Manual 9

Hemostasis Determinations

The National Heart, Lung, and Blood Institute
of the National Institutes of Health

Atherosclerosis Risk in Communities Study Protocol

Manual 9

Hemostasis Determinations

Visit 2

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1.0 INTRODUCTION

1.1 Objective

The objective of the hemostasis component of the ARIC study is to determine the association of a group of selected hemostatic factors with (a) atherosclerosis measured by ultrasound and (b) new coronary heart disease (CHD) events or stroke in a prospective, longitudinal study of a cohort population of approximately 16,000 men and women, ages ranging from 45-64 in four diverse communities. The ultimate goal is to use the hemostatic factors for identifying individuals who are at high risk of atherosclerotic vascular diseases and for whom antithrombotic therapy may be appropriate.

The secondary objective is to correlate the hemostatic factors with other risk factors (lipids, hypertension, and diabetes) in this cohort. The information will pave the way for investigation of the mechanisms by which changes in the hemostatic factors occur and lead to a better understanding of the pathogenesis of atherosclerotic disorders in humans.

During the first period (Visit I) of investigation (1986-1989), seven hemostatic factors (Fibrinogen, factor VII, VIII, von Willebrand factor, antithrombin III, protein C and activated partial thromboplastin time) were measured in each participant. Details were described in Vol. 9 of the ARIC Procedure Manual. This manual is an updated version which describes the work scope of the second period of investigation.

For the Visit II ARIC Study, the following measurements will be performed:

1. Ultrasound cases and controls. Samples stored from Visit I: fibrinopeptide A (FPA), ß-thromboglobulin (ßTG), platelet factor 4 (PF 4), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), protein S and D-dimer.

   Blood samples from Visit II. In addition to the measurements listed above, fibrinogen, factor VII, VIII, von Willebrand factor, protein C, antithrombin III, and aPTT will be performed.

2. Clinical cases and controls. Only the specialized tests (FPA, ßTG, PF4, tPA, PAI-1, protein S and D-dimer) will be performed on stored samples from Visit I.

1.2 Rationale

There is compelling evidence that hemostatic factors play a pivotal role in the pathogenesis of human coronary heart disease (CHD) and cerebrovascular disease (CVD). The hemostatic factors comprise an array of cellular and protein constituents generally categorized into four major areas:

1. endothelium and its products
2. the blood platelet and its products
3. coagulation factors
4. fibrinolytic system

Normally, these factors exist in inactive forms. Maintenance of the normal hemostatic state is attributed to an intact endothelium complemented by the presence of natural inhibitors, notably protein C, protein S and antithrombin III (AT-III). Once the endothelium integrity is disrupted, there is a rapid activation of platelets and coagulation factors. A chain of chemical and cellular reactions ensue, leading to the formation of platelet-fibrin thrombi. Thrombus formation on the atherogenesis and vascular surface appears to play a

crucial role in causing acute vascular events (1,2).

Activated platelets contribute to atherogenesis and vasospasm. Once activated by subendothelial collagen and thrombin, arachidonic acid (AA) is liberated from the membrane phospholipid and rapidly converted into several biologically active compounds (3). Thromboxane A2 (TXA2), produced via the cyclooxygenase pathway, is a potent stimulator of the platelet release reaction and secondary aggregation as well as a vasoconstrictor (4). 12-Hydroxyeicosatetraenoic acid (12-HETE) [produced via the lipoxygenase pathway] has recently been shown to induce vascular smooth muscle cell migration (5). Migration of smooth muscle cells to the surface of the damaged vessel wall permits the platelet-derived growth factor (PDGF), released from alpha granules when platelets are activated, to induce smooth muscle cell proliferation, an important step in atherogenesis (6). In addition to TXA2, platelets secrete serotonin and ADP to promote vasospasm. Two types of granules are released by platelets. The dense granules which contain ADP, Ca++ and serotonin are involved in promoting platelet aggregation and vasospasm. The α-granules containing PDGF and coagulation factors, notably fibrinogen, VIII, vWF and V are important in atherogenesis and fibrin generation. The α-granules also contain two platelet specific proteins, ß-thromboglobulin (ßTG) and platelet factor 4 (PF-4). These proteins are useful markers for studying platelet activation in vivo (8).

Fibrin is crucial for consolidation of platelet aggregates which accumulate on the damaged vessel wall. Fibrin formation is the end result of at least 2 pathways:

1. Activation of factor XII by subendothelial tissues ("intrinsic" pathway) and

2. expression of tissue factor ("extrinsic" pathway).

Both pathways involve stepwise enzymic conversion of coagulation proteins (i.e., XII, XI, IX, VIII, X, V, and II) with the eventual conversion of the substrate, fibrinogen, into fibrin. Activated platelet surface and Ca++ are required at several steps to ensure an adequate rate of clot formation. Hence when the coagulation system is activated, there are active coagulation factors as well as their inactive precursors in the blood. Measurement of the active factors, (e.g., Xa), is a fairly reliable way of detecting thrombus formation. However, measurement of Xa and other active factors is cumbersome and unsuitable for population studies. The preferred approach is to detect the early activation peptides of fibrinogen. Fibrinopeptide A (FPA) and fibrinopeptide B are such markers useful for early detection of fibrin formation (9, 10). Other coagulation activation peptides such as prothrombin peptides (F1+2), factor IX peptides, etc. are also useful markers (11).

