intravascular coagulation, shock, polycythemia vera, cerebrovascular disorders, extra-corporeal circulation, diabetes, cardiovascular disease, renal disease, malignancy, and during the post-operative period. Endothelial cell injury also may result in increased levels of PF4. Low normal to absent PF4 levels are observed in patients with gray platelet syndrome, a type of platelet storage pool defect.

Clinical Significance

Increased levels of PF4 indicate in vivo or in vitro platelet activation. Accelerated platelet turnover can result in PF4 levels that are 10 times normal.

Assay Principle

PF4 is measured by an enzyme immunoassay utilizing a sandwich technique. The patient sample containing PF4 is placed into a microtiter plate coated with rabbit anti-human platelet factor 4 antibodies. Rabbit anti PF4 antibody coupled with peroxidase conjugate is added forming a sandwich. The conjugate hydrolyzes a color-producing substrate. The intensity of color formation is directly proportional to the amount of PF4 in the sample.

Performance Characteristics

The validity of platelet marker assays depends on the quality of specimen collection and processing. This assay is valid only when the specimen is collected as described in "specimen requirements." The specimen collection must be performed to avoid all ex-vivo activation of the platelets. Traumatic venipuncture, prolonged stasis, or inadequate centrifuging may invalidate results. It is important to determine that the elevated levels reflect in vivo activation and are not the result of improper sample collection. This assay does not determine the presence or absence of heparin-induced platelet antibodies.
Reference Range

PF4 is normally present in very low concentrations of <36 IU/mL.

Specimen Requirements

The validity of platelet marker assays depends on the quality of specimen collection and processing. Specimen collection must be handled with extreme care in order to avoid all ex-vivo activation of the platelets. A special anticoagulant mixture (CTAD) which stabilizes the platelet must be used. When using CTAD vacutainers, break the vacuum of these tubes before sample collection. Prepare a bath of crushed ice and water deep enough to cool the entire contents of the sample collection tube. Uncentrifuged specimens are stable for one hour when placed in a crushed ice/water bath. Perform the venipuncture with two syringes and a 19g butterfly needle, using the tourniquet only to locate the vein. Remove the tourniquet as soon as the first drops of blood appear in the syringe. Collect and discard the first 2 mL of blood. Change syringes and collect the next 5.0 mL of blood into a syringe. Transfer 4.5 mL of blood gently down the side of the CTAD tube containing 0.5 mL of special anticoagulant. Cap and mix by gently inverting 3 times. Allow the tube to cool in the bath of crushed ice and water for at least 15 minutes. Within the hour, centrifuge the tube at 2,500g for 30 minutes in a refrigerated centrifuge. As soon as the centrifugation is completed, collect one-third the volume of the plasma supernatant by aspiration from the middle region of the liquid portion, making sure not to aspirate too near the top surface where some light platelets may be found, or too near the platelet layer that sits on top of the cell layer.

Test Request Information

PF4 (#300504) can be ordered separately from the Esoterix Service Directory. CPT Code 83520. This assay does not determine the presence or absence of heparin-induced platelet antibody.

Reflex Recommendations

Elevated PF4 levels may accompany increased levels of beta thromboglobulin indicating platelet activation.

References

PROTEIN C

Physical Characteristics

Protein C (PC) is a 62,000 Dalton vitamin K-dependent glycoprotein produced in the liver. PC's plasma concentration averages 0.5 mg/dL and its plasma biological half-life is 6 to 8 hours. Once formed, activated protein C (APC) has a plasma half-life of approximately 15 minutes.

Physiology

Protein C is a vitamin K-dependent zymogen that, when converted to its enzymatically active serine proteinase form, APC, functions as an anticoagulant that inactivates factors Va and VIIIa. Protein C localizes to the endothelial surface due to epithelial protein C receptor (EPCR). In the activation of protein C, thrombin first binds to thrombomodulin, a structural protein on the intact endothelial cell surface. PC is localized to the membrane surface through binding to EPCR. The thrombin/thrombomodulin complex then converts bound PC to APC. For APC to bind and cleave factors Va and VIIIa, the plasma cofactor, Protein S (PS) must also be available. APC, once released from the endothelial cell, combines with PS and this complex assembles with the target procoagulants Va and VIIIa on a phospholipid surface, such as a platelet or endothelial cell membrane. The APC then cleaves Va and VIIIa, limiting their participation in the procoagulant pathway. APC also reduces platelet prothrombinase activity.

Incidence of Deficiency

Congenital PC deficiency in the heterozygous state may be detected in one to three out of 1000 individuals and is the probable cause of 2%–5% of the cases of recurrent venous thrombosis. Homozygous PC deficiency is rare, being reported in 1 in every 500,000 to 750,000 births. Acquired PC deficiency is observed more frequently than congenital deficiency as it occurs in a number of clinical conditions.

Etiology of Deficiency

Because PC is synthesized in the liver, is vitamin K dependent, and has a short plasma half life, its activity is diminished in several conditions. PC activity is decreased in almost all cases of disseminated intravascular coagulation due to consumption. PC levels usually are diminished in severe acute and chronic liver disease, nephrotic syndrome, malignancy, inflammatory bowel diseases, sepsis, severe preeclampsia, and adult respiratory distress syndrome. Functional PC production is decreased with vitamin K deficiency or warfarin therapy, and in L-asparaginase therapy or fluorouracil therapy. It is also decreased in the acute phase following thrombotic events or surgery. Neonatal plasma levels are typically 50%-90% of adult levels. Adult levels are not achieved until approximately 12 to 16 years of age.

Congenital PC deficiency is inherited as one of two types of autosomal dominant traits. The majority are quantitative, or “type I” deficiencies, in which structurally normal PC molecules are produced at diminished plasma levels. Type I deficiencies are usually the result of deletion of a major portion of the PC gene or of a small deletion that generates a premature stop codon. Qualitative, “type II,” deficiencies, in which functionally abnormal molecules are present at normal or near normal plasma levels, are less common. A point mutation may cause a defective PS binding site, phospholipid binding site, or a defective enzymatic site. In type II deficiencies, PC antigen assay results are normal, but clot-based activity levels are diminished. Chromogenic PC activity assays may miss certain qualitative abnormalities. As there are many mutations associated with PC deficiency, genetic analysis is not a feasible means to diagnose congenital deficiency. Diagnosis, therefore, relies largely on the evaluation of plasma levels of functional PC.

Clinical Significance of Deficiency

Approximately 30% of individuals with heterozygous PC deficiency suffer venous thromboembolism, usually deep venous thrombosis and/or pulmonary emboli. Nearly 50% of individuals with symptomatic heterozygous PC deficiency and 10% of their relatives experience thrombotic episodes by age 45, the initial event commonly occurring between 20 and 30 years of age. Thrombosis may occur at unusual sites such as mesenteric and axillary veins. In one-third of congenital deficiencies, thrombosis is spontaneous; in the remainder it may be traced to trauma,
surgery, pregnancy, oral contraceptive usage, or other events. Heterozygous PC deficiency may also be associated with placental thrombosis resulting in fetal loss, infants born small for gestational age, preeclampsia and placental abruption.

**Anti-vitamin K Therapy**

Warfarin-induced skin necrosis is associated with heterozygous PC deficiency. Progressive, purpuric, necrotic skin lesions may occur during the first few days of warfarin therapy in individuals deficient in PC due to rapid plasma depletion of already low PC activity. PC’s short half-life (6–8 hours) causes it to become depleted sooner than other vitamin K dependent proteins, especially before prothrombin and factor X become proportionately depressed, creating a transient prothrombotic state. This can be prevented by avoiding loading doses of warfarin therapy or by initiating warfarin therapy while the patient is on another anticoagulant, such as heparin.

Purpura fulminans, DIC, and life-threatening thrombosis occur in homozygous deficient neonates. This condition is life threatening unless treatment is promptly initiated.

**Chromogenic Activity Assay Principle**

Test plasma is mixed with a venom collected from *Agkistrodon contortrix contortrix*. The venom activates PC. Chromogenic substrate, provided in a second reagent, is then hydrolyzed by the APC. The degree of hydrolysis, measurable by colored product intensity, is proportional to the activity of PC in the test plasma.

**Chromogenic Activity Assay Performance Characteristics**

The chromogenic substrate test for plasma PC activity detects both quantitative and qualitative PC deficiencies except for those rare qualitative deficiencies that arise from the congenital modification of the PS, or phospholipid-binding regions of the PC molecule. The chromogenic activity may be falsely increased above its true value in patients on anti-vitamin K therapy due to interference by non-carboxylated, inactive PC. It is not affected by direct thrombin inhibitors.

**Clottable Activity Assay Principle**

Activated PC prolongs the activated partial thromboplastin time (APTT) assay, PT assay or the Russell’s Viper Venom assay. Test plasma is mixed with PC-depleted normal plasma to ensure normal levels of all factors except PC. A solution of *Agkistrodon contortrix contortrix* with particulate activators or Russell’s reagent is added, then calcium chloride, and the interval to clot formation is measured. Prolongation is proportional to plasma PC activity. This is the preferred screening assay for PC deficiency.

**Clottable Activity Assay Performance Characteristics**

Calibration is necessary with each run of assays, as the inter-assay CV% is about 5%. The presence of unfractionated heparin levels above 1.0 IU/mL leads to over-estimation of PC. Reagents usually contain a heparin neutralizer and generally have the ability to neutralize up to 1.0 IU/mL unfractionated heparin. Factor VIII levels above 250%, as may be found in inflammation, have been reported to lead to under-estimation of PC in an APTT but not a PT or dRVVT based assay. The presence of a lupus anticoagulant may cause over-estimation of PC activity using the clotting method as will the presence of direct thrombin inhibitors.
**Antigen Assay Principle**

Enzyme immunoassay is used to measure PC antigen. Rabbit anti-human PC is coated on microtiter plate wells to capture test plasma PC. Next, rabbit anti-human PC conjugate with peroxidase marker is added. Third, a peroxidase substrate is added. The intensity of the color product is proportional to the plasma PC antigen.

**Antigen Assay Performance Characteristics**

The PC antigen concentration assay detects most acquired deficiencies and quantitative congenital deficiencies, but does not detect qualitative congenital abnormalities. The chromogenic or clottable PC assays detect essentially all types of deficiencies.

**Chromogenic, Clottable, or Antigen Assay Performance Characteristics**

Because it is vitamin K dependent, the functional PC level begins to decrease six to twelve hours after oral anti-vitamin K therapy is begun. Following cessation of warfarin therapy, vitamin K-dependent coagulation factors remain decreased for approximately two weeks. For several days following surgery or a thrombotic event, the PC will be diminished even in the absence of warfarin therapy, so during this period plasma-based assays cannot be used to establish a congenital deficiency. The PC antigen/factor VII antigen ratio may be used to determine a presumptive PC deficiency if clinical indications prevent suspension of warfarin therapy. A ratio of 0.5 or less indicates Protein C deficiency, however, deficiency is not absolutely ruled out when the ratio is 0.6 or above as the antigen assay may not detect a dysfunctional protein C molecule. Evaluation for PC deficiency while individuals are on warfarin therapy is not advised. Warfarin therapy should be discontinued for 14 – 30 days before PC levels are evaluated.

**Reference Ranges**

Adult PC activity and antigen levels range from 55%-150%. PC activity levels in infants are in the range of 10 – 50 % and, although they rise to functional levels within the first three months of life, they do not reach adult normal levels until 12 to 16 years of age.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference Range</th>
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<tbody>
<tr>
<td>Protein C Activity (chromogenic)</td>
<td>55 – 140%</td>
</tr>
<tr>
<td>Protein C Antigen</td>
<td>60 - 150%</td>
</tr>
<tr>
<td>Protein C Ag/Factor VII Ag Ratio</td>
<td>0.5 – 2.2 ratio</td>
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</table>

**Specimen Requirements**

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.
**Protein C Test Request Information**

Protein C activity (chromogenic) (#300035), protein C antigen (#300036) and protein C Ag/factor VII Ag ratio (#300067) may be ordered separately from the Esoterix Service Directory. CPT Code: Protein C activity (chromogenic) 85303, Protein C antigen 85302, Protein C Ag/factor VII Ag ratio 85302, 85230.

**Reflex Recommendations**

If the plasma PC activity result is below 55%, the plasma PC antigen assay may be performed to distinguish qualitative from quantitative deficiencies. Since therapy for type I and type II deficiencies does not vary, such an evaluation may not be cost effective. Acquired causes of PC deficiency should be excluded. If there is a clinical reason to suspect a qualitative PC deficiency despite a normal chromogenic PC activity assay and plasma PC antigen assay results, a clottable PC assay may be used to detect an uncommon mutation of either the PS or phospholipid binding sites. A clottable PC result may give a more accurate result in patients on anti-vitamin K therapy.

After initial detection, all presumed PC deficiencies should be confirmed by repeat analysis on a new plasma sample after several weeks before a diagnosis of PC deficiency is made. The kindred of PC deficient individuals should be screened for deficiency both to confirm its hereditary nature and to assess thrombotic risk to family members.

**Therapy of Protein C Deficiency**

Acute thrombotic episodes are managed by heparin therapy followed by warfarin prophylaxis, maintaining an INR of 2-3. In PC deficient individuals, warfarin-induced skin necrosis may occur early in therapy due to rapid depletion of PC before the procoagulant factors, such as thrombin and factor X, become depressed. This may be controlled by eliminating the warfarin loading dose or using heparin early in warfarin therapy. In severe conditions where the PC level is very low, such as purpura fulminans, fresh frozen plasma or PC concentrate are necessary to prevent life-threatening thrombosis.

**References**

PROTEIN S AND C4b BINDING PROTEIN

Physical Characteristics

Protein S (PS) is a 69,000 Dalton vitamin K-dependent glycoprotein produced by the liver, megakaryocytes, and endothelial cells. Total PS plasma concentration averages 20 to 25 mg/L and its biological half-life is 60 hours. In blood PS exists in a free and bound state. Sixty to seventy percent of plasma PS circulates complexed to C4b binding protein (C4bBP), a 570,000 Dalton complement system regulator. The remaining PS, called free PS, in molar excess to C4bBP, is the functionally active form of PS.

Physiology

PS is an essential cofactor of the Protein C (PC) pathway. PC, when converted to its enzymatically active serine proteinase form, Activated Protein C (APC), inactivates FVa and FVIIIa. PS also has anticoagulant activity independent of PC by inactivating FXa, although the physiological importance of this is unknown.

In the intact blood vessel, thrombin first binds thrombomodulin, an endothelial membrane receptor. The thrombin-thrombomodulin complex then converts circulating plasma PC to APC. For APC to bind and cleave FVa and FVIIIa, the vitamin K dependent plasma cofactor, PS must be available. APC and free PS assemble with their target procoagulants FVa and FVIIIa on a phospholipid surface such as a platelet or endothelial cell membrane, where the stabilized APC cleaves FVa and FVIIIa, limiting thrombin production. PS also upregulates the hydrolysis rate of FVa.