There exist several powerful systems to defend against excessive activation of the hemostatic system. These defensive factors act at different levels. For example, protein C and protein S retard clot formation by digesting factors V and VIII (12-15). AT-III, on the other hand, is a specific antagonist of thrombin and factor Xa and hence limits thrombus formation (16). Prostacyclin, produced by the vascular endothelium, inhibits platelet aggregation and dissolves platelet aggregates (17). The fibrinolytic system provides the ultimate defense mechanism, lysing fibrin clots by generation of a powerful proteolytic enzyme, plasmin (18,19). Plasmin digests fibrin and fibrinogen into several discrete fragments. One of the degradation products, D-dimer appears to be a useful plasma marker for fibrinolysis. Fibrinolysis activation peptides such as fibrinopeptide B (15-42), and (1-42), appear to be also useful indicator of fibrinolysis (20).

In view of the intimate involvement of hemostatic factors in the pathogenesis of CHD, there has been an interest in defining the association of hemostatic factors with CHD events. Most of the studies reported in the literature are based on a retrospective case-control design, in which blood is studied in cases after the occurrence of the clinical event. This approach is of limited value because these factors are either acute-phase reactants or are altered in the presence of clinical CHD events. Hence, the prospective design of ARIC is particularly important for the study of hemostasis. The Northwick Park Heart Study (a prospective study) reported by Meade et al from England, included 1510 white men aged 40-64 at recruitment (21). Factors V, VII, VIII:C, fibrinogen, AT-III, fibrinolytic activity, platelet adhesiveness, platelet count, $\alpha 2$ macroglobulin, cholesterol and blood pressure were measured. Their findings indicate that the mean levels of fibrinogen, VII and VIII:C were significantly higher in those who died of cardiovascular disease than in those who survived. Their data further showed that the independent associations of factor VII and fibrinogen with cardiovascular death were at least as strong as the association of blood cholesterol with cardiovascular death. These findings were confirmed in part by a prospective study conducted in Gothenburg, Sweden by Wilhelmsen, Tibblin and associates (22). They followed 792 men aged 54 at recruitment for 13.5 years. Their results indicate that fibrinogen represents an important risk factor for myocardial infarction and ischemic stroke. In this study, the factor VIII:C levels of those who had MI or stroke and those who had neither event were not statistically different. Since Factor VII was measured as a component of factor II-VII-X complexes, the data with respect to factor VII as a risk factor are difficult to interpret. Nevertheless, both studies provide a strong basis for including coagulation factors, particularly factors I (fibrinogen), VII, and VIII:C in the ARIC prospective study. Data obtained from ARIC will not only be valuable for delineating the differences between American white and European white populations but will also be uniquely informative in understanding whether there exist distinctive racial and sex differences in hemostatic factors as risk factors month the cohort population in diversified American communities.

The ARIC Visit I Study is scheduled to be completed in December 1989. The data are too early for definitive analysis. However, once completed, the sample size is large enough for statistical analysis. In Visit II study, focus is centered upon the investigation of association of hemostasis-activation products with carotid artery atherosclerosis detected by ultrasound and with clinical events. Factor VIII activity will be measured on each participant in Visit II because of its instability. Blood samples will otherwise be obtained and stored at -70°C. Coagulation factors may be measured in Visit II cohorts when indicated.

Hemostasis activation peptides to be included in the Visit II case-control studies are as follows:

1. platelet activation peptides, ßTG & PF4
2. coagulation activation peptides, FPA and F1+2 if necessary
3. fibrinolysis activation peptides, D-dimer.

In addition tPA, PAI-1 and protein S will also be measured. Selection of these factors is based on:

1. availability of accurate and precise assay
2. coverage of each aspect of hemostatic mechanisms
3. stability

The specific rationale for selecting these factors is briefly described below.

VIII:vWF von Willebrand factor is essential for platelet adhesion to damaged vessel wall (23). Its physiologic and pathophysiologic role in hemostasis and thrombosis is therefore important. In addition, VIII:vWF is completed with and provides stability to VIII:C, a coagulation factor found to be associated with CHD events in the British prospective study. The factor can be accurately measured by an enzyme-linked immunosorbant assay (ELISA) for vWF:Ag and bioassay (ristocetin-induced platelet agglutination) for VIII:vWF.

AT-III, Protein C. Both factors are naturally occurring anticoagulants. Defects in these factors have been shown be associated with recurrent deep vein thrombosis and pulmonary embolism (24-26). Functional and immunologic assays have become available for measuring these factors. The hypothesis for including these factors is that reduced levels of AT-III or protein C may represent risk factors for CHD events. Protein S, another vitamin K dependent protein, which serves as a cofactor for activated protein C (14). Deficiency of protein S has also been shown to predispose to recurrent venous thrombosis and pulmonary embolism (27). Is present in plasma as bound and free forms. We will measure the free (active) form and total protein S.

In vivo coagulation activation may be determined in several ways:

1. measuring activated coagulation factors, i.e., Xa and thrombin
2. measuring fibrin monomers
3 measuring coagulation activation peptides

The activation fibrinogen peptides such as FPA or FPB have been extensively investigated. Fibrinopeptide A appears to be most suitable. Its measurement by RIA or ELISA is sensitive and specific for detection of deep vein thrombosis and disseminated intravascular coagulation (9). It should provide a sensitive index for detecting in vivo coagulation activation in this prospective study.

In vivo platelet activation may be determined by several techniques:

1. platelet aggregation, either spontaneous or induced (29)
2 circulating platelet aggregates (CPA) by the technique of Wu and Hoak (30)
3. measurement of released products such as ßTG and PF-4 (31-34)
4. measurement of platelet TXB2 production (35)

The platelet aggregation technique is insensitive, subject to a high degree of variability and probably does not reflect in vivo platelet activation. Although measuring CPA correlates well with plasma ßTG and PF-4 levels (36), CPA measurement is impractical for population studies. On the other hand, ßTG and PF-4 can be reliably measured by RIA or ELISA (34,37) and their levels have been shown by case-control studies to be elevated in CHD (38-39). Reduced fibrinolytic activity is associated with postoperative venous thrombosis and recurrent venous thrombosis (40). The importance of reduced fibrinolytic activity in CHD is suggested by a case-control study in post-myocardial infarction patients. Techniques for measuring fibrinolysis in humans remain problematic. We plan to measure plasma D-dimer as a general indicator of fibrinolysis. Besides, we will measure tPA and PAI-1. By measuring the regulators of fibrinolysis (tPA, PAI-1 and protein C) and the specific early products of fibrinolysis, ARIC will gain insight into the role of defective fibrinolysis in CHD events.

1.3 Blood Drawing and Processing

Blood samples are obtained and processed at each field center and then shipped on dry ice to the Central Hemostasis Laboratory. Correct blood drawing and

processing techniques are critical for the hemostasis tests performed in ARIC and require substantial coordination. The techniques are described in a separate ARIC manual, entitled "Blood Collection and Processing" (ARIC Manual of Procedures Vol. No. 7).

1.4 Temporary Storage and Shipment

Care in the handling and shipping of samples from the ARIC field centers to the Central Hemostasis Laboratory is crucial. Certain coagulation factors such as VIII:C, vWF, V, and fibrinogen are unstable such that changes in the storage conditions, e.g. temperature, are likely to cause variation in the test results. Therefore, the storage and shipping conditions must be standardized. Blood samples are collected and processed at the field centers according to protocol defined in ARIC manual 7. The plasma is then stored at -70°C for up to one week following collection before being shipped to the central laboratories. The field centers ship samples weekly by overnight courier to the Central Hemostasis laboratory. There they are immediately unpacked and carefully inspected to ensure that the samples have remained frozen and that sample identification is correct. The factor VIII tests are done within one week following receipt of the samples. In the interim, the samples are kept at -70°C. For a more detailed description, see the Manual of Blood Collection and Processing (ARIC Manual Volume No. 7).

1.5 Sample Storage at the Central Hemostasis Laboratory

After bags are received at the Central Hemostasis Laboratory, the ID numbers are logged in the computer and samples are carefully inspected. The bags containing the rest of the samples for case-control studies are stored in long-term freezers (-70°C). For the entire period of study, two 20 ft3 freezers are needed for short-term storage and eight freezers for long-term storage. The long term sample bags are placed in boxes that can hold up to 10 bags. The boxes are then placed in metal racks, holding nine to eighteen boxes each. For the purpose of rapid retrieval, each freezer is assigned a Letter (A-H). Each compartment is designated 1-6. Each rack of boxes is assigned a letter (a-d) and the boxes in each holder are given an Arabic number 1-18. Each bag has an I.D. number and the tubes in the bags are color, as well as, number coded. For example, a number of A2b3 indicates that the samples are located in the long-term freezer number (A) compartment 2, rack (b) and the third box of the holder. This system substantially reduces the time needed for retrieving samples and reduces the risk of partial thawing.

All the freezers are equipped with alarm and back-up systems. The names, telephone numbers and beeper numbers of responsible persons of the Central Hemostasis Laboratory (Dr. Wu and Ms. Stinson are listed with the security office). When the alarm signal is activated, the security guard immediately checks the system and contacts the responsible person. In view of the tight alarm and back-up system, the chance of malfunction is extremely slim. The system is further guarded by emergency generator power and backup liquid nitrogen tanks.

Freezing of the samples at -70°C ensures stability of the hemostatic factors.

2.0 ASSAY PROCEDURES

2.1 Activated Partial Thromboplastin Time (aPTT)

2.1.1 Principle

Activated Partial Thromboplastin Time is a general assay for all factors in the intrinsic coagulation system, excluding PF3. Partial thromboplastin

(phospholipids) is mixed with the plasma to be tested in the presence of a particulate activator and incubated for a specific period of time at 37°C. CaCl is added to initiate the reaction, and the time required for clot formation is measured in seconds.

2.1.2 Reagents/Preparation

1. Automated aPTT Reagent (General Diagnostics): Automated aPTT reagent contains rabbit brain phospholipids together with micronized silica in N-2hydroxyethylpiperazine-N-2- ethanesulfonic acid buffer. Reconstitute with 5 ml of distilled water. Shake vigorously. Add stirring bar and place in the stir cool well of the Coag-A-Mate. Reagent is stable for one week at 2°-8° C.

2. Calcium Chloride (General Diagnostics): 0.025 M Calcium Chloride (10 ml). Store at room temperature.

3. Universal Coagulation Reference Plasma (UCRP Pacific Hemostasis) Reconstitute with 1.0 ml distilled water. Wait 15 minutes, then swirl gently before use.

2.1.3 Quality Control Material

Universal Coagulation Reference Plasma (UCRP) is the internal quality control material.