Incidence of Deficiency

Congenital PS deficiency is an autosomal dominant disorder and in the heterozygous state occurs in 2% of unselected patients with venous thrombosis. Frequency in the population of heterozygous deficiency is about 1 in 700. Homozygous deficiency is very rare. In contrast, acquired PS deficiency is observed more frequently and occurs in a number of clinical conditions.

Etiology of Deficiency

Acquired PS deficiency may be the results of decreased PS synthesis, or increased PS consumption. C4bBP is an acute phase reactant, thus its plasma concentration increases with inflammation and hormonal changes, but this does not result in increased PS binding nor a relative deficiency of free PS. C4bBP is elevated in inflammation, pregnancy, estrogen and progestin administration, diabetes mellitus, systemic lupus erythematosus, AIDS, renal allograft rejection, and smoking (see table). Functional PS synthesis is diminished in vitamin K deficiency, liver disease, chemotherapy, warfarin therapy, and L-asparaginase therapy. PS consumption is increased in acute thrombosis, polycythemia vera, sickle cell disease, essential thrombocythemia, and disseminated intravascular coagulation. PS levels decrease in pregnancy and this decrease is related to gestation.

Congenital PS deficiency is inherited as one of three types of autosomal dominant traits. About 90% are quantitative, or "type I" deficiencies, in which structurally normal PS molecules are produced at diminished plasma levels. In quantitative PS deficiency, total and free PS antigen levels and clottable PS activity levels are all below 65% (see table). Qualitative, "type II" deficiencies, in which functionally abnormal molecules are present at normal plasma levels, are rare. In the few reported cases of qualitative deficiency, total and free PS antigen levels are normal, but the clottable PS activity level is low. In "type III" congenital abnormalities, PS mutations lead to increased C4bBP binding. The total PS antigen concentration is normal, but the free PS antigen and clottable PS activity levels are low.

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Total PS antigen</th>
<th>Free PS antigen</th>
<th>Clottable PS activity</th>
<th>C4b BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>&lt;65%</td>
<td>&lt;65%</td>
<td>&lt;65%</td>
<td>60 – 150%</td>
</tr>
<tr>
<td>Type II</td>
<td>&gt;65%</td>
<td>&gt;65%</td>
<td>&lt;65%</td>
<td>60 – 150%</td>
</tr>
<tr>
<td>Type III</td>
<td>&gt;65%</td>
<td>&lt;65%</td>
<td>&lt;65%</td>
<td>60 – 150%</td>
</tr>
<tr>
<td>Inflammation</td>
<td>&gt;65%</td>
<td>&lt;65%</td>
<td>&lt;65%</td>
<td>&gt;150%</td>
</tr>
</tbody>
</table>
**Clinical Significance of Deficiency**

Venous thrombosis most commonly manifests as deep vein thrombosis and pulmonary emboli, and may occur when PS levels drop to 50% or below. The relationship of PS deficiency to risk of arterial thrombosis is uncertain. Half of individuals with heterozygous PS deficiency experience thrombotic episodes by age 45, the initial event commonly occurring between 20 and 30 years of age. Thrombosis may occur at unusual sites such as mesenteric and axillary veins; also recurrent events are likely. In many instances of congenital deficiencies, thrombosis is spontaneous; in the remainder it may be traced to trauma, surgery, pregnancy, oral contraceptive usage, or other events.

Purpura fulminans, DIC, and life-threatening thrombosis have been described in homozygous neonates, but there are few confirmed cases of homozygous deficiency. Symptoms include purpura in all areas of the body, deep vein thrombosis, mental retardation, blindness, and delayed psychomotor development. Death occurs unless treatment is initiated.

**Clottable Assay Principle**

PS in the presence of normal plasma, APC, and FVa prolongs the activated partial thromboplastin time (APTT) test. Test plasma is mixed with PS-depleted normal plasma to ensure normal levels of all factors but PS. APC and bovine FVa are added, then calcium chloride, and the interval to clot formation is measured. Prolongation is proportional to plasma PS activity.

**Activity (Clottable) Assay Performance Characteristics**

Calibration is necessary with each run of assays, as the inter-assay CV% may exceed 5%. The presence of heparin levels above 1.0 IU/mL and lupus anticoagulants lead to over-estimation of PS. APC resistance, as can be seen in patients with FV Leiden, will interfere causing an artifactual decrease in PS activity. Due to these variables, the PS activity assay is not the recommended screening assay to determine PS deficiency.

**Activity and Antigen Reference Ranges**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS Activity (clottable)</td>
<td>50 - 140%</td>
</tr>
<tr>
<td>PS Antigen Total</td>
<td>60 - 150%</td>
</tr>
<tr>
<td>PS Antigen Free</td>
<td>62 - 146%</td>
</tr>
<tr>
<td>C4b Binding Protein</td>
<td>50 - 130%</td>
</tr>
<tr>
<td>PS Ag/Factor VII Ag Ratio</td>
<td>0.5 – 2.2 ratio</td>
</tr>
</tbody>
</table>

C4bBP levels range from 60% to 150%. In newborns, total PS is low; however, free PS levels exceed adult levels because C4bBP is also low. Adult levels of total and free PS and C4bBP are reached at six months of age. Adult females under 45 years old have slightly lower plasma PS levels than men.

**Total PS Antigen Assay Principle**

Enzyme immunoassay is used to measure total PS antigen. Rabbit anti-human PS is coated on microtiter plate wells to capture test plasma PS. Next, rabbit anti-human PS conjugate with peroxidase marker is added. Third, ortho-phenylenediamine substrate is added in the presence of hydrogen peroxide. The intensity of the color produced is proportional to the total plasma PS.
Total PS Antigen Assay Performance Characteristics

The PS antigen concentration assay detects most quantitative congenital deficiencies, but does not detect qualitative (type II) or type III congenital abnormalities. Total PS levels are influenced by a number of factors and levels are decreased in pregnancy and in women on oral contraceptives. Women have consistently lower PS levels than men.

Free PS Antigen Assay Principle

Enzyme immunoassay is used to measure free PS antigen. Monoclonal anti-free PS is coated on microtiter plate wells to capture test plasma free PS. Next, rabbit anti-human PS conjugate with peroxidase marker is added. Third, ortho-phenylenediamine substrate is added in the presence of hydrogen peroxide. The intensity of the color produced is proportional to the free plasma PS.

Free PS Antigen Assay Performance Characteristics

Assays for free PS are superior to those for total PS in predicting PS deficiency. This is consistent with the fact that free PS is the functionally active form of the protein. Measurement of free PS is recognized by ISTH and WHO guidelines as the appropriate assay to screen for PS deficiency. The free PS antigen concentration assay detects most quantitative (type I) and most type III congenital or acquired (inflammatory) deficiencies, but does not detect the rare qualitative (type II) congenital abnormalities. The clottable PS assay detects all deficiencies.

C4b Binding Protein Antigen Assay Test Principle

The assay for C4b-BP is a latex immunoassay. Microlatex particles are coated with antibody and when a sample contains the antigen being tested for, the latex particles agglutinate. A beam of monochromatic light is passed through the sample and there is a direct relationship between absorbance value and the concentration of antigen.

C4b Binding Protein Antigen Assay Performance Characteristics

The C4bBP antigen assay detects elevated C4bBP secondary to inflammation or estrogen elevation.

PS Clottable Activity and Antigen Assay Limitations in Anticoagulant Therapy

Because it is vitamin K dependent, the PS level begins to decrease 60 hours after oral anticoagulant warfarin therapy is begun. When oral anticoagulant is stopped, its effects on PS may be seen for up to two weeks. For several days after surgery or a thrombotic event, the PS levels may be diminished even in the absence of Coumarin-based anticoagulant therapy; during this period assays cannot be used to establish a congenital deficiency. The total PS antigen concentration assay/FVII antigen concentration assay ratio (#300059) may be used to determine a presumptive PS deficiency in patients on oral anticoagulant therapy if clinical indications prevent suspension of this therapy. A ratio of 0.5 or less suggests PS deficiency; however deficiency cannot be ruled out when the ratio is 0.6 or above as a functional PS deficiency will not be detected using this method.
Specimen Requirements

Citrated plasma specimen is required for all PS assays and the C4bBP assay. Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure the evacuated blood tube is filled. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

PS Test Request Information

The clottable plasma PS activity test (#300088), the total PS antigen (#300038), the free PS (#300087), and C4bBP (#300160) assays may be ordered separately from the Esoterix Service Directory. The C4bBP assay is approved for research use only.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference Range</th>
<th>CPT Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS Activity (clottable)</td>
<td>55 – 160%</td>
<td>85306</td>
</tr>
<tr>
<td>PS Antigen Total</td>
<td>60 – 150%</td>
<td>85305</td>
</tr>
<tr>
<td>PS Antigen Free</td>
<td>62 – 146%</td>
<td>85306</td>
</tr>
<tr>
<td>C4b Binding Protein</td>
<td>50 – 130%</td>
<td>83520</td>
</tr>
<tr>
<td>PS Ag/Factor VII Ag Ratio</td>
<td>0.5 – 2.2</td>
<td>85305, 85230</td>
</tr>
</tbody>
</table>

Reflex Recommendations

When screening for PS deficiency, the free PS Antigen assay is recommended. If the clottable plasma PS activity result is below 65%, the total and free plasma PS antigen assays and the C4bBP assay may be performed to distinguish types I, II, and III deficiencies.

After initial detection, all presumed PS deficiencies should be confirmed by repeat analysis after several weeks. The kindred of PS deficient individuals should be screened for deficiency to confirm its hereditary nature and to assess thrombotic risk to family members.

In addition to PS deficiency, antithrombin and PC deficiency, APC resistance, Prothrombin Gene G20210A mutation, hyperhomocysteinemia, elevated lipoprotein(a), or the presence of antiphospholipid antibodies may promote the development of venous thrombosis.

Therapy of PS Deficiency

Acute thrombotic episodes are managed by heparin therapy followed by long-term warfarin prophylaxis, maintaining an INR of 2-3. In severe conditions where the PS level is very low, such as purpura fulminans, fresh frozen plasma may be necessary to prevent life-threatening thrombosis.
References


Physical Characteristics

Prothrombin fragment 1+2 (F 1+2) is the amino terminus of the prothrombin molecule. It is a polypeptide with a half-life of approximately 90 minutes.

Physiology

Evaluation of F 1+2 in plasma is a determinant of in vivo thrombin generation. F 1+2 is released from prothrombin when prothrombin is converted to thrombin by the prothrombinase complex. The prothrombinase complex consists of activated factors X and V, calcium ions, and phospholipid.

Clinical Significance

F 1+2 has been utilized to assess thrombotic risk and monitor anticoagulant therapy. Increases of F 1+2 occur as a result of increased conversion of prothrombin to thrombin. F 1+2 levels are elevated in patients with deep venous thrombosis, pulmonary embolism, disseminated intravascular coagulation, sepsis, preeclampsia, eclampsia, and trauma. Elevated levels following discontinuation of anticoagulant therapy may predict increased risk for recurrence of venous thrombosis. F 1+2 also may be elevated in asymptomatic patients with hereditary protein C or protein S deficiency. Likewise, F 1+2 is elevated as much as two times normal in asymptomatic patients with antithrombin deficiency. Levels may be increased in patients with underlying active malignancy and may predict survival.

F 1+2 may be decreased below the reference range in patients receiving therapeutic anticoagulant therapy and levels may be used to assess efficacy of anticoagulant therapy.

Assay Principle

F 1+2 is measured by an enzyme immunoassay based on the sandwich principle. F 1+2 in the sample binds to anti-F 1+2 antibodies fixed to the microtiter plate during an incubation period. Peroxidase conjugated antibodies are then added and a second incubation takes place. A chromophore is added and color formation is directly proportional to the concentration of F 1+2.

Performance Characteristics

Traumatic venipuncture or prolonged stasis may falsely elevate results. Improper mixing of the specimen after venipuncture can falsely elevate F 1+2 levels.

Reference Range

The normal reference range for F 1+2 is 87 – 325 pmol/L.

Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). If necessary, transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.
PROTHROMBIN FRAGMENT 1+2 (cont.)

Test Request Information

F 1+2 (#300718) can be ordered separately from the Esoterix Service Directory. CPT Code 83520.

Reflex Recommendations

F 1+2 may be accompanied by increases in other markers of clot formation and lysis such as thrombin-antithrombin complex and D-dimer.

References


**Physical Characteristics**

A G-to-A substitution in nucleotide position 20210 of the 3’ untranslated region of the prothrombin gene is responsible for a common polymorphism of factor II protein. Individuals who inherit one abnormal allele are considered heterozygous and those with two mutant alleles are homozygous for G20210A.

**Physiology**

Prothrombin is the precursor of thrombin, a pivotal serine protease in hemostasis that exhibits both anticoagulant and procoagulant properties.

**Incidence of Factor II G20210A Mutation**

The G20210A mutation is found in 8% of patients with confirmed deep venous thrombosis. This mutation has been reported in 18% of patients with a positive personal or family history of thrombosis. The mutation also has been reported in up to 2.3% of healthy individuals. The G20210A mutation appears to be more consistently distributed in the African and Caucasian populations than the Factor V Leiden mutation.

**Clinical Significance**

The presence of one prothrombin G20210A allele (heterozygous) is associated with at least a 3-6 fold increased risk of deep venous thrombosis for all ages and both genders. Homozygous deficiency further increases risk but the magnitude is not known. Risk of thrombosis is further increased by up to 80 times normal in individuals who carry both the prothrombin defect and factor V Leiden. Increased levels of prothrombin may be seen in individuals that carry the prothrombin G20210A mutation and appear to be the cause of increased risk of thrombosis. Prothrombin antigen and/or activity levels cannot be used to test for the presence of the mutation, however, as these assays are not sufficiently sensitive or specific for the G20210A polymorphism.

**Assay Principle**

The assay for prothrombin G20210A uses polymerase chain reaction (PCR). The patient’s DNA is isolated from white blood cells.

**Assay Performance Characteristics**

Patients can be characterized as normal for this specific mutation, heterozygous, carrying the mutation on only one copy of the prothrombin gene, or homozygous, carrying the mutation on both copies of the prothrombin gene.

**Reference Range**

In unaffected persons, the assay is negative (wild type).
**Specimen Requirements**

EDTA whole blood maintained at room temperature, do not freeze: Collect blood into a lavender (EDTA) stopper tube, ensure complete fill, and invert gently six times immediately after filling. The specimen should be kept at room temperature and should not be centrifuged. Label and place specimen in container to be shipped at room temperature so that it is received within 5 days of collection.

**Test Request Information**

Factor II G20210A mutation (#120736) can be ordered separately from the Esoterix Service Directory. CPT Codes 83891, 83894, 83900, 83912

**Reflex Recommendations**

The kindred of individuals with prothrombin G20210A mutation should be considered for screening to assess thrombotic risk to family members.

**References**

**Physiology**

The prothrombin time (PT) is the clotting time in seconds of a mixture of thromboplastin (tissue factor) reagent and citrated plasma in the presence of calcium ions. The PT tests for deficient activity of procoagulants of the extrinsic or “tissue factor” and common coagulation pathways, including, in the order of their reactions, factors VII, X, V, II (FVII, FX, FV, FII), and fibrinogen. Deficiency or inhibition of any extrinsic or common pathway factor may prolong the PT result. PT may also be prolonged in the presence of a dysfibrinogenemia or when the fibrinogen concentration falls below 100 mg/dL. The PT is more sensitive to deficiencies of FII, FV and FX than the APTT.

The Stypven time is the clotting time in seconds of a mixture of Russell's viper venom reagent and citrated plasma and tests the common pathway factors, bypassing the requirement for FVII. Deficiency or inhibition of any common pathway factor, therefore, prolongs the Stypven time result.

Tissue factor is an integral membrane protein found on the plasma membrane of most nonvascular cells. It can be expressed by monocytes and endothelial cells following exposure to certain stimuli. The coagulation process begins with exposure of plasma to tissue factor thus triggering FVII activation. FVIIa then forms a complex with the exposed tissue factor and nearby cell membrane phospholipids. This complex activates FX. FXa in the presence of Ca++ FVa and phospholipid (prothrombinase complex) cleaves prothrombin (FII) to form thrombin (FIIa). Thrombin cleaves the fibrinogen molecule and fibrin monomer is formed. Polymerization of fibrin monomers follows and a soluble fibrin clot is formed.

The tissue FVIIa complex also activates FIX, forming a complex with FVIIa, also activating FX. This in vivo pathway is bypassed in the prothrombin time test, thus FVIII and IX are not considered to be part of the extrinsic pathway.

The extrinsic pathway’s activity is short-lived. Soon after tissue factor and FVIIa join with their substrate, FX, the FVIIa-Xa complex is neutralized by plasma tissue factor pathway inhibitor (TFPI).

**Incidence and Etiology of Factor Deficiencies**

The PT is sensitive to FVII deficiency and is moderately sensitive to deficiencies of FX, V, and II. The PT generally prolongs only when the fibrinogen is < 100 mg/dL. PTs are often ordered to establish the cause of bleeding or to disclose possible coagulation defects in those about to undergo an invasive procedure. Congenital deficiencies of the extrinsic and common pathways cause hemorrhagic disorders of varying severity. These deficiencies are rare with both quantitative and qualitative abnormalities having been described.

Acquired multiple factor deficiencies may be seen during warfarin therapy, with vitamin K deficiency, disseminated intravascular coagulation (DIC) and in liver disease. During anti-vitamin K therapy or with vitamin K deficiency FII, VII, IX and X activities diminish, prolonging the PT result. In the other listed conditions, nearly all factors are deficient due to either consumption or lack of production, respectively.

**Coumarin-Based Therapy and Prothrombin Time**

Oral anticoagulants based on vitamin K antagonism, variously named warfarin, sodium warfarin, dicumarol, coumarin, or Coumadin™, are employed to prevent the propagation of thrombi in venous and arterial thrombotic disease and to prevent thrombus development. Warfarin's therapeutic range in plasma is narrow; overanticoagulation causes hemorrhage, while inadequate dosages permit rethrombosis. It takes approximately two weeks for warfarin to achieve a stable therapeutic range in an individual. Duration of therapy, several months to years, is dependent on location of thrombus, age of patient at time of development and underlying cause of thrombosis (i.e. acquired or congenital risk factors). Laboratory monitoring is essential throughout the use of warfarin therapy in order to keep the dosage within the therapeutic range. Warfarin is a vitamin K antagonist that suppresses the normal production of carboxylated FII, FVII, FIX, and FX. Because it is sensitive to deficiencies of FII, FVII, and FX, the PT is most often used to monitor warfarin therapy. The results are expressed as international normalized ratio (INR) values to normalize for the variable responsiveness of thromboplastin reagents.
Recommended Therapeutic Range for Oral Anticoagulant Therapy**

<table>
<thead>
<tr>
<th>INR</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 - 1.5</td>
<td>Prevention of catheter-related venous thrombosis</td>
</tr>
<tr>
<td>2.0 - 3.0</td>
<td>• Prophylaxis of venous thrombosis</td>
</tr>
<tr>
<td></td>
<td>• Treatment of venous thrombosis</td>
</tr>
<tr>
<td></td>
<td>• Treatment of pulmonary embolism</td>
</tr>
<tr>
<td></td>
<td>• Prevention of systemic embolism</td>
</tr>
<tr>
<td></td>
<td>Tissue heart valves</td>
</tr>
<tr>
<td></td>
<td>AMI (to prevent systemic embolism)*</td>
</tr>
<tr>
<td></td>
<td>Valvular heart disease</td>
</tr>
<tr>
<td></td>
<td>Atrial Fibrillation</td>
</tr>
<tr>
<td></td>
<td>Bileaflet mechanical valve in aortic position</td>
</tr>
<tr>
<td>2.5 - 3.5</td>
<td>Mechanical prosthetic valves (high risk)</td>
</tr>
</tbody>
</table>

* If oral anticoagulant therapy is elected to prevent recurrent MI, an INR of 2.0 to 3.0 is recommended, consistent with Food and Drug Administration recommendations.

** Chest 2004; 126 (3): 401S-428S.

Cytochrome P450 2C9 Genetic Polymorphisms and Oral Anticoagulant Therapy

Warfarin is one of the most frequently prescribed medications in the world. It is metabolized by the liver through the cytochrome P450 system. A major complication of warfarin therapy is bleeding and this risk is significantly enhanced in those individuals that demonstrate impaired warfarin metabolism. Genetic variants due to single nucleotide variations in the cytochrome P450 2C9 system, called polymorphisms, have recently been identified that result in diminished warfarin metabolism/clearance causing an increased bleeding risk.

The CYP2C9 isoenzyme is the principle enzyme responsible for the metabolism of warfarin as well as several other drugs. Six variants of CYP2C9 have been identified, each the result of a single amino acid variation. The two variants most frequently identified are CYP2C9*2 (CYS144/IL359) and CYP2C9*3 (Arg144/LEU359). The frequencies of the CYP2C9 polymorphisms vary among ethnic groups. CYP2C9*3 has been identified in a wide variety of ethnic groups with the following allelic frequencies in the homozygous state: 6-10% in Caucasian, 1.7-5% in Asians (Chinese and Japanese) and 0.5-1.5% in African-Americans. CYP2C9*2 is not common in Asians but homozygous CYP2C9*2 has been detected in 8-19% of Caucasian and 1-3.6% of African-American populations. Approximately 20% of the Caucasian population or 1 in 5 persons carries a CYP2C9-defective allele, and 1% of Caucasians carry 2 defective CYP2C9 alleles.

Individuals with these genetic polymorphisms demonstrate an increased dose response with warfarin administration and are therefore at an increased risk for hemorrhagic complications during therapy. Patients should be tested for these polymorphisms prior to warfarin administration to avoid the potential for severe hemorrhagic complications/morbidity in affected individuals.

Prolonged Prothrombin Time and Plasma Inhibitors

Specific inhibitors of extrinsic or common pathway coagulation factors are extremely rare, but may be reflected in prolonged prothrombin times. Lupus anticoagulant (LA), present in 1% of the unselected population, may cause a slightly prolonged PT. LA, despite its name, is associated with venous and arterial thrombosis, and is a member of the antiphospholipid protein complex family of antibodies. High titer
lupus anticoagulants may bind and partially neutralize PT reagent phospholipid to cause occasional prolongation of the PT test results. The laboratory employs mixing studies to distinguish between lupus anticoagulant, presence of warfarin, and single or multiple factor deficiencies followed by confirmatory tests as indicated. These patients must be monitored by another test such as the chromogenic FX assay which is insensitive to the effects of a lupus anticoagulant. Patients with lupus anticoagulants occasionally develop antibodies to prothrombin which result in clearance of the antibody-prothrombin complex. These patients typically have a significantly increased PT, drastically reduced FII levels and a potential for hemorrhagic diathesis.

**Assay Principle**

Two of the types of commonly available PT test reagents are: 1) a mixture of tissue factor and phospholipids extracted from rabbit brain and 2) a mixture of affinity-purified or recombinant human placental or brain (available in Europe) tissue factor with synthetic phospholipids. Human thromboplastin reagents are more sensitive to factor deficiencies than are the animal-derived thromboplastins. In either case, prothrombin time reagent containing a measured amount of tissue thromboplastin and calcium chloride is mixed with the patient plasma and the mixture is timed until clot formation occurs. The time interval is proportional to the degree of deficiency or inhibition of the extrinsic pathway procoagulant.

**Assay Characteristics**

Because PT reagents vary in their responsiveness to coagulation factor deficiencies, the international normalized ratio (INR) system was developed to report PT results in patients on stable oral anticoagulant therapy. The PT result is first expressed as a “PT ratio,” specifically the ratio of the patient PT result to the geometric mean of the PT normal range for the specific reagent/instrument system in use:

\[
\text{PT Ratio} = \frac{\text{Patient PT}}{\text{Geometric Mean of the PT Reference Range}}
\]

\((\text{Geometric mean is calculated on a minimum of 20 normal individual specimens using an institution's own test system})\)

The PT ratio is then raised to the power of the international sensitivity index (ISI), an expression of the PT reagent responsiveness. Highly sensitive thromboplastin reagents have low ISI values (1.0 to 1.8) while insensitive thromboplastins have high ISI values (1.9 or greater). This calculation yields the INR, which is the PT ratio that would be obtained if the World Health Organization (WHO) reference reagent had been used to perform the PT: \(\text{INR} = (\text{PT Ratio})^{1/ISI}\)

The ISI is the slope of a regression equation comparing the PT results of a set of reference plasmas using a manufacturer’s thromboplastin reagent to the PT results using the WHO reagent whose ISI, by definition, is 1.0. The ISI determination and computation is usually made by the reagent manufacturer. The lower the ISI, the more responsive is the reagent.

Prothrombin reagent responsiveness to plasma coagulation factor deficiencies is also influenced by the type of instrument and by local specimen collection variables. For this reason, many coagulation laboratories perform local ISI calibration by purchasing reference plasmas whose INRs have been established and testing them with the local reagent-instrument system. If the calibrated ISI varies by more than 10% of the manufacturer’s generated ISI, the computed ISI should replace the reagent manufacturer’s ISI in subsequent PT tests.

**Prothrombin Time Mixing Studies**

An isolated prolonged PT result with a normal APTT implies a specific FVII deficiency or inhibitor, although this could occur with a mild deficiency of FV, X or II. An abnormal PT and APTT suggests the presence of warfarin, vitamin K deficiency, a lupus anticoagulant, or a deficiency or specific factor inhibitor of the common pathway factors (FX, FV, FII or fibrinogen). Unless the cause of the prolongation is known, as in warfarin therapy, mixing studies are necessary to identify the cause of the prolongation.

To perform mixing studies, the patient specimen is mixed 1:1 with normal platelet free plasma and with veronal buffered saline. PTs are performed on these mixtures. An aliquot of the normal plasma/patient plasma is incubated at 37°C for one to two hours and then a PT is performed to further clarify the cause of the initial prolongation. Normal platelet free plasma and patient plasma are also incubated separately and then mixed after incubation as a control for factor degradation during incubation.
PROTHROMBIN TIME/PROTHROMBIN TIME MIXING STUDIES/STYPVEN TIME (cont.)

Mixing Studies Assay Characteristics

If the result obtained with the immediate normal plasma mixture corrects to within the reference range and the saline mixture result increases dramatically, a factor deficiency or warfarin therapy is suspected. If the result obtained with the immediate normal plasma mixture shows only partial or no correction and the result obtained with the saline mixture shows near correction to the reference range or only mild prolongation, then an inhibitor such as LA, or a factor specific inhibitor, is suspected.

After incubation at 37°C, a PT result equivalent to that of the original mixture is indicative of a factor deficiency. If the result of the PT on the mixture demonstrates further prolongation (with the control remaining equivalent to the original result) a time dependent specific factor inhibitor is indicated.

Factor deficiencies should be further identified by performing specific factor assays. Plasma suspected of containing an LA should be further tested with other LA specific tests [i.e. Dilute Russell's Viper Venom (DRVVT), Platelet Neutralization Procedure (PNP), or the Hexagonal Phospholipid Neutralization Procedure] to confirm the presence of a lupus anticoagulant. Specific factor inhibitors such as anti-FV, anti-FX, anti-FII and anti-FVII should be identified and assayed using the Bethesda titer methodology.

Stypven Time Assay Principle

Reagent containing a specified amount of Russell's viper venom and calcium chloride is mixed with the patient test plasma and the mixture is timed until clot formation occurs. Russell's viper venom activates the common pathway by activating FX directly, bypassing the action of FVII. The degree of prolongation is proportional to the degree of deficiency or inhibition of common pathway procoagulants.

Stypven Time Assay Characteristics

The Stypven Time assay is no longer a widely used assay. It should not be confused with the “Dilute” Russell's Viper Venom Time (DRVVT) used in lupus anticoagulant (LA) testing. The Stypven Time may be used in differentiating deficiencies due to FVII and FX.

Reference Ranges

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin Time (PT, “Protime”)</td>
<td>10.0 – 13.0 seconds</td>
</tr>
<tr>
<td>PT (International Normalized Ratio)</td>
<td>See Recommended Therapeutic Range for</td>
</tr>
<tr>
<td>Oral Anticoagulant Therapy</td>
<td></td>
</tr>
<tr>
<td>PT Mixing Studies</td>
<td>See Text</td>
</tr>
<tr>
<td>Stypven Time (Russell's viper venom time)</td>
<td>16.1 – 29.8 seconds</td>
</tr>
</tbody>
</table>

Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). If necessary, transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.
Test Request Information

The Prothrombin Time (PT/INR) (#300080), PT mixing studies (#300116), Stypven time (#300611) and CYP 2C9 (#300749) may be ordered separately from the Esoterix Service Directory. CPT Code: PT/INR 85610; PT mixing studies 85610, 85611 x 2, Stypven time 85612; CYP 2C9 83891, 83892 x 2, 83894 x 2, 83898 x 2, 83912

Reflex Recommendations

A prolonged PT result implies the presence of anti-vitamin K therapy, vitamin K deficiency, specific factor inhibitor, or a factor deficiency. Unless the cause of the prolongation is known, as in the case of warfarin therapy, mixing studies are necessary.