2.1.4 Equipment and Supplies

Coag-A-Mate X-2
Polystyrene test tubes (12X75 mm)
Test tube racks
Melting ice bath
Pipette and tips (100 μl)
Reagent trays
Stir bar
1 ml Class A volumetric pipette

2.1.5 Procedure

2.1.5.1 Sample Collection

Collect blood into 3.8% sodium citrate anticoagulant solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution as described in ARIC Manual 7. Invert the tube gently 8 times and place in a melting ice bath. Spin at 4° C for 10 minutes at 3000 x g. Separate the plasma from the red cells immediately and store frozen at -70° C.

2.1.5.2 Set-up

1. Set-up a worksheet with UCRP as the control in the first two work stations, followed by test plasmas and an additional set of controls at the end of the tray. Samples and controls should be run undiluted in duplicate.

2. Reconstitute reagents and control material. Let stand at room temperature for 15 minutes.

3. At the end of 15 minutes thaw the samples at 37° C, and place the controls, reagents, and samples in a melting ice bath.

2.1.5.3 Assay

1. Situate reagent tubing assemblies in the appropriate pump position (See chart on Coag-A-Mate for aPTT). Insert the tubing ends into the vials, ensuring that the pick-up tip touches the bottom of the vial and that the stir bar is rotating properly.

2. Prime lines with the reagents, delivering a slight amount of excess reagents into a suitable receptacle. Inspect the tubing for bubbles and reprime if necessary.

3. Pipette 100 µl of undiluted controls and samples into the appropriate wells of the sample tray.

4. Position and seat test tray on incubation test plate, matching the notch in the tray with the notch in the hub. Lower the reagent incubation arm.

5. Key in correct "END TEST" number. Press INDEX until desired starting station is reached.

6. Activate deprime mode by pressing ENTER, DEPRIME and ENTER keys. (This step is not necessary if more than one run will be performed.)

7. Lower light shield and press aPTT key.

8. Press the START key. The instrument operates automatically until the final test is complete.

9. If the automatic deprime mode was not activated, do not allow the reagent to remain in the tubing for more than 30 minutes after a run is complete.

2.1.6 Back-up procedure in case of instrument failure.

The laboratory has access to another Coag-A-Mate X-2.

2.1.7 Computation of Results

The two duplicate values for each control and sample are averaged and reported in seconds. Results must be repeated if:

1. Duplicates do not match within 10%.

2. If the unknown value is greater than 35 seconds or less than 25 seconds.

3. The tray control values fall outside established limits.

2.2 Fibrinogen

2.2.1 Principle

In this assay thrombin is added to the plasma samples, converting the fibrinogen present to fibrin. All samples are diluted 1:10 and run against a concentration curve prepared from a calibrated reference. The length of time for clot formation is measured in seconds and reported in mg/dl from the concentration curve.

2.2.2 Reagents/Preparation

1. Thrombin Reagent (General Diagnostics): Thrombin reagent contains approximately 100 NIH units/ml of bovine thrombin with stabilizers and buffer. Reconstitute with 3.0 ml of water and mix gently. Reagent is stable at 2°-8°C for up to 3 days.

2. Veronal Buffer, pH 7.35: Dissolve 5.875 g sodium dimethyl barbiturate and 7.335 g NaCl in 215 ml 0.1 N HCl. Add deionized water to 900 ml and adjust pH to 7.35. Adjust volume to 1.0 liter. Store at 4° C. Prepare fresh buffer every three months.

3. Universal Coagulation Reference Plasma (UCRP Pacific Hemostasis) Reconstitute with 1 ml water. Wait 15 minutes then swirl gently to mix before use.

4. Fibrinogen Calibration Reference (Fibriquik General Diagnostics): Lyophilized human plasma with stabilizers and buffer. Reconstitute with 1.0 ml of water. Allow to stand 30 minutes. Swirl gently to mix before use. Discard after 24 hours.

2.2.3 Quality Control Material

Universal Coagulation Reference (UCRP) is the internal quality control material.

2.2.4 Equipment and Supplies

Pipette with tips
Coag-A-Mate X-2
Polystyrene test tubes (12x75 mm)
Test tube racks
Reagent trays
1 ml volumetric pipette

2.2.5 Procedure

2.2.5.1 Sample Collection

Collect blood into 3.8% sodium citrate anticoagulant solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution as described in ARIC Manual 7. Invert tube gently 8 times and place in a melting ice bath. Spin at 4° C for 10 minutes at 3000 x g. Separate plasma immediately and store frozen at -70°C.

2.2.5.2 Set-up

1. Reconstitute reagents and controls.

2. Thaw samples at 37° C.

3. Set up worksheet with standards (1:6, 1:7.5, 1:10, 1:15, 1:20). Run 1 standard curve in duplicate on each tray. Run controls and samples at 1:10 in duplicate.

4. Dilute Calibration Material as follows:

	Dilution	Reference	Buffer
A	1:6	300 µl	1500 µl
B	1:7.5	300 µl	1950 µl
C	1:10	300 µl	2700 µl
D	1:15	700 µl (B)	700 µl
E	1:10	700 µl (C)	700 µl

Fibrinogen values for each dilution of the calibration curve must be calculated with each new lot number according to the 1:10 dilution.