If mixing studies indicate the possibility of a factor deficiency or inhibitor, an APTT should be performed. The combination of normal APTT and prolonged PT result implies FVII deficiency and a FVII activity assay should be performed. The combination of prolonged APTT and PT implies a deficiency or inhibitor of the common pathway FX, FV, FII, or fibrinogen or multiple factor deficiencies. Factor assays should be performed to detect single or multiple factor deficiency/inhibition. The Stypven time test may be used to rule out an isolated FVII deficiency.

If a lupus anticoagulant is suspected as a result of the mixing study, the APTT-based mixing studies as well as confirmatory tests such as DRVVT, PNP and/or Hexagonal Phospholipid Neutralization should be performed. A patient with initial positive lupus anticoagulant testing should be retested after eight weeks to determine if the antibody is persistent, as those that do persist over time are thought to be instigators of hypercoagulability.

References

THROMBIN - ANTITHROMBIN COMPLEX

Physical Characteristics

Thrombin-antithrombin complexes (TAT) form covalently following thrombin generation and have a plasma half-life of 10-15 minutes. The presence of TAT indicates ongoing thrombin formation and the consumption of antithrombin.

Physiology

Upon activation of coagulation, antithrombin complexes with thrombin as well as other serine proteases. The complex formation is greatly enhanced by the presence of heparin or other glycosaminoglycans. The reaction initially is reversible, but becomes irreversible following the formation of a covalent bond between antithrombin and thrombin. This binding results in complete inhibition of thrombin's activity.

Etiology

Elevated levels of TAT may be associated with advancing age, pregnancy, septicemia, disseminated intravascular coagulation, multiple trauma, acute pancreatitis, acute and chronic leukemia, pre-eclampsia, acute and chronic liver disease, and other predisposing causes of thrombosis. Increased levels also are reported during heparin and fibrinolytic therapy. TAT levels are markedly reduced in the first 24 hours after receiving oral anticoagulants.

Clinical Significance

TAT levels are a marker of in vivo thrombin generation. The TAT assay can detect this intravascular generation of thrombin and provides valuable information in the diagnosis of thrombotic events. Decreasing TAT levels can also indicate the resolution of a thrombotic event. A normal TAT level in the presence of an elevated D-dimer may indicate an old thrombus.

Assay Principle

TAT is measured by enzyme immunoassay utilizing a sandwich technique. The patient sample containing TAT is incubated with antibodies against thrombin, and the unbound constituents are removed by washing. Enzyme-conjugated antibodies to antithrombin are then added to the reaction, and the excess antibodies are removed by washing. The remaining (bound) enzymatic activity acts upon a chromophore. The resulting color intensity is proportional to the TAT in the sample.

Assay Performance Characteristics

Traumatic venipuncture, prolonged stasis, or inadequate centrifugation may falsely elevate results. Inadequate mixing of the patient sample and the citrate in the collection tube may result in falsely elevated levels. TAT levels may be elevated secondary to inflammation and microvascular thrombosis in surgical patients or those patients in acute distress.

Reference Range

TAT is normally present in very low concentrations of <5.1 ng/mL.
**Specimen Requirements**

Citrate plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count < 10 x 10^9/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

**Thrombin-Antithrombin Complex Test Request Information**

The TAT assay (#300714) can be ordered separately from the Esoterix Service Directory. CPT Code 83520.

**Reflex Recommendations**

Elevated TAT measurements may be accompanied by increased levels of prothrombin fragment 1+2, fibrinogen degradation products, and D-dimer. Quantitative D-dimer has a greater sensitivity for excluding the presence of acute deep venous thrombosis.

**References**

Physical Characteristics

Thromboxane B₂ (TXB₂) is the stable, inactive product of prostaglandin metabolism formed from thromboxane A₂. It is cleared renally and can be measured in the urine.

Physiology

Platelets are activated when agonists such as epinephrine or thrombin bind a membrane receptor. Activation results in the liberation of arachidonic acid from the inner surface of the platelet membrane. Arachidonic acid is ultimately converted to Thromboxane A₂ (TXA₂) through the action of two enzymes, cyclo-oxygenase and thromboxane synthase. TXA₂ is unstable in the plasma and is rapidly hydrolyzed to a stable plasma product, TXB₂. TXB₂ is excreted renally.

Clinical Significance

Studies have shown that TXB₂ is a sensitive indicator of platelet activation. Since platelets participate in atherogenesis and contribute to acute, ischemic atherosclerotic complications, elevated TXB₂ levels may reflect ongoing cardiovascular, peripheral vascular, and cerebrovascular disease processes. Additionally, TXB₂ may be of interest in conditions with increased platelet turnover such as disseminated intravascular coagulation or immune thrombocytopenia. Studies of patients with diffuse atherosclerotic disease show that TXB₂ may be a more sensitive measure of platelet activation than other platelet specific proteins. Serum or plasma TXB₂ assays are typically limited to a research setting because of the significant in vitro platelet instability. Urinary TXB₂ is of interest in monitoring the anticoagulant response of aspirin therapy since aspirin inhibits the formation of TXB₂ in platelets. In those patients responsive to aspirin therapy and taking an adequate dose, urinary 11-dehydro TXB₂ levels should be reduced below a predetermined cut-off when compared to control values.

Assay Principle

The assay is a competitive assay between the urine 11-dehydro TXB₂ and an added urinary 11-dehydro TXB₂ tracer (linked to acetylcholinesterase). The compound in the patient urine and the tracer compound compete for a limited number of 11-dehydro TXB₂ rabbit antiserum binding sites attached to microtest wells. The tracer concentration is held constant while the patient concentration is varied. Therefore, the amount of tracer that can bind to the rabbit antiserum is inversely proportional to the concentration of 11-dehydro TXB₂ in the specimen urine. The test wells are washed and conjugated substrate is added. The color production is inversely proportional to the amount of 11-dehydro TXB₂ present in the patient urine.

Assay Performance Characteristics

The assay shows no significant cross reactivity with other prostaglandins or prostaglandin metabolites.

Reference Ranges

To normalize for variations in urine concentration, results are expressed as pg of 11-dehydro TXB₂/mg creatinine. Baseline values apply only to individuals not currently on aspirin therapy and those individuals who have not taken platelet-modifying products for at least ten days. Values greater than 1000 pg 11-dehydro TXB₂/mg creatinine are consistent with levels observed in individuals not taking aspirin. Individuals on aspirin with levels greater than 1000 pg 11-dehydro TXB₂/mg creatinine may be resistant to aspirin at that dosage or they may be non-compliant. Values less than 400 pg 11-dehydro TXB₂/mg creatinine are consistent with a typical, normal response to aspirin. In patients on aspirin with results in the 400 - 600 PURR range, repeat analysis on a new urine sample is recommended.
Specimen Requirements

First morning urine. Mix well and aliquot to an ASPIRINcheck™ specific plastic transport tube containing a preservative tablet. Fill tube to fill line (about 9mL); minimum permissible fill is 6mL. Seal tube and invert until preservative is dissolved. Samples with preservative are stable at room temperature for 24 hours and may be shipped overnight at ambient temperature. If unable to ship overnight, preserved urine samples must be frozen and shipped on dry ice. Samples without preservative must be frozen within 2 hours of collection and shipped on dry ice overnight. Please include aspirin dosage, or zero if not on aspirin.

Test Request Information

ASPIRINcheck™, urinary 11-dehydro TXB₂ (#300250) can be ordered separately from the Esoterix Service Directory. CPT Codes: 83520, 82570.

References

Physiology

The TT (thrombin clotting time, TCT) measures the amount of time to fibrin clot formation following the addition of thrombin to citrated plasma. The TT reagent, which is bovine-derived thrombin, cleaves fibrinopeptides A and B from plasma fibrinogen leading to clot formation. Thrombin clotting times can also be performed using human-derived thrombin if this is specially requested.

The reptilase time (RT) measures the time to fibrin clot formation after the addition of reptilase to citrated plasma. The reagent is derived from the venom of Bothrops atrox and cleaves only fibrinopeptide A from the fibrinogen molecule which also results in clot formation.

Etiology

Prolongations of either the thrombin time and/or reptilase time may reflect abnormalities in fibrinogen concentration or structure or occur when there is interference with fibrin polymerization as may occur in the presence of fibrin degradation products or monoclonal antibodies. Afibrinogenemia (the complete absence of fibrinogen), hypofibrinogenemia (decreased levels of fibrinogen) and dysfibrinogenemia (a functional abnormality of the fibrinogen molecule) are congenital disorders that may be associated with a bleeding diathesis. Both the thrombin time and reptilase time will be prolonged in these disorders. The TT and RT are generally both prolonged when functional fibrinogen is less than 100 mg/dL. Acquired decreases in fibrinogen levels or abnormal function may be seen in chronic liver disease, renal disease, systemic amyloidosis, multiple myeloma, disseminated intravascular coagulation (DIC) or in thrombolytic therapy. Fibrinogen levels above 600 mg/dL may also cause a prolonged TT.

Therapeutic unfractionated heparin prolongs the thrombin time but not the reptilase time. Direct thrombin inhibitors such as Argatroban, hirudin and ximelagatran will also prolong the TT. Paraproteins and fibrin degradation products, especially fragments D and E, interfere with fibrin polymerization thus prolonging both the TT and reptilase time. Heparin, paraproteins and fibrin(ogen) degradation products (FDPs) may also prolong the results of the prothrombin time (PT) and the activated partial thromboplastin time (APTT) assays as all three clot-based tests depend upon unimpeded fibrin polymerization.

Bovine thrombin inhibitors (anti-bovine thrombin antibody) may develop in patients previously treated with “fibrin glue” during surgical procedures (most fibrin glue products contain bovine thrombin). This acquired inhibitor prolongs the (bovine-derived reagent) thrombin time, but does not prolong the reptilase time. A thrombin time using human-derived thrombin reagent will generally not be prolonged but can be in the presence of a bovine thrombin inhibitor.

Thrombin Time Assay Principle

When bovine thrombin is added to test plasma, fibrinopeptides A and B are cleaved from plasma fibrinogen to create fibrin monomer, which immediately polymerizes to form a detectable clot. The time interval from addition of thrombin to clot formation is measured.

Reptilase Time Assay Principle

Reptilase, a reagent derived from the venom of Bothrops atrox, cleaves fibrinopeptide A, but not fibrinopeptide B, from the fibrinogen molecule resulting in clot formation. The time interval from addition of reptilase reagent to clot formation is measured.

Assay Performance Characteristics

TT and reptilase time tests are both qualitative assays of fibrinogen function and do not always indicate a specific disorder; thus additional testing may be required for clarification of prolongation in test results.
Reptilase is a thrombin-like enzyme. The reptilase clotting time may be used in place of or in conjunction with the TT to measure fibrin formation. In contrast to thrombin, reptilase is not affected by the presence of heparin, heparinoids or hirudin and may be a useful tool in evaluating test plasma for their presence. A prolonged thrombin time associated with a normal reptilase time, suggesting heparin therapy or contamination, may be further clarified by the addition of protamine sulfate or another heparin neutralizer to the test plasma resulting in normalization of the thrombin time result in heparin's presence.

**Thrombin Time Mixing Studies**

To distinguish fibrinogen deficiency from something inhibiting fibrin polymerization, the test plasma is mixed 1:1 with normal platelet-free plasma and a thrombin time is performed on the mixture. If the prolongation is not corrected, the presence of heparin, direct thrombin inhibitor, FDPs or paraproteins is suspected. To confirm the presence of FDPs, a D-dimer assay should be performed. The presence of plasma paraproteins is confirmed through serum protein electrophoresis and may be associated with hematologic disorders such as multiple myeloma.

If the TT on the 1:1 mixture is corrected to normal or near normal, fibrinogen deficiency or dysfunction may be confirmed by the combination of the clot-based fibrinogen activity assay and the fibrinogen antigen immunoassay.

### Reference Range

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin Time, bovine</td>
<td>8.0 – 14.0 seconds</td>
</tr>
<tr>
<td>Thrombin Time, human</td>
<td>10.2 – 12.7 seconds</td>
</tr>
<tr>
<td>Thrombin Time Mixing Studies</td>
<td>8.0 – 14.0 seconds</td>
</tr>
<tr>
<td>Reptilase Time</td>
<td>13.5 – 19.5 seconds</td>
</tr>
</tbody>
</table>

### Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. The plasma must be platelet free (platelet count <10 x 10⁹/L). Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

### Test Request Information

The thrombin time (#300807), the thrombin mixing studies (#300813), and the reptilase time (#300610) may be ordered separately from the Esoterix Service Directory. CPT code: thrombin time 85670, thrombin time mixing studies 85670 X 3, and reptilase time 85635.

### Reflex Recommendations

The TT should be ordered when both the PT and the APTT are prolonged. A reptilase time may be performed to evaluate for the presence or absence of unfractionated heparin, or a bovine thrombin inhibitor. The presence of unfractionated heparin is further elucidated by adding a heparin neutralizer and retesting or by performing a heparin assay. If the TT is prolonged and heparin is not present, a fibrinogen activity and
fibrinogen antigen may be used to confirm and quantitate afibrinogenemia, hypofibrinogenemia and dysfibrinogenemia. If DIC is suspected, D-dimer and/or FDP assays may be used for elucidation. If a bovine thrombin (anti-IIa) inhibitor is suspected, a thrombin time using human-derived thrombin as the reagent should be performed. If a paraprotein disorder is suspected, a serum protein electrophoresis may be used as a diagnostic tool.

References

**Physical Characteristics**

Tissue plasminogen activator (tPA) is a serine protease synthesized in the endothelium of the vasculature. TPA exists as one and two chain molecules, both forms being capable of activating plasminogen. Single chain tPA has a molecular weight of 70,000 Daltons and a short half-life of two to six minutes.

**Physiology**

Tissue plasminogen activator is a major regulator in the fibrinolytic system and it physiologically serves to activate plasminogen to plasmin. Plasmin then degrades fibrin to soluble fibrin degradation products. TPA is stored in the endothelial cells and is rapidly released following exercise, venous occlusion, and injection of substances such as epinephrine and DDAVP. TPA is primarily inhibited by plasminogen activator inhibitor-1 (PAI-1).

**Clinical Significance**

In the resting state, tPA is present at very low levels and these basal levels are not an accurate reflection of gene expression. tPA is released from the endothelial cell following stimulation. In the evaluation of the role that tPA plays in the fibrinolytic response, analysis is usually performed on plasma samples obtained after some form of stimulation. Diminished tPA response post stimulation has been reported in individuals and families with a history of venous thromboembolic disease. In a review by Brandt, he notes that it cannot be certain if this represents a true hereditary defect or is an acquired trait related to the venous thrombosis. Brandt also notes that there have been no reports of documented mutations in the tPA gene leading to tPA deficiency. Other studies have been unable to demonstrate abnormalities of tPA synthesis or release in families with a history of venous thrombosis.