Example: If reference material possesses 280 mg/dl of fibrinogen:

Dilution	Fibrinogen (mg/dl)		
1:6	466.6	10/6 x 280	= 466.6
1:7.5	373.3	10/7.5 x 280	= 373.3
1:10	280	10/10 x 280	= 280
1:15	186.6	10/15 x 280	= 186.6
1:20	140	10/20 x 280	= 140

5. Make a 1:10 dilution of samples and controls by adding 100 µl of plasma to 900 µl of buffer.

2.2.5.3 Assay

1. Situate reagent tubing assemblies on Coag--A-Mate in appropriate pump positions (see chart on Coag-A-Mate for FIBRINOGEN). Insert tubing ends into reagent vial, insuring that the pick-up tip touches the bottom of the vial. Do not add stir bar.

2. Prime lines with reagent. Deliver a slight amount of excess reagent into a suitable receptacle. Inspect tubing for bubbles and reprime if necessary.

3. Pipet 200 µl diluted plasma, control, or calibration material into appropriate wells of sample tray.

4. Position and set test tray on incubation test plate, matching notch in tray with notch in hub. Lower reagent incubation arm.

5. Key in correct END TEST number. Press INDEX key until desired starting station is reached.

6. Activate deprime mode by pressing ENTER, DEPRIME, and ENTER keys. (This step is not necessary if more than one run must be performed.)

7. Lower light shield. Press FIBRINOGEN key.

8. Press START key. Instrument operates automatically until final test is complete.

9. Do not allow reagent to remain in tubing for more than 30 minutes after run is completed.

2.2.6 Back-up procedure in case of instrument failure.

The laboratory has access to another Coag-A-Mate X-2.

2.2.7 Computation of results

Results are calculated using Best Linear Fit Program on the Coag-A-Mate. Results must be repeated on a new sample if:

1. Duplicates do not match within 10%.

2. The unknown value is greater than the highest standard or lower than the lowest standard. Repeat assay using an appropriate dilution and multiply result by the corresponding dilution factor.

3. Tray control value exceeds 2 SD of the mean.

2.3 Factor VII Activity

2.3.1 Principle

This quantitative assay is based on the ability of the test plasma to correct a Factor VII deficient substrate. After adding the deficient substrate to diluted samples, the test is run as a prothrombin time (PT). All samples are diluted 1:20 and run against a concentration curve prepared from the UCRP standard. Thromboplastin and calcium are mixed with plasma, and the length of the time required for clot formation is measured in seconds. This is converted to percent activity by comparing unknown clot times to the calibration curve clot times.

2.3.2 Reagents/Preparation

1. Thromboplastin (Thromboscreen, Pacific Hemostasis): Lyophilized extract of rabbit brain in buffered saline with stabilizers and calcium chloride. Reconstitute the Thromboplastin with distilled water according to the instructions on the vial label.

2. Factor VII Deficient Plasma (Pacific Hemostasis) Assayed Factor VII activity is less than 1%. Reconstitute with 5 ml distilled water. Wait 15 minutes and swirl gently before use.

3. Veronal Buffer, pH 7.35: Dissolve 5.875 g sodium dimethyl barbiturate and 7.335 g NaCl in 215 ml 0.1 N HCl. Add deionized water to 900 ml and adjust pH to 7.35. Adjust volume to one liter. Store at 4°C. Make fresh every three months.

4. Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis) UCRP is assayed pooled human plasma : Reconstitute with 1 ml distilled water, wait 15 minutes then swirl gently to mix before use.

2.3.3 Quality Control Material

Universal Coagulation Reference Plasma (UCRP) is used for both calibration and quality control. The calibration UCRP lot number must be different from the UCRP used for quality control. Values for the calibration curve must be calculated with each new lot number according to the value at 1:20.

2.3.4 Equipment and Supplies

Coag-A-Mate X-2
Polystyrene test tubes (12X75 mm)
Test Tube Racks
Pipette and tips 100 μl-1000 μl and 10 μl-100 μl
Coag-A-Mate Reagent trays

Stirring bar
1 ml volumetric pipette
Eppendorf repeater pipette and tips

2.3.5 Procedure

2.3.5.1 Sample Collection and Preparation

Blood is collected by flawless venipuncture in a 3.8% sodium citrate anticoagulant solution, in the proportion of 9 parts blood to 1 part anticoagulant solution as described in ARIC Manual 7. Invert the tube gently 8 times and place in a melting ice bath. Centrifuge at 4°C for 10 minutes at 3,000 x g. Separate plasma immediately and store frozen at -70°C. Plasma is thawed rapidly at 37°C immediately prior to use. After that perform analysis within 2 hours.

2.3.5.2 Set Up

1. Reconstitute reagents and controls.

2. Thaw samples.

3. Set up worksheet with standards (1:10, 1:15, 1:20, 1:30, 1:40, :80). Run 1 standard curve in duplicate on each tray. Run controls and samples at 1:20 in duplicate.

4. Dilute Calibration Material as follows:

	Dilutions	UCRP (µl)	Buffer (µl)
A	1:10	100	900
B	1:15	100	1400
C	1:20	500 (A)	500
D	1:30	500 (B)	500
E	1:40	500 (C)	500
F	1:80	500 (E)	500

Values for the calibration curve must be calculated with each new lot number of UCRP according to the value at a 1:20 dilution. For Example: If UCRP posses 93% Factor VII activity:

Dilution	Factor VII (%)	
1:10	186.0	20/10 x 93% =186.0%
1:15	124.0	20/15 x 93% =124.0%
1:20	93.0	20/20 x 93% = 93.0%
1:30	62.3	20/30 x 93% = 62.3%
1:40	46.5	20/40 x 93% = 46.5%
1:80	23.3	20/80 x 93% = 23.3%

5. Make a 1:20 dilution of samples and controls by adding 50 µl of plasma to 950 µl buffer. Vortex all dilutions to mix well.