**Tissue Plasminogen Activator Activity Assay Principle**

Sample tPA is captured by monoclonal antibodies coated on the microtest wells. The wells are washed, a chromogenic substrate is added, and the color development is proportional to functional tPA in the sample.

**Tissue Plasminogen Activator Activity Assay Performance Characteristics**

Results may be spuriously elevated due to tPA release during specimen procurement as may occur with prolonged or excessive use of a tourniquet during phlebotomy or following strenuous exercise. Plasminogen and fibrinogen do not interfere with the assay. Plasmas collected using routine EDTA or citrate blood collection tubes may show low levels of tPA due to the presence of PAI-1.

**Tissue Plasminogen Activator Antigen Assay Principle**

Tissue plasminogen activator contained in the sample is added to a microtest well coated with goat anti-tPA containing soluble non-immune goat IgG. After incubation, HRP labeled anti-tPA Fab fragments are added. These react with the bound tPA and the wells are washed to remove unbound conjugate. The peroxidase substrate is added and the amount of color produced is directly proportional to the quantity of tPA present in the sample.

**Tissue Plasminogen Activator and Antigen Performance Characteristics**

Results may be spuriously elevated due to tPA release during specimen procurement as may occur with prolonged or excessive use of a tourniquet during phlebotomy or following strenuous exercise. As the resting tPA antigen is low, more clinical information may be gained by evaluating levels pre and post stimulation (such as venous occlusion).
TISSUE PLASMINOGEN ACTIVATOR (cont.)

Tissue Plasminogen Activator Reference Range

The normal reference range for tPA activity is < 1.1 IU/mL and the normal reference range to tPA antigen is < 14.1 ng/mL.

Tissue Plasminogen Activator Activity Specimen Requirements

This assay requires that specimens be drawn into special tubes, specifically Stabilyte™ evacuated tubes as acidification of the plasma sample is necessary to obtain accurate results.

Plasma specimen collected in Stabilyte™ tubes: Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2500g for fifteen minutes. Transfer the plasma to a plastic centrifuge tube using a plastic pipette, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Tissue Plasminogen Activator Antigen Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. The plasma must be platelet free (platelet count < 10 x 10^9/L). If necessary, transfer the plasma to a plastic centrifuge tube using a plastic pipette, then recentrifuge for ten additional minutes. Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.

Test Request Information

tPA activity (#300134) and tPA antigen (#300125) can be ordered separately from the Esoterix Service Directory, CPT Code: 85415.

Reflex Recommendations

Elevations of tPA can be accompanied by elevations of other markers of cardiovascular or cerebrovascular disease including homocysteine, lipoprotein (a), factor VIIa and plasminogen activator inhibitor-1.

References

von Willebrand Disease Tests

Physical Characteristics

von Willebrand factor (vWF) is a glycoprotein synthesized in both the megakaryocyte and endothelial cell. In plasma, vWF circulates bound to factor VIII (FVIII) in a non-covalent fashion. vWF from the megakaryocyte is stored in the platelet alpha granules. Conversely, endothelial cell-produced vWF is stored in the cells’ Wiebel-Palade bodies.

vWF consists of a series of multimers that range in size from 500,000 to greater than 20 million Daltons. Multimer size is directly linked to hemostatic efficacy, the largest multimers have the highest functional activity. The half-life of von Willebrand factor in plasma is approximately 24 hours.

Physiology

vWF has two major functions. It serves as a carrier protein for FVIII in the circulation and stabilizes FVIII preventing its clearance. In effect, vWF increases the half-life of FVIII. vWF also mediates platelet adhesion to regions of endothelial damage, e.g. when there is exposure of the subendothelium. vWF forms a bridge between glycoprotein Ib on the platelet surface and subendothelial collagen, anchoring the platelet to the site of damage.

vWF is an acute phase reactant protein. Elevated levels of vWF antigen and activity can occur in a variety of conditions, including pregnancy, following administration of estrogen (e.g. hormone replacement therapy or oral contraceptive pills), during periods of inflammation, acute infection, post-surgery or following physical exercise or a stressful situation. Individuals with blood group O have less circulating vWF than persons with blood groups A, B, or AB. This is thought to be due to increased clearance of the von Willebrand protein in blood type O individuals compared to the other blood types. Due to these variables, a diagnosis of von Willebrand Disease (vWD) can be difficult and may require repeated laboratory analysis, knowledge of the patient’s blood type and clinical correlation.

Etiology of von Willebrand Disease

vWD is an inherited, or rarely acquired, bleeding disorder represented by a qualitative or quantitative deficiency of vWF. vWD is the most common inherited bleeding disorder with an incidence estimated at 1 in 100. Type 1 vWD is most commonly inherited in an autosomal dominant manner. Type 2 vWD may be inherited in a dominant or recessive manner. The less common severe type 3 vWD is inherited in an autosomal recessive manner in which the carriers (parents) are heterozygous for vWD and each passes along an abnormal von Willebrand gene.

vWD may also be an acquired condition. This can be due to reduced synthesis or enhanced clearance/proteolysis of vWF or the presence of an autoantibody to vWF. Autoimmune von Willebrand disease, in which antibodies are directed against vWF, is seen primarily in elderly patients with multiple myeloma, lymphoproliferative disorders, collagen vascular disease or may occur in those with no underlying disorder. Hypothyroidism has been associated with decreased vWF antigen and activity and a normal multimeric pattern. With treatment and the realization of euthyroidism, vWF results return to normal. Occasionally, acquired vWD is seen in patients with Wilms’ tumor, or congenital cardiovascular defects. In these situations, the vWF is apparently bound to the tumor or a portion of the vWF is consumed.

von Willebrand Disease Classification Based on Laboratory Evaluation

Before a diagnosis of vWD is made, a correlation with the patient’s clinical picture must be considered. The diagnosis should be made only in individuals with signs and symptoms consistent with the diagnosis. In 1994 reclassification of vWD was established by the International Society on Thrombosis and Haemostasis. This reclassification was an effort to simplify the previous classification system that contained more than 28 subtypes. The scheme listed below is based on the 1994 ISTH classification.

Type 1 vWD is characterized by a partial quantitative deficiency of vWF. vWF antigen, function, and FVIII activity are generally decreased in a proportional fashion. The multimeric fractions of vWF in plasma and platelets are normal with a typical distribution although typically reduced in quantity.
Type 2 vWD is characterized as qualitative deficiency of vWF. Type 2 vWD can be further sub-classified into types 2A, 2B, 2N and 2M based on comparison of vWF and FVIII levels, and multimeric analysis. In general type 2 vWD is characterized by a discordance between the vWF antigen and activity levels, with a typical ratio of antigen to activity of 2:1.

Type 2A vWD is characterized by the lack of large and intermediate multimers of vWF. Decreased concentrations of vWF antigen is observed but vWF activity is decreased to a greater degree. Results of collagen binding assay are typically abnormal yielding a ratio of vWF antigen to collagen binding activity less than 0.5. vWD subtype determination can be confirmed using molecular techniques to identify the gene mutation.

Type 2B vWD is characterized by qualitative variants with increased affinity for platelet glycoprotein Ib (Gp 1b). FVIII and vWF antigen show variable but normal to reduced concentrations in plasma. vWF activity is decreased to a greater degree. Results of the collagen binding are typically abnormal yielding a ratio of vWF antigen to collagen binding activity less than 0.5. Increased low dose ristocetin platelet aggregation (LDRIPA) is observed in vitro (see below). vWD subtype determination can be confirmed using molecular techniques to identify the gene mutation.

Type 2M vWD is characterized by decreased platelet-dependent function not caused by the absence of high molecular weight multimers. Multimeric analysis is essentially normal or may show unusual satellite banding patterns using high-resolution gels. FVIII and vWF antigen show variable but normal to reduced concentrations in plasma. vWF activity is decreased when evaluated by ristocetin agglutination but normal with the collagen-binding assay. vWD subtype determination can be confirmed using molecular techniques to identify the gene mutation.

Type 2N vWD refers to all qualitative variants with decreased affinity for FVIII. The vWF functions normally and is present in normal quantity but the protein cannot bind and therefore stabilize FVIII. In type 2N vWD, vWF antigen and activity are normal to reduced while FVIII is disproportionately decreased. Results of the collagen-binding assay are normal as is multimeric analysis. A vWD 2N binding assay is typically low. vWD subtype determination can be confirmed using molecular techniques to identify the gene mutation.

Type 3 vWD represents essentially a homozygous deficiency with virtually complete deficiency of vWF antigen, activity and FVIII. Type III is the most severe form of vWD. Multimeric analysis shows essentially absence of multimers.

Platelet-type pseudo von Willebrand disease is a rare disorder due to an abnormal GP1b receptor that spontaneously binds plasma vWF, particularly the high molecular weight forms. Clinically, these patients resemble type 2B vWD and in fact, it can be difficult to distinguish these subtypes. This can be done using DNA sequencing or a platelet-binding assay using the patient vWF and normal fixed platelets.

The laboratory abnormalities observed in acquired vWD vary and depend on the underlying etiology of the abnormal vWF. The presence of antibodies directed against vWF can be detect using mixing studies in only about 10% of patients with acquired vWD. Most commonly, the clinical picture resembles type 2 vWD, as acquired antibodies may cause rapid disappearance of the highest molecular weight multimers. In addition, in patients with cardiovascular defects or certain malignancies, the largest molecular weight multimers may be lost.

<table>
<thead>
<tr>
<th>vWF Type</th>
<th>VIII</th>
<th>vWF Activity Ristocetin</th>
<th>vWF Activity CBA</th>
<th>vWF Antigen CBA/Antigen Ratio</th>
<th>LDRIPA</th>
<th>Multimers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
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<td>↓</td>
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<td>↓</td>
<td>↓</td>
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<td>N</td>
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<td>N</td>
<td>Usually N</td>
</tr>
</tbody>
</table>

N = Normal    Abn = Abnormal

This table represents the typical pattern seen in the various forms of vWD.
Clinical Significance of von Willebrand Disease

Patients with vWD have marked variability in the severity of their bleeding episodes. This variability may exist between families, among the affected family members within a given family, or for an individual over time. Because of the variety and severity of symptoms, patients with vWD may experience their first bleeding episode at any age. Bleeding is generally characterized as mucosal in nature and patients typically suffer epistaxis, menorrhagia, gingival bleeding, easy bruising and exaggerated bleeding with trauma or surgical procedures. Epistaxis is the most common manifestation and often the first indication of a bleeding disorder in boys with vWD, while menorrhagia is commonly the first manifestation in girls. For heterozygous type 1 vWD patients, clinical manifestations are typically mild, but in type 2 vWD the bleeding may be substantial. Type 3 patients may present with severe symptoms and can sustain significant spontaneous bleeding. A severe deficiency can also be associated with post-surgical bleeding, hemarthrosis, or rarely, uncontrollable gastrointestinal hemorrhage.

Activity Assay Principle

This assay, formerly called the ristocetin cofactor activity assay, uses the patient’s plasma vWF to agglutinate formalin fixed platelets in the presence of ristocetin. By comparing the rate of agglutination against a normal reference curve, the vWF activity can be quantitated. The lower limit of precision for the assay is approximately 14% and values below this cannot be accurately determined.

Quantitative Immunoturbidometric (Latex Immunoassay [LIA]) Antigen Assay

This is the standard vWF antigen assay performed. Antibody-coated latex particles bind vWF protein in patient samples forming aggregates. Monochromatic light passed through the sample is absorbed in a manner proportionate to the degree of aggregation and, therefore, vWF antigen level in the test plasma.

Antigen Assay Principle - ELISA

The vWF antigen may be quantitated by an ELISA method. Immobilized rabbit anti-human vWF antibodies bind the vW antigen present and rabbit anti-vWF conjugated with an enzyme is added. The color production is directly proportional to the vWF antigen levels in the plasma specimen. The ELISA vWF antigen assay is typically performed only when the latex immunoassay antigen assay shows a very high result and possible interference is suspected.

Collagen Binding Assay Principle

This assay evaluates the ability of high molecular weight multimers present in the patient’s sample to bind type III or type IV collagen. Collagen is coated on microtiter plates and the amount of vWF bound is measured using peroxidase-conjugated antibodies to human vWF. Following the addition of substrate, color intensity is proportional to the high molecular weight multimers present in the sample. Results are expressed as percent collagen binding activity and as a ratio of percent collagen binding activity (CBA) to total vWF antigen. In type 2M vWD, the collagen binding activity is typically normal while the ristocetin activity is reduced.

2 N Assay Principle

Patient vWF is captured in a microtiter well coated with monoclonal antibody to vWF; the patient FVIII is essentially removed and the vWF allowed to bind to added recombinant FVIII. The bound recombinant FVIII is measured using a chromogenic assay. The patient vWF is independently quantitated by ELISA and compared to the quantity of bound FVIII, a ratio of FVIII to vWF is reported.
Multimers Assay Principle

vWF multimeric analysis is performed using SDS (sodium dodecyl sulfate)-agarose gel electrophoresis to separate the vWF multimers followed by detection with immunoblotting. The plasma is diluted in a buffer and the multimers are separated by electrophoresis in a 1.8% SDS-agarose gel. The vWF multimer bands are then transferred from the gel onto a nitrocellulose membrane using immunoblotting. Following the transfer, the membrane is incubated with a vWF antibody linked to HRP and then stained with a chromogenic substrate to reveal the multimeric bands. This Western Blot technique distinguishes high and low molecular weight multimers, allowing the identification of type 1 and type 2 vWD and in most instances, permitting accurate sub-classification of type 2 vWD.

Ristocetin-Induced Platelet Aggregation Assay Principle (LDRIPA)

Hyper-responsiveness to ristocetin-induced platelet aggregation may be seen in Type 2B and platelet-type vWD. Platelet rich plasma from these patients will induce hyper-aggregation in response to low dose ristocetin. Normal individuals will not demonstrate aggregation at this concentration of ristocetin. Testing for LDRIPA must be performed on a fresh sample that is less than 1 hour old.

Assay Performance Characteristics

Due to the variability of vWD, not all assay values will be abnormal at any given time. Repeat testing may be required to confirm the diagnosis.

von Willebrand Factor Assay Reference Ranges

The normal range for FVIII activity, von Willebrand factor activity, von Willebrand factor antigen and vWF collagen binding activity is 50 – 150%. The normal ratio for the collagen binding assay is 0.6 to 5.0.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Willebrand Factor Activity (Ristocetin Cofactor)</td>
<td>50 – 150%</td>
</tr>
<tr>
<td>Collagen Binding Activity</td>
<td>50 – 150%</td>
</tr>
<tr>
<td>Collagen Binding Ratio</td>
<td>0.6 – 5.0 ratio</td>
</tr>
<tr>
<td>von Willebrand Factor Antigen</td>
<td>50 – 150%</td>
</tr>
<tr>
<td>von Willebrand Factor Multimers</td>
<td>Normal pattern and distribution</td>
</tr>
<tr>
<td>Low-dose Ristocetin-induced Platelet Aggregation</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Specimen Requirements

Citrated plasma specimen: Collect blood into a blue-stopper collection tube containing 3.2% (0.109M) buffered sodium citrate. Ensure complete fill of the evacuated tube. Invert gently six times immediately after filling. Centrifuge the capped tube at 2000g for ten minutes. The plasma must be platelet free (platelet count <10 x 10^9/L). If necessary, transfer the plasma to a plastic centrifuge tube using a plastic pipette, then re-centrifuge for ten additional minutes. Transfer the plasma to a second plastic tube, seal, label, and freeze immediately at -20°C or lower. Ship the frozen specimen overnight on dry ice.
von Willebrand Disease Tests (cont.)

Test Request Information

von Willebrand Disease Profile (#300910) includes APTT, FVIII activity, vWF antigen, vWF activity, vWF collagen binding activity and collagen binding assay. APTT (#300040), FVIII activity (#300103), von Willebrand Factor Activity (#300114), von Willebrand Factor Antigen (#300113), collagen binding assay (300314) and von Willebrand Factor Multimers (#300117) can be ordered separately from the Esoterix Service Directory. Ristocetin-induced platelet aggregation is available on a local basis only. CPT codes: APTT 85730; FVIII activity 85240; von Willebrand Factor antigen 85246; von Willebrand Factor activity 85245; collagen binding assay 83520; and von Willebrand Factor multimers 85247.

Reflex Recommendations

vWD, especially type 2N, may be confused with milder forms of classic hemophilia. Patients with vWD often have decreased FVIII levels in addition to an expected decrease in vWF. As inheritance of vWD is autosomal, it affects both men and women whereas hemophilia is X-linked. Due to the inherited nature of this disorder, it is important to test family members of individuals who are diagnosed with vWD.

Therapy of von Willebrand Disease

The goal of therapy in patients with vWD is to bring the vWF and FVIII activity levels to hemostatically sufficient levels if they are decreased or dysfunctional. A variety of treatment modalities have been used. Since many factor replacement therapies carry a potential risk of viral transmission, other therapies are used initially when possible. The first choice in mild vWD is compression for a local bleed. Another agent, 1-deamino-8-D-arginine vasopressin (DDAVP), provides temporary benefit by inducing the release of von Willebrand factor from endothelial stores. This therapy is most effective in patients with type I vWD and in some persons with type II vWD. Type III vWD patients have limited or no response to DDAVP. One common drug that can be used as adjunctive therapy in the management of vWD is epsilon-aminocaproic acid (Amicar). This agent blocks the activity of plasmin. In addition, intermediate-purified FVIII concentrate preparations (such as Humate P) are given which contain both von Willebrand factor and FVIII including the largest hemostatically functioning von Willebrand factor multimers.

References

Activated Protein C Resistance – an acquired or inherited condition associated with an increased risk of venous thrombosis. It is most commonly associated with a mutation in the factor V gene. Patients with activated protein C resistance have limited in vitro anticoagulant response to activated protein C.

Activated partial thromboplastin time (APTT) - the time required for clot formation in recalcified plasma after the addition of contact activators, such as kaolin, ellagic acid, or silica, and platelet substitutes (phospholipids). Measures the factors in the intrinsic and common pathways of blood coagulation including factor XII, high molecular weight kininogen, prekallikrein, and factors XI, IX, VIII, X, V, prothrombin, and fibrinogen. The APTT is commonly used to monitor heparin therapy.

Aggregometry - a quantitative assessment of platelet cohesion in a photometer by the detection of changes in light transmission through a suspension of platelets in plasma. The light transmission pattern is analyzed in response to a variety of agonists.

Agonist - a substance that stimulates platelet aggregation.

Alpha granule - a platelet storage organelle that contains hemostatic proteins.

Anticoagulant - substances that interfere with the various mechanisms of clot formation can be endogenous or exogenous. Exogenous agents are used in the prophylaxis and treatment of blood clotting disorders. Includes the oral anticoagulants which inhibit synthesis of vitamin K-dependent clotting factors and parenteral agents such as heparin, low molecular weight heparin, and direct thrombin inhibitors.

Antiphospholipid antibodies - plasma IgG, IgM, or IgA antibodies directed against protein phospholipid complexes.

Alpha 2 antiplasmin - a major inhibitor of plasmin produced and secreted by hepatocytes and stored in platelet alpha granules.

Antithrombin - formerly called antithrombin III, ATIII. A naturally occurring anticoagulant that inactivates thrombin, coagulation factors Xa, IXa, XIa, and kallikrein. Antithrombin’s anticoagulant activity is enhanced 1000-fold in the presence of heparin.

AVK - Anti Vitamin K - see Warfarin

Atherosclerosis - the deposition of cholesterol and lipids within the lining of large and medium-sized arteries. A common form of arteriosclerosis, which is a group of diseases characterized by thickening and subsequent loss of elasticity of the arterial walls.

Beta thromboglobulin - a platelet specific alpha granule protein that can weakly neutralize heparin. This protein is used to monitor early platelet activation.

Bernard Soulier Syndrome - a rare congenital platelet disorder characterized by large gray platelets. Platelets from patients with this disorder demonstrate abnormal adhesion and no aggregation response to ristocetin even in the presence of von Willebrand factor.

Bethesda titer - titer units (BU) used to measure coagulation factor inhibitors. Persons with titers of less than 5 BUs are generally considered low responders, while individuals with titers of greater than 10 BUs are classified as high responders.

Bleeding time - the time for a standard wound inflicted, using a template device that creates a standard incision, to stop bleeding under constant intracapillary pressure. This test is an initial screen of platelet function, capillary integrity, and von Willebrand factor function.

Chromogenic Assay – also called amidolytic. An activity assay that utilizes a small substrate that fits the active site pocket and the enzyme to be measured. In general, is not phospholipid dependent and therefore is not sensitive to the presence of lupus anticoagulants.
GLOSSARY (cont.)

CLSI - Clinical Laboratory Standards Institute, formerly called NCCLS - National Committee for Clinical Laboratory Standards. This committee is made up of professionals, governmental staff, and industry representatives who create standards for the improvement of laboratory quality and efficiency.

Coagulation cascade - a theory of coagulation that explains in vitro fibrin clot formation as a series of interdependent enzymatic reactions occurring in a specific sequence or cascade.

Contact activation - a process that initiates the intrinsic pathway of coagulation through contact with a variety of negatively-charged surfaces.

Coumadin™ – See warfarin

Coumarin - a vitamin K antagonist administered orally that is routinely used in the clinical management of thrombosis.

D-dimer – a terminal degradation product of the fibrin clot. One of several peptides released as a result of the action of plasmin on fibrin.

Deep vein thrombosis - also known as DVT, the formation of a thrombus in the deep veins typically of the lower extremities. A common complication of DVT is pulmonary embolism.

DRVVT - Dilute Russell’s Viper Venom Time – a snake venom based assay that initiates clotting at the common pathway and uses a dilute concentration of phospholipid making it sensitive to the presence of lupus anticoagulants.

Disseminated intravascular coagulation - a syndrome associated with accelerated destruction and increased consumption of coagulation factors and platelets. Occurs secondarily to a variety of initiating events such as infection or malignancy and most commonly leads to hemorrhage but on rare occasions thrombotic complications.

ELISA – Enzyme Linked Immunosorbent Assay

Embolism - the abrupt blocking of a blood vessel by a clot or foreign material brought to the site through blood flow.

Emboli - a portion or entirety of a blood clot that breaks loose from a thrombus and travels with the flow of blood.

Extrinsic pathway – the clotting pathway initiated through activation of the clotting mechanism through expression of tissue factor. The only factor unique to this pathway is factor VII.

Factor II mutation - a G to A substitution at nucleotide position 20210 in the 3’ untranslated region of the prothrombin gene that leads to an increased risk of thrombosis.

Factor V mutation (Leiden) – a substitution of Gln for Arg in the 506 amino acid position of the factor V gene. This results in resistance to activated protein C and accompanying risk of thrombosis.

Fibrin - a protein formed from the precursor fibrinogen by the proteolytic activity of thrombin during clot formation. Fibrin is an essential part of the blood clot.

Fibrinogen - a blood coagulation factor also known as factor I that serves as a precursor of fibrin.

Fibrinolysis - the lysis of fibrin clot by enzymatic activity.
**Fibrinopeptide** - fibrinopeptide A and fibrinopeptide B are produced by the cleavage of fibrinogen by thrombin. One of the molecular markers of thrombosis, fibrinopeptide levels are an early indication of clot formation.

**G**

**Glycosaminoglycan** - a class of high molecular weight polysaccharides that have disaccharide repeating units containing N-acetylhexosamine and hexose or hexuronic acid. Heparin, heparan sulfate, chondroitin sulfate, and dermatan sulfate are included in this group.

**H**

**Haplotype** - refers to closely linked genetic markers that are inherited together.

**Hemophilia** - a group of inherited blood coagulation disorders characterized by decreased or dysfunctional clotting factors.

**Hemorrhage** - the escape of blood from an artery, vein, or capillary into the skin or surrounding area.

**Hemostasis** - the control of bleeding by the physiological processes of vasoconstriction, coagulation factors, and platelets or by surgical procedures that result in the preservation of blood vessel integrity and prevention of hemorrhage.

**Heparin** - one of the glycosaminoglycans used in the prevention and treatment of thromboembolism. Heparin's anticoagulant activity is the result of its interaction with antithrombin and other activated serine proteases and less importantly heparin cofactor II.

**HIT** - Heparin Induced Thrombocytopenia

**Heparin Cofactor II** - a thrombin inhibitor that in a manner similar to antithrombin, demonstrates activation in the presence of heparin.

**Heparin-associated thrombocytopenia (HAT)** - also known as heparin-induced thrombocytopenia. It may present as a nonimmune, self limiting form of thrombocytopenia or an immune based antibody - mediated, more severe form, and may be associated with thrombosis.

**Homocysteine** - a thiol containing non-essential amino acid that is produced by demethylation of methionine. Hyperhomocysteinemia is a risk factor for both arterial and venous thrombosis.

**HPA** - Human Platelet Antigen - this is the term used to refer to antigens expressed on the surface of platelets.

**I**

**Imune thrombocytopenia purpura** - a marked decrease in platelet number as a result of circulating antiplatelet antibodies. The disorder is acquired and may present acutely or in chronic form.

**Intrinsic pathway** - the portion of the coagulation cascade that initiates with factor XII activation by a negatively charged surface. Factors unique to the intrinsic pathway include factors XII, XI, IX and VIII.

**L**

**Lipoprotein(a)** - a low density lipoprotein containing structures similar to the plasminogen molecule. Increased lipoprotein(a) has been identified as an independent risk factor for coronary disease, stroke and venous thrombosis.

**Low molecular weight heparin (LMWH)** - a low molecular weight fraction of a heparin preparation that has more anti-Xa activity than anti-IIa and more predictable dose response than standard, unfractionated heparin.

**Lupus anticoagulant** – an acquired circulating inhibitor that causes prolongation of phospholipid dependent clotting assays. The lupus anticoagulant is an important cause of thrombosis, both venous and arterial.
**Plasmin** - a serine protease that is derived from the precursor plasminogen. Plasmin degrades a variety of coagulation factors including fibrinogen, and factors V and VIII.

**Plasminogen** - the precursor of plasmin that is cleaved by plasminogen activators to form the active serine protease plasmin.

**Plasminogen activator** - a group of serine proteases that cleave plasminogen to form plasmin. This group includes tissue plasminogen activator, streptokinase, and urokinase.

**Plasminogen activator inhibitor** - a class of glycoproteins known as serpins (serine protease inhibitors) that function to modulate the fibrinolytic system; includes PAI-1, PAI-2, PAI-3 and protease nexin.

**Platelet** - the cellular component of hemostasis produced in the bone marrow from the megakaryocyte.

**Platelet antibodies** - antibodies to platelet antigens that are IgG or IgM isotype and are called platelet associated immunoglobulin or PAIg. The presence of increased PAIg on the platelet surface is an indication of immune thrombocytopenia.

**Platelet antigen** - platelet specific antigens that are located on the platelet glycoproteins GPIa/IIa, GPIb/IX, and GP IIb/IIIa. Platelet antigen PL A1 is the most common antigen seen in alloimmune thrombocytopenia and in autoimmune thrombocytopenic purpura.

**Platelet factor 4** - a high-affinity heparin-binding protein that is stored in platelet alpha granules.

**Platelet rich plasma** - the preparation of whole blood by centrifugation to produce a supernatant fraction of plasma with a high platelet content.

**Polymorphism** – a genetic mutation with a frequency in the population of at least 1%.

**Protein C** - a vitamin K-dependent naturally occurring anticoagulant protein produced in the liver that, when enzymatically converted to activated protein C, cleaves factors Va and VIIIa.

**Protein S** - a vitamin K-dependent naturally occurring anticoagulant glycoprotein produced in the liver that serves as a cofactor to the activities of protein C.

**Prothrombin** - a central coagulation factor, known as factor II, which is converted to thrombin.

**Prothrombin time** - an assay to detect the deficient activity of coagulation factors of the extrinsic or tissue factor pathway including factors VII, X, V, II, and fibrinogen. Because it is sensitive to deficiencies of factor II, VII, and X, the prothrombin time is the most common assay used to monitor warfarin therapy.

**Pulmonary embolism** - the sudden occlusion of the pulmonary artery or its branches by an embolus.

**Russell’s Viper Venom (Stypven)** - tissue thromboplastin derived from snake venom than can activate the coagulation cascade at factor X. This reagent is used to detect deficiency of factor X.

**Serine protease** - proteins whose enzymatic activity selectively hydrolyzes peptide bonds containing arginine or lysine. Typically, these enzymes circulate in a precursor or zymogen form, being regulated by specific activators and inhibitors.

**Single nucleotide polymorphism** – a common genetic mutation involving a single nucleotide and therefore altering the amino acid encoded.
Sodium citrate - the anticoagulant contained in the standard evacuated tubes used for coagulation testing. When evacuated tubes are filled to proper volume, they contain the recommended ratio of 9 parts blood to 1 part anticoagulant. Usually present at 3.2% or 3.8% concentration. A 3.2% concentration is generally recommended.