2.3.5.3 Assay

1. Prime lines with reagent, delivering slight amount of excess reagent into a suitable receptacle. Inspect tubing for bubbles and reprime if necessary.

2. Situate reagent tubing assemblies in appropriate pump positions. (See

chart and Coag-A-Mate for PT'S.) Insert tubing ends into Thromboplastin vial, ensuring that the pick-up tip touches the bottom of the vial and the stirring bar is rotating correctly.

3. Pipette 100 µl diluted Calibration material, control, or unknown plasma into appropriate well of sample tray.

4. Using Eppendorf repeater pipette, dispense 100 µl Factor VII deficient plasma into each well used for assay.

5. Gently mix tray by rotating on counter top, taking care not to splash any sample from wells. Position and set test tray on incubation plate, matching notch in tray with notch on hub. Lower reagent incubation arm.

6. Key in correct END TEST number. Press INDEX key until desired starting station is reached.

7. Activate deprime mode by pressing ENTER, DEPRIME, and ENTER keys. (This step is not necessary if more than one run must be performed.

8. Lower light shield. Press PT key.

9. Press START key. Instrument operates automatically until final test is complete.

10. Do not allow reagent to remain in tubing for more than 30 minutes after run is completed.

2.3.6 Back-up procedure in case of instrument failure.

The laboratory has access to another Coag--A-Mate X-2.

2.3.7 Computation of Results

Results are calculated using the log-log function of a linear regression program. Clotting times in seconds are plotted on the abscissa (x-axis) and corresponding factor VII values in % are plotted on the ordinate (y-axis). Participant and control values diluted 1:20 are read directly from the curve. Results must be repeated if:

1. Duplicates do not match within 10%.

2. The unknown value is greater than the highest standard or lower than the lowest standards. Repeat assay using an appropriate dilution and multiply result by the corresponding dilution factor.

3. Tray control value falls outside established limits.

2.4 Factor VIII Activity

2.4.1 Principle

This quantitative assay is based on the ability of the test plasma to correct a Factor VIII deficient substrate. After adding the deficient substrate to diluted samples, the test is run as an aPTT. All samples are diluted 1:20 And run against a concentration curve prepared from the UCRP standard. APTT reagent and 0.025M calcium chloride are mixed with the diluted plasma, and the length of time for clot formation is measured in seconds. This is converted to percent activity by comparing unknown clot times to the concentration curve clot times.

2.4.2 Reagents/Preparation

1. Automated aPTT Reagent (General Diagnostics): Rabbit brain phospholipids together with micronized silica in N-2-hydroxyethyl-piperazine-N-2ethanesulfonic acid buffer. Reconstitute with distilled water according to instructions on vial label. Agitate gently until reagent is completely dissolved. Add stir bar and place in the stir-cool well of Coag-A-Mate X2.

2. Calcium Chloride (General Diagnostics): 0.025M Calcium Chloride (10 ml). Store at room temperature.

3. Factor VIII Deficient Plasma (Pacific Hemostasis): Factor VIII deficient plasma has less than 1% activity. Reconstitute with 5 ml distilled water. Wait 15 minutes and swirl gently before use.

4. Veronal Buffer, pH 7.35: Dissolve 5.875 g sodium dimethyl barbiturate and 7.335 g NaCl in 215 ml 0.1 N HCl. Add deionized water to 900 ml and adjust pH to 7.35. Adjust volume to one liter. Store at 4°C. Make fresh every three months.

5. Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis) UCRP is an assayed pooled normal human plasma. Reconstitute with 1 ml distilled water, wait 15 minutes then swirl gently to mix before use.

2.4.3 Quality Control Material

Universal Coagulation Reference Plasma (UCRP) is used for both calibration and quality control. The calibration UCRP lot number must be different from the UCRP used for quality control. Values for the calibration curve must be calculated with each new lot number according to the value at a 1:20 dilution.

2.4.4 Equipment and Supplies

Coag-A-Mate X-2
Polystyrene test tubes (12X75 mm)
Test Tube Racks
Pipette and tips 100-1000 μl
Coag-A-Mate Reagent Trays
Stir Bar
1 ml class A volumetric pipette
Eppendorf repeater pipette and tips

2.4.5 Procedure

2.4.5.1 Sample Collection and Preparation

Blood is collected by flawless venipuncture in a 3.8% sodium citrate anticoagulant solution, in the proportion of 9 parts blood to 1 part anticoagulant solution as described in ARIC Manual 7. Invert the tube gently 8 times and place in a melting ice bath. Centrifuge at 4°C for 10 minutes at 3,000 x g. Separate plasma immediately and store frozen at -70°C. Plasma is thawed rapidly at 37°C immediately prior to use, mixed and placed in a melting ice bath.

2.4.5.2 Set-up

1. Set up worksheet with standards (1:8, 1:10, 1:16, 1:20, 1:32, 1:64).

Run standard curve in duplicate on each tray. Run controls and samples at 1:20 in duplicate.

2. Reconstitute reagents, control material and standard material and let stand at room temperature for 15 minutes.

3. At the end of 15 minutes thaw the samples at 37°C. Place reagents, controls, standards, and samples in a melting ice bath.

4. Dilute the calibration material as follows:

Dilutions		UCRP	Buffer
A	1:8	250 µl	1750 µl
B	1:10	200 µl	1800 µl
C	1:16	1000 µl	1000 µl
D	1:20	1000 µl	1000 µl
E	1:32	1000 µl	1000 µl
F	1:64	1000 µl	1000 µl

5. While tubes are in a melting ice bath prepare 1:20 dilutions of samples and controls by adding 50 µl of plasma to 950 µl of buffer. Vortex all dilutions to mix well.

2.4.5.3 Assay

1. Prime lines with reagents, delivering a slight amount of excess reagent into a suitable receptacle. Inspect the tubing for bubbles and reprime if necessary.

2. Situate reagent tubing assemblies in the appropriate pump position (See chart on Coag-A-Mate for aPTT's). Insert tubing ends into the reagent vials, ensuring that the pick-up tip touches the bottom of the vial and the stirring bar is rotating correctly.

3. Pipette 100 µl diluted Calibration material, control, or unknown plasma into the appropriate well of the sample tray.

4. Using the Eppendorf repeater pipette, dispense 100 µl Factor VIII deficient plasma into each well used for assay.

5. Gently mix tray by rotating it on counter top, taking care not to splash any sample from the wells. Position and set test tray on incubation plate, matching the notch on the tray with the notch on the hub. Lower incubation arm.

6. Key in correct END TEST number. Press INDEX key until desired starting station is reached.

7. Activate deprime mode by pressing ENTER, DEPRIME, and ENTER keys. (This step is not necessary if more than one run must be performed).

8. Lower light shield. Press aPTT key.

9. Press START key. Instrument operates automatically until the final test is complete.

10. Do not allow reagents to remain in tubing for more than 30 minutes after the run is complete.

2.4.6 Back-up procedure in case if instrument failure.

The laboratory has access to another Coag-A-Mate X-2.

2.4.7 Computation of Results

Results are calculated using the semi-log function of a linear regression program. Clotting times in seconds are plotted on the abscissa (x-axis) and the corresponding Factor VIII values in % are plotted on the ordinate (y-axis). Unknown and control values diluted at 1:20 are read directly from the curve.

Results must be repeated if:

1. Duplicates do not match within 10%.

2. The unknown value is greater than the highest standard or lower than the lowest standard. Repeat assay using an appropriate dilution and multiply or divide the results by the corresponding dilution factor.

3. Tray control values falls outside established limits.

2.5 von Willebrand Factor Antigen (vWF)

2.5.1 Principle

Plasma Factor VIII is composed of two portions: a procoagulant fraction (VIII:C) and a von Willebrand factor (vWF:Ag). Von Willebrand factor mediates adhesion of platelets to damaged vessel wall. It complexes with factor VIII:C protein and regulates plasma levels of VIII:C. Von Willebrand factor is measured by enzyme immunoassay. Von Willebrand factor is bound via one of its antigenic determinations to F(ab) fragments coating the microliter plate wells. This is revealed by anti-vWF peroxidase, which is then reacted with the peroxidase substrate. O.D. readings are taken at 410 nm and results compared to the calibration curve.

2.5.2 Reagent Preparation

1. Ant-vWF Coating (American Bioproducts Co., Diagnostica Stago, NJ): F(ab')2 fragments specific for von Willebrand factor, in coating buffer are freeze-dried. Reconstitute with 20 ml distilled water. Coat plates as described in procedure section. Coating may be stored up to one month at 2°-8°C. (Cover completely with masking tape, label, date, and store flat.)

2. Anti-vWF-Peroxidase (Stago): Anti-vWF rabbit immunoglobulins coupled with peroxidase, freeze-dried. Reconstitute with 20 ml dilution buffer just before use. Stable 24 hours at 2°-8°C.

3. Dilution Buffer (Stago): Phosphate buffer containing bovine serum albumin and Tween 20, concentrated 10 fold, 20 ml per vial. Reconstitute to 200 ml with distilled water in a graduated cylinder. Store in a clear plastic bottle. Stable 15 days at 2°-8°C. Label and date the bottle.

4. Washing Solution (Stago): Saline solution containing Tween 20, concentrated 20 fold, in a 50 ml vial. Reconstituted in a graduated cylinder up to the 1000 ml mark. Store in a clean plastic bottle. Stable 15 days at 2°-8°C. Label and date the bottle. NOTE: If crystals are observed in dilution buffer or washing solution, incubate

10-30 minutes at 37°C until crystals are completely dissolved.

5. Peroxidase Substrate, ABTS (Kirkegaard & Perry): (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]. The solution is ready for use as received. The substrate should be clear and free of any particulate material. Discard any remaining solution. Store at 2°-8°C.

6. Sodium dodecyl sulfate (SDS) (Sigma): Dissolve 50 g of SDS in 1000 ml water. Store at room temperature. Discard if solution becomes turbid.

7. Universal Coagulation Reference Plasma (UCRP): Reconstitute UCRP with 1.0 ml water using a class A volumetric pipet. Swirl gently until all of the dry cake is under water. Do not invert. Let stand for 15 minutes at room temperature before use.

2.5.3 Quality Control Material

Universal Coagulation Reference Plasma (UCRP) is used for both calibration and quality control. The calibration UCRP lot number must be different from the UCRP used for quality control. Values for the calibration curve must be calculated with each new lot number according to the value at a 1:20 dilution.