Thrombin - the enzyme derived from the precursor prothrombin that converts fibrinogen to fibrin.
Thrombin Time - the period of time required for fibrinogen to generate a fibrin clot after the addition of thrombin.
Thrombocytopenia - a decrease in the platelet count typically defined as a platelet count below 150,000/mm3.
Thromboembolism - an obstruction of a vessel formed by a thrombus carried by the blood stream from the site of origin.
Thrombolysis - the lysing of thrombi by a series of events, most significantly involving the action of plasmin on the thrombus.
Thromboxane - a potent aggregating substance formed from arachidonic acid through the cyclo-oxygenase pathway.
Thrombus - a gelatinous blood clot formed by the aggregation of platelets and fibrin which may also contain other elements including red blood cells. Thrombi commonly cause vascular obstruction at the point of formation.

von Willebrand Disease - an autosomal bleeding disorder characterized by quantitative or qualitative decreases in von Willebrand factor. The typical hemorrhagic manifestation is bleeding from mucous membranes.

Warfarin - a coumarin derivative administered orally for the prevention of thrombosis. Coumadin™ is a brand name of this compound. This is the most common form of anti-vitamin K therapy administered in North American.
APPENDICES

Coagulation Assays Affected by Anticoagulants  A

Pediatric Reference Ranges (Diagnostica Stago)  B

Component Tests of Coagulation Profiles  C

Glossary of Assays containing the term Factor X (FX)  D

Assay Menu by Disease or Testing Category  E
### Appendix A

## Coagulation Assays Affected by Anticoagulants

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>UNFRACTIONATED HEPARIN (UFH)</th>
<th>LOW MOLECULAR WEIGHT HEPARIN (LMWH)</th>
<th>COUMADIN Anti-Vitamin K</th>
<th>DIRECT THROMBIN INHIBITOR*</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clot-based assays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRVVT confirm</td>
<td>Reagent contains heparin neutralizer*</td>
<td>No effect on ratio</td>
<td>Frequently causes weakly positive ratio result spuriously</td>
<td>Prolongs</td>
<td></td>
</tr>
<tr>
<td>PNP</td>
<td>Spurious (false) positive</td>
<td>May cause false positive</td>
<td>No effect</td>
<td>Pre-neutralization APTT likely above measurable range</td>
<td></td>
</tr>
<tr>
<td>Hexagonal phase phospholipid</td>
<td>Reagent contains heparin neutralizer**</td>
<td>May increase</td>
<td>No effect</td>
<td>Pre-neutralization APTT likely above measurable range</td>
<td></td>
</tr>
<tr>
<td>Kaolin clotting time</td>
<td>Prolongs</td>
<td>May prolong</td>
<td>Prolongs</td>
<td>Prolongs</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>
</tr>
<tr>
<td>Antithrombin activity*</td>
<td>Usually decreased physiologically</td>
<td>May decrease levels physiologically</td>
<td>May increase</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Protein C activity* Chromogenic</td>
<td>No effect</td>
<td>No effect</td>
<td>Activity is decreased but is over-estimated due to PIVKA* interference</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Protein S or Protein C clot-based activity*</td>
<td>May increase spuriously; some reagents contain heparin neutralizer**</td>
<td>May increase spuriously</td>
<td>Activity is decreased physiologically</td>
<td>Increases result spuriously</td>
<td>Spuriously increased in the presence of lupus anticoagulant (LA) PS decreases in pregnancy and with endogenous estrogen therapy</td>
</tr>
<tr>
<td>APCR with FV deficient (2nd generation assay)</td>
<td>Reagent contains heparin neutralizer**; May increase ratio (false negative)</td>
<td>May increase ratio (false negative)</td>
<td>No effect</td>
<td>May increase ratio (false negative) LA – false decrease</td>
<td></td>
</tr>
<tr>
<td>APCR no FV deficient (1st generation assay)</td>
<td>May increase ratio (false negative)</td>
<td>May increase ratio (false negative)</td>
<td>May increase ratio (false negative)</td>
<td>May increase ratio (false negative)</td>
<td>Ratio may be falsely low in the presence of LA</td>
</tr>
<tr>
<td><strong>Immunogenic assays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein C or Protein S antigen*</td>
<td>No effect</td>
<td>No effect</td>
<td>Decreases physiologically</td>
<td>No effect</td>
<td>PS decreases in pregnancy and with endogenous estrogen therapy</td>
</tr>
<tr>
<td>Antithrombin II antigen*</td>
<td>Usually decreased physiologically</td>
<td>May decrease levels physiologically</td>
<td>May increase physiologically</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Other antiphospholipid antibodies* (ACA, APTS, B2GP1)</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Titters may fluctuate over time</td>
</tr>
</tbody>
</table>

* Levels may be decreased following an acute thrombotic event secondary to “consumption”.
# Proteins induced in vitamin K absence/antagonism
++ Most heparin neutralizers will neutralize up to 1 U/mL of UFH. Heparin levels of > 1 U/mL in plasma may affect some of these tests.
Appendix B

**PEDIATRIC COAGULATION RANGE**

### Reference Values for Coagulation Screening Tests in Infants, Children, and Adults†

<table>
<thead>
<tr>
<th>Test</th>
<th>1 Day</th>
<th>1 Month</th>
<th>6 Months</th>
<th>1-5 Years</th>
<th>6-10 Years</th>
<th>11-16 Years</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT</td>
<td>13.0 ± 1.43</td>
<td>11.8 ± 1.25</td>
<td>12.3 ± 0.79</td>
<td>11.0 (10.6-11.4)</td>
<td>11.1 (10.1-12.1)</td>
<td>11.2 (10.2-12.0)</td>
<td>12.0 (11.0-14.0)</td>
</tr>
<tr>
<td>INR</td>
<td></td>
<td></td>
<td></td>
<td>1.0 (0.96-1.04)</td>
<td>1.01 (0.91-1.11)</td>
<td>1.02 (0.93-1.10)</td>
<td>1.10 (1.00-1.30)</td>
</tr>
<tr>
<td>TCT</td>
<td>23.5 ± 2.38</td>
<td>24.3 ± 2.44</td>
<td>25.5 ± 2.86</td>
<td></td>
<td></td>
<td></td>
<td>25.0 ± 2.66</td>
</tr>
<tr>
<td>aPTT</td>
<td>42.9 ± 5.80</td>
<td>40.4 ± 7.42</td>
<td>35.5 ± 3.71</td>
<td>30.0 (24-36)</td>
<td>31 (26-36)</td>
<td></td>
<td>32 (26-37)</td>
</tr>
</tbody>
</table>

### Reference Values for Coagulation Factor Assays in Infants, Children, and Adults‡

<table>
<thead>
<tr>
<th>Factor</th>
<th>1 Day</th>
<th>1 Month</th>
<th>6 Months</th>
<th>1-5 Years</th>
<th>6-10 Years</th>
<th>11-16 Years</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>283 ± 58</td>
<td>270 ± 54</td>
<td>251 ± 68</td>
<td>276 (170-405)</td>
<td>279 (157-400)</td>
<td>300 (154-448)</td>
<td>278 (156-400)</td>
</tr>
<tr>
<td>II</td>
<td>48 ± 11</td>
<td>68 ± 17</td>
<td>88 ± 14</td>
<td>94 (71-116)</td>
<td>88 (67-107)</td>
<td>83 (61-104)</td>
<td>108 (70-146)</td>
</tr>
<tr>
<td>V</td>
<td>72 ± 18</td>
<td>98 ± 18</td>
<td>91 ± 18</td>
<td>103 (79-127)</td>
<td>90 (63-116)</td>
<td>77 (55-99)</td>
<td>106 (62-150)</td>
</tr>
<tr>
<td>VIII</td>
<td>66 ± 19</td>
<td>90 ± 24</td>
<td>87 ± 20</td>
<td>82 (53-116)</td>
<td>85 (52-120)</td>
<td>83 (58-115)</td>
<td>105 (67-143)</td>
</tr>
<tr>
<td>VIII</td>
<td>100 ± 39</td>
<td>91 ± 33</td>
<td>73 ± 18</td>
<td>90 (59-142)</td>
<td>95 (58-132)</td>
<td>92 (53-131)</td>
<td>99 (50-149)</td>
</tr>
<tr>
<td>IX</td>
<td>53 ± 19</td>
<td>51 ± 15</td>
<td>86 ± 25</td>
<td>73 (47-101)</td>
<td>75 (63-89)</td>
<td>82 (59-122)</td>
<td>109 (55-163)</td>
</tr>
<tr>
<td>X</td>
<td>40 ± 14</td>
<td>59 ± 14</td>
<td>78 ± 20</td>
<td>88 (58-116)</td>
<td>75 (55-101)</td>
<td>79 (50-117)</td>
<td>106 (70-152)</td>
</tr>
<tr>
<td>XI</td>
<td>38 ± 14</td>
<td>53 ± 13</td>
<td>86 ± 24</td>
<td>97 (56-150)</td>
<td>86 (52-120)</td>
<td>74 (50-97)</td>
<td>97 (67-127)</td>
</tr>
<tr>
<td>XII</td>
<td>53 ± 20</td>
<td>49 ± 16</td>
<td>77 ± 19</td>
<td>93 (64-129)</td>
<td>92 (60-140)</td>
<td>81 (34-137)</td>
<td>108 (52-164)</td>
</tr>
<tr>
<td>PK</td>
<td>37 ± 16</td>
<td>57 ± 17</td>
<td>85 ± 15</td>
<td>95 (63-130)</td>
<td>99 (66-131)</td>
<td>99 (53-145)</td>
<td>112 (62-162)</td>
</tr>
<tr>
<td>HK</td>
<td>54 ± 24</td>
<td>77 ± 22</td>
<td>82 ± 23</td>
<td>98 (64-132)</td>
<td>93 (60-130)</td>
<td>91 (63-119)</td>
<td>92 (50-136)</td>
</tr>
<tr>
<td>vWF Ag</td>
<td>15 ± 67</td>
<td>128 ± 59</td>
<td>107 ± 45</td>
<td>82 (60-120)</td>
<td>95 (44-144)</td>
<td>100 (46-153)</td>
<td>92 (50-158)</td>
</tr>
</tbody>
</table>

### Reference Values for Coagulation Inhibitors (%)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>N = 21</th>
<th>N = 22</th>
<th>N = 30</th>
<th>N = 45</th>
<th>N = 55</th>
<th>N = 90</th>
<th>N = 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin</td>
<td>76 (58-90)</td>
<td>74 (60-89)</td>
<td>109 (72-134)</td>
<td>116 (101-131)</td>
<td>114 (95-134)</td>
<td>111 (96-126)</td>
<td>96 (66-124)</td>
</tr>
<tr>
<td>PC Chromogenic</td>
<td>36 (24-44)</td>
<td>44 (28-54)</td>
<td>71 (31-112)</td>
<td>96 (65-127)</td>
<td>100 (71-129)</td>
<td>94 (66-118)</td>
<td>104 (74-164)</td>
</tr>
<tr>
<td>PC Clotting</td>
<td>32 (24-40)</td>
<td>33 (24-51)</td>
<td>77 (28-124)</td>
<td>94 (50-134)</td>
<td>94 (64-125)</td>
<td>88 (59-112)</td>
<td>103 (54-166)</td>
</tr>
<tr>
<td>PS Clotting</td>
<td>36 (28-47)</td>
<td>49 (33-67)</td>
<td>102 (29-162)</td>
<td>101 (67-136)</td>
<td>106 (64-154)</td>
<td>103 (65-140)</td>
<td>75 (54-103)</td>
</tr>
</tbody>
</table>

† All values are expressed in seconds. Values are expressed as the mean ± 1 SD for ages 1 day to 6 months and as the mean followed by the range encompassing 95% of normal controls for ages 1 year to adult.

‡ All of the above factors are expressed as % in normal plasma except for fibrinogen, which is expressed as mg/dL.

◆ Pediatric values have reached adult levels.

* Published reference range is significantly different from range used at Esoterix Coagulation. All other published reference ranges are similar.

**Abbreviations:** aPTT, activated partial thromboplastin time; HK, high molecular weight kininogen; INR, international normalized ratio; PK, prekallikrein; PC, protein C; PS, protein S; PT, prothrombin time; TCT, thrombin clotting time; vWF, von Willebrand factor.

ESOTERIX- COLORADO COAGULATION • 3176 S. Peoria Court • Aurora CO 80014-3114 • Phone 303-399-3336 • 1-800-444-9111 • Fax 303-399-3338 Rev. Feb. 2006
### Appendix B

**PEdiatric Coagulation Range**

#### Reference Values for Inhibitors of Coagulation in Infants, Children, and Adults++

<table>
<thead>
<tr>
<th>Antigen</th>
<th>1 Day</th>
<th>1 Month</th>
<th>6 Months</th>
<th>1-5 Years</th>
<th>6-10 Years</th>
<th>11-16 Years</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antithrombin</td>
<td>63 ± 12</td>
<td>78 ± 15</td>
<td>104 ± 10◆</td>
<td>111 (82-139)</td>
<td>111 (90-131)</td>
<td>105 (77-132)</td>
<td>100 (74-126)</td>
</tr>
<tr>
<td>Heparin Co-Factor II</td>
<td>43 ± 25</td>
<td>47 ± 20</td>
<td>120 ± 35</td>
<td>88 (48-128)#</td>
<td>86 (40-132) #</td>
<td>91 (53-129) #</td>
<td>108 (66-126)</td>
</tr>
<tr>
<td>Protein C</td>
<td>35 ± 9</td>
<td>43 ± 11</td>
<td>59 ± 11</td>
<td>66 (40-92)#</td>
<td>69 (45-93)#</td>
<td>83 (55-111)#</td>
<td>96 (64-128)</td>
</tr>
<tr>
<td>Protein S Total</td>
<td>36 ± 12</td>
<td>50 ± 14</td>
<td>87 ± 16◆</td>
<td>86 (54-118)</td>
<td>78 (41-114)</td>
<td>72 (52-92)</td>
<td>81 (60-113)</td>
</tr>
<tr>
<td>Protein S Free</td>
<td></td>
<td></td>
<td></td>
<td>45 (21-69)◆</td>
<td>42 (22-52)◆</td>
<td>38 (26-55)◆</td>
<td>45 (27-61)◆</td>
</tr>
</tbody>
</table>

#### Reference Values for Fibrinolytic Tests in Infants, Children, and Adults++++

<table>
<thead>
<tr>
<th>Test</th>
<th>1 Day</th>
<th>1 Month</th>
<th>6 Months</th>
<th>1-5 Years</th>
<th>6-10 Years</th>
<th>11-16 Years</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGN Act</td>
<td>50% ± 16</td>
<td>50% ± 16</td>
<td>90% ± 20</td>
<td>98 (78-118)</td>
<td>92 (75-108)</td>
<td>86 (68-103)</td>
<td>99 (70-130)</td>
</tr>
<tr>
<td>t-PA Ag</td>
<td></td>
<td></td>
<td></td>
<td>2.15 (1.0-4.5)</td>
<td>2.42 (1.0-5.0)</td>
<td>2.16 (1.0-4.0)</td>
<td>4.9 (0.5-14)</td>
</tr>
<tr>
<td>α2-AP</td>
<td>85 ± 15</td>
<td>100 ± 12</td>
<td>111 ± 21</td>
<td>105 (93-117)</td>
<td>99 (89-110)</td>
<td>98 (78-118)</td>
<td>102 (80-150)</td>
</tr>
<tr>
<td>PAI Act</td>
<td>5.42 (1-10)◆</td>
<td>6.79 (2-12)◆</td>
<td>6.07 (2-10)◆</td>
<td>3.6 (0-11)◆</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

++ All values are expressed as the mean ± 1 SD in % normal plasma.
++++ Values are expressed as the mean followed by the range encompassing 95% of the normal controls for ages 1 year to adult, and as the mean ± 1 SD for ages 1 day to 6 months. PGN and α2-AP are expressed as % normal plasma. t-PA values are expressed as nanograms per milliliter. Values for PAI are given as units per milliliter.
# Published reference range is significantly different from adults.
◆ Values are significantly different from adults.

Abbreviations: α2-AP, α2-antiplasmin; PAI, plasminogen activator inhibitor; PGN, plasminogen; t-PA, tissue-type plasminogen activator.

References:
COMPONENT TESTS OF COAGULATION PROFILES

Abnormal PT or APTT Reflexive Evaluation (300908)

- APTT (300040)
- PT/INR (300080)
- Thrombin Time (300807)

(Additional assays will be performed as necessary to determine the cause of the abnormality)

Antiphospholipid Syndrome Comprehensive (300918)

- APTT (300040)
- APTT Mixing Studies (300806)
- Anticardiolipin Antibody, IgG/M/A (300034)
- Anti B-2 Glycoprotein I, IgG/M/A (300166)
- Antiphosphatidylserine, IgG/M (300153)
- Antiprothrombin Ab, IgG/M (300230)
- Dilute Russell’s Viper Venom Test (300057)
- Hexagonal Phospholipid Neutralization (300144)
- Platelet Neutralization Procedure (300805)

Bleeding Diathesis With A Normal APTT/PT (300909)

- Factor VIII Activity (300103)
- Factor IX Activity (300104)
- Factor XI Activity (300106)
- von Willebrand Factor Antigen (300113)
- von Willebrand Factor Activity (300114)
- Factor XIII Activity (300108)
- PAI-1 Activity (300126)
- Euglobulin Lysis Time (300403)
- Alpha 2 Antiplasmin (300039)

Extrinsic Pathway Evaluation (300920)

- Factor II Activity (300100)
- Factor V Activity (300101)
- Factor VII Activity (300102)
- Factor X Activity (300105)
Fibrinogen Evaluation (300925)
- Fibrinogen Activity (300200)
- Fibrinogen Antigen (300199)
- Reptilase Time (300610)
- Thrombin Time (300807)
- Thrombin Time Mixing Studies (300813)

Fibrinolysis Evaluation (300913)
- alpha-2-Antiplasmin (300039)
- D-Dimer Quantitative (300415)
- Euglobulin Lysis Time (300403)
- FDP (300201)
- PAI-1 Activity (300126)
- Plasminogen Activity (300400)
- tPA Antigen (300125)

Heart Disease/Stroke Risk (300917)
- Fibrinogen Activity (300200)
- hsCRP (300312)
- Homocysteine (300727)
- Lipoprotein (a) (300715)
- von Willebrand Factor Antigen (300113)

Heparin Antibody Evaluation (300935)
- Serotonin Release Assay (300082)***
- Heparin Induced Plt Ab (300522)

Intrinsic Pathway Evaluation (300921)
- Factor VIII Activity (300103)
- Factor IX Activity (300104)
- Factor XI Activity (300106)
- Factor XII Activity (300107)
Lupus Anticoagulant/Antiphospholipid (300906)

- APTT (300040)
- APTT Mixing Studies (300806)
- PT/INR (300080)
- Anticardiolipin Antibody, IgG/M (300165)
- DRVVT (300057)
- Thrombin Time (300807)
- Hexagonal Phospholipid Neutralization (300144)

Lupus Anticoagulant Confirmatory (300916)

- APTT (300040)
- APTT Mixing Studies (300806)
- PT/INR (300080)
- Anticardiolipin Antibody, IgG/M (300165)
- DRVVT (300057)
- Hexagonal Phospholipid Neutralization (300144)
- Platelet Neutralization Procedure (300805)
- Thrombin Time (300807)

Menorrhagia Evaluation (300934)

- APTT (300040)
- PT/INR (300080)
- Factor VIII Activity (300103)
- von Willebrand Factor Activity (300114)
- von Willebrand Factor Antigen (300113)
- Factor XI Activity (300106)

Oral Contraceptive/HRT Thrombotic Risk (300933)

- Antithrombin Activity (300030)
- Factor II Gene Mutation (120736)
- Factor V Leiden (120719)
- Protein C Activity (300035)
- Protein S Antigen, Free (300087)
Protein S Evaluation (300928)

- C4b binding protein *RUO* (300160)
- Protein S Activity (300088)
- Protein S Antigen, Free (300087)
- Protein S Antigen, Total (300038)

Recurrent Miscarriage/Fetal Demise (300915)

- APTT (300040)
  (Reflex to APTT Mixing Study if APTT is abnormal)
- Anti B-2 Glycoprotein I, IgG/M/A (300166)
- Anticardiolipin Antibody, IgG/M/A (300034)
- Antiphosphatidylserine, IgG/M (300153)
- Antithrombin Activity (300030)
- DRVVT (300057)
- Factor II Gene Mutation (120736)
- Factor V Leiden (120719)
- Hexagonal Phospholipid Neutralization (300144)
- Homocysteine (300727)
- Protein C Activity (300035)
- Protein S Antigen, Free (300087)

ThrombAssureSM (129260)

- Factor II Gene Mutation (120736)
- Factor V Leiden (120719)

ThrombAssureSM Inherited (300932)

- Factor II Gene Mutation (120736)
- Factor V Leiden (120719)
- Antithrombin Activity (300030)
- Protein C Activity (300035)
- Protein S Antigen, Free (300087)
APPENDIX C (cont.)

**ThrombAssureSM Enhanced (300931)**
- Factor II Gene Mutation (120736)
- Factor V Leiden (120719)
- Anticardiolipin Antibody, IgG/M/A (300034)
- Antithrombin Activity (300030)
- DRVVT (300057)
- Hexagonal Phospholipid Neutralization (300144)
- Homocysteine (300727)
- Protein C Activity (300035)
- Protein S Antigen, Free (300087)

**Thrombotic Marker (300907)**
- D-Dimer Quantitative (300415)
- Fibrin Monomer (300202)
- Prothrombin Fragment 1+2 (300718)
- Thrombin-Antithrombin Complex (300714)

**Venous Thrombosis, Hypercoagulability (300901)**
- APC-Resistance (300716)
  (Reflex to FV Leiden if APCR is abnormal)
- Anticardiolipin, IgG/M (300165)
- Antithrombin Activity (300030)
- DRVVT (300057)
- Factor II Gene Mutation G20210A (120736)
- Hexagonal Phospholipid Neutralization (300144)
- Homocysteine (300727)
- Protein C Activity (300035)
- Protein S Antigen, Free (300087)
- Factor VIII Activity (300103)

**Venous Thrombosis, Hypercoagulability (300903)**
(Patients on Anti-vitamin K Therapy)
- APC-Resistance (300716)
  (Reflex to FV Leiden if APCR is abnormal)
Anticardiolipin, IgG/M (300165)
Antithrombin Activity (300030)
Dilute Russell's Viper Venom Test (300057)
Factor II Gene Mutation G20210A (120736)
Hexagonal Phospholipid Neutralization (300144)
Homocysteine (300727)
Protein C Antigen (300036)
Protein S Antigen, Total (300038)
Factor VII Antigen (300112)
Protein C Ag/Factor VII Ag Ratio (300067)
Protein S Ag/Factor VII Ag Ratio (300059)
Factor VIII Activity (300103)

Venous Thrombosis Appended (300905)
Factor IX Activity (300104)
Factor XI Activity (300106)
Fibrinogen Activity (300200)
Fibrinogen Antigen (300199)
HR2 Haplotype (300734)
Lipoprotein (a) (300715)
Plasminogen Activity (300400)

von Willebrand Disease Evaluation (300910)
APTT (300040)
Collagen Binding Assay (300314)
Factor VIII Activity (300103)
von Willebrand Factor Activity (300114)
von Willebrand Factor Antigen (300113)
APPENDIX D

Assays Containing the Term Factor X

Please see below for brief descriptions of all the different assays offered by Esoterix Coagulation involving Factor X.

300105 (Factor X Activity) – Clot based assay measuring the amount of functional factor X in plasma.

300206 (Factor X Antigen) – ELISA based assay measuring immunologic level of factor X quantity in a sample. (Measures only quantity of factor X in plasma, not amount that is functional.)

300211 (Factor X Chromogenic) – A factor X activity assay based on chromogenic methodology rather than the typical clotting based activity assay. Does not show Lupus Anticoagulant (LA) interference and can be used to monitor oral anticoagulant therapy in patients having a lupus anticoagulant. – Therapeutic range: 20-44%.

300215 (Factor X Chromogenic) - Chromogenic based assay measuring the amount of functional factor X in a sample. (Used if chromogenic method desired to measure X activity in individuals without Lupus Anticoagulant– Reference range: 75-150%)

300600 (Heparin Anti-Xa UFH) – Assay used to monitor unfractionated heparin therapy based on the heparin’s ability to inhibit factor Xa in the presence of antithrombin.

300602 (Heparin Anti-Xa LMWH) – Assay used to monitor Low Molecular Weight Heparin therapy based on the heparin’s ability to inhibit factor Xa in the presence of antithrombin.

300864 (Heparin Anti-Xa Fondaparinux) (Arixtra-brand name) – Assay used to monitor Fondaparinux therapy based on the drug’s ability to inhibit factor Xa in the presence of antithrombin.

(All of the anti-Xa assays are done by adding factor X in excess to see how much can be inhibited by the drug, they do not in any way measure the level of factor X in a patient’s plasma.)
Key Capabilities

Antiphospholipid Antibody Detection
- Anticardiolipin Antibody
- Anti-Phosphatidylserine Antibody
- beta-2 Glycoprotein I Antibody
- Anti-Prothrombin Antibody
- Anti-Annexin-V Antibody

Antiplatelet Antibody Detection
- Heparin-Induced Antibody
- Heparin-Induced Antibody Titer
- Serotonin Release Assay
- Platelet Antibody Screen (Indirect)
- Platelet Antibody (Direct)
- Platelet Specific Antibody
- Platelet Antibody Drug-Induced

Aspirin Resistance
- ASPIRINcheck™: 11-dehydro Thromboxane B2/creatinine ratio

Cardiovascular Risk
- 11-dehydro Thromboxane B2/Creatinine ratio
- Activated Partial Thromboplastin Time
- PL-A Polymorphism
- C-reactive Protein, High Sensitivity
- Factor VII Activity
- Fibrinogen Activity
- Homocysteine
- Lipoprotein (a)
- Plasminogen Activator Inhibitor 1 Activity
- von Willebrand Factor Antigen

Factor Analysis
- Factor II Activity
- Factor V Activity
- Factor VII Activity and Antigen
- Factor VIII Activity and Antigen
- Factor VIII Chromogenic Assay
- Factor IX Activity and Antigen
- Factor X Activity and Antigen
- Factor X Chromogenic Assay
- Factor XI Activity
- Factor XII Activity
- Factor XIII Activity
- Fibrinogen Activity and Antigen
- Fletcher Factor (Prekallikrein) Activity
- High Molecular Weight Kininogen Activity

Fibrinolytic Evaluation
- Alpha-2-Antiplasmin
- D-Dimer Quantitative
- Euglobulin Lysis Time
- Fibrin Monomer Qualitative
- Fibrinogen Degradation Products
- Plasminogen Activity and Antigen
- Plasminogen Activator Inhibitor-1 (PAI-1) Activity, Antigen
- Tissue Plasminogen Activator Activity and Antigen

Inhibitor Screens and Titers*
- Factor Inhibitor Screen
Lupus Anticoagulant Testing
- Dilute Russell's Viper Venom Test
- Hexagonal Phospholipid Neutralization
- Kaolin Clotting Time
- Platelet Neutralization Procedure
- Tissue Thromboplastin Inhibition Test

Platelet Evaluation
- beta-Thromboglobulin
- Bleeding Time (Local Clients Only)
- Platelet Aggregation (Local Clients Only)
- Platelet Factor 4
- Sticky Platelet Evaluation (Local Clients Only)
- PL\textsuperscript{A2} Polymorphism

Routine
- Activated Partial Thromboplastin Time
- APTT Mixing Studies
- Prothrombin Time
- Prothrombin Time Mixing Studies
- Stypven Time (Russell Viper Venom)
- Reptilase Time
- Thrombin Time
- Thrombin Time Mixing Studies

Thrombin Generation Markers
- Prothrombin Fragment 1+2
- Thrombin-Antithrombin Complex

Therapeutic Monitoring
- Ecarin Clotting Time
- Factor VIII Chromogenic Assay
- Factor X Chromogenic Assay
- Heparin Anti-Xa Fondaparinux
- Heparin Anti-Xa Unfractionated
- Heparin Anti-Xa LMWH
- PIVKA-II
- Thrombin Generation Assay

Thrombotic Risk Evaluation
- Activated Protein C Resistance with & without Factor V
- Antithrombin Activity and Antigen
- C4b-Binding Protein
- Factor V Mutation (Leiden)
- Heparin Cofactor II
- Homocysteine
- Lipoprotein (a)
- HR2 Haplotype
- Methyleneoehydrofolate Reductase (MTHFR) C677T and A1298C Mutation
- PAI 4G/5G Insertion/Deletion Polymorphism
- Prothrombin Gene 20210 Mutation
- Protein C Activity and Antigen
- Protein S Activity, Antigen Free and Antigen Total

von Willebrand Disease Evaluation
- Collagen Binding Assay
- Factor VIII Activity
- von Willebrand Factor Activity
- von Willebrand Factor Antigen
- von Willebrand Factor Multimeric Analysis
- von Willebrand Factor 2N Assay

Miscellaneous
- Cryofibrinogen
- Methylmalonic Acid Urine, Serum
- von Willebrand Cleaving Protease Activity, Inhibitor

*Specific Factor Inhibitor Titers are available for all coagulation factors