2.5.4 Equipment and Supplies

Multichannel pipette
Reagent Reservoir
Dynatech Immulon 2 Microliter plates
100-1000 µl digital Eppendorf pipette
10-100 µl digital Eppendorf pipette
200 µl fixed Eppendorf pipette
Culture-tek Vaccupette/96 plate washer with 50 ml syringe attached
Microplate mixer
MR 5000/7000 ELISA Reader (Dynatech, Slater, Va)
Stopwatch
Serological pipette
Class A volumetric pipette

2.5.5 Procedure

2.5.5.1 Sample Collection

Blood is collected at 1:10 dilution in 3.8% sodium citrate as described in ARIC Manual 7. The sample is centrifuged at 2°-8°C for 10 minutes and the plasma is separated from the red cells. The plasma is frozen rapidly and stored at -70°C until shipping to the CHL. Rapid thawing (37°C) is recommended. After thawing perform test within 8 hours.

2.5.5.2 Coating of Microliter Plates

Add 200 µl of Coating Buffer to each well of a microwell plate. Cover with tape and incubate overnight at room temperature on a level surface.

2.5.5.3 Wash

1. Fill Vaccupette trough with wash buffer.

2. Decant plate completely.

3. Fill dispenser by pulling to the 22 ml mark.

4. Line up wells on the plate with dispenser.

5. Deliver wash solution by gently pushing syringe in. Do not allow dispenser tips to touch solution.

6. Decant and drain briefly on paper towels.

2.5.5.4 Standard Preparation

Use UCRP to make the following dilutions:

	Dilutions	UCRP	Buffer
A	1:60	25 µl	1.475 ml
B	1:80	25 µl	1.975 ml
C	1:120	500 µl (A)	500 µl
D	1:160	500 µl (B)	500 µl
E	1:240	500 µl (C)	500 µl
F	1:480	500 µl (E)	500 µl

Values for the Calibration curve must be calculated according to the value of vWF at a 1:120 dilution.

For example: If UCRP posses 115% activity:

Dilution	vWF	
1:60	230.0%	120/60 x 115% = 230.0%
1:80	172.0%	120/80 x 115% = 172.0%
1:120	115.0%	120/120 x 115% = 115.0%
1:160	86.2%	120/160 x 115% = 86.2%
1:240	57.5%	120/240 x 115% = 57.5%
1:480	28.8%	120/480 x 115% = 28.8%

The same 1:120 dilution may be used be used for both von Willebrand Factor and Protein C if both assays are performed at the same time.

Double the amounts when running Protein C and von Willebrand factor assays together to assure sufficient volume.

2.5.5.5 Assay

1. Wash the previously coated microwell plate.

2. Pipet 200 µl each diluted standard, control, and unknown plasma into an appropriate well of the microwell plate. Run at least one standard curve in duplicate per tray, Run diluted unknowns and controls in duplicate. Include at least one control per column.

3. Cover the plate with tape and incubate at room temperature for 3 hours on the minimis.

4. Shortly before the end of the incubation, prepare the immunoconjugate and pour into the reagent reservoir marked vWF immunoconjugate. Wash the plate 5 times and drain completely.

5. Immediately add 200 µl of immunoconjugate to all wells using the 8 channel pipet. Cover and incubate for 2 hours at room temperature on the minimix.

6. Before the end of the incubation time bring the ABTS to room temperature. Pour a sufficient amount of ABTS in a reservoir (approximately 20 ml) and shield from light until ready for use. Pour a sufficient amount of 5% SDS in a reservoir (approximately 10 ml) and cover until ready for use. Place a stop watch in a handy location, as well as two 8 channel pipets. Set one pipette to deliver 200 µl and set one to deliver 100 µl.

7. At the end of the incubation wash the plate 5 times with washing solution. Drain completely. Place plate on the minimix.

8. Add 200 µl ABTS substrate at precisely timed intervals. 20 seconds are recommended.

9. Stop the reactions with 100 µl of 5% SDS at the same precisely timed intervals used in step 8, beginning when the O.D. of Reference dilution "A" reaches 0.800-0.900 (approximately 4 minutes).

10. Allow the plate to mix for 10 minutes at room temperature before final reading.

11. Read microwell plate on MR5000/7000.

    a) Turn on MR5000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test #, (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
    e) Press "Enter"
    f) Enter plate ID # by pressing the key immediately below the cursor, when the ID is complete, press enter to accept it.
    g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.5.6 Back-up procedure in case of instrument failure

The laboratory has access to another Microelisa Reader in case of instrument malfunction.

2.5.7 Computation of Results

Calculate results using a log linear regression program. Read results from 1:120 dilutions directly from the printout. Dilute samples containing elevated amounts of vWF and multiply by the appropriate dilution factor. Results must be repeated if:

1. Duplicates do not match within 10%

2. The unknown values fall outside the calibration curve

3. Repeat all results in a column if a column controls fall outside the established limits.

2.6 Antithrombin III Activity (AT III)

2.6.1 Principle

Antithrombin III (AT-III) is the primary plasma protein responsible for the

inactivation of thrombin. AT-III complexes with heparin and inactivates thrombin by binding with thrombin and becoming irreversibly associated. It also acts as an inhibitor to factors Xa, IXa, and XIIa. In this assay, plasma in the presence of heparin is incubated with an excess of thrombin. The remaining amount of thrombin is determined by its amidolytic activity on the synthetic chromogenic substrate CBS 34.47. This quantity is inversely proportional to the quantity of Antithrombin-III in the unknown, which is compared against a standard curve and reported as % activity.

2.6.2 Reagents/Preparation

1. CBS 34.47: 1.8 umole/vial of lyophilized 2 AcOH.H-D-CHG-But-Arg-pNA. Freeze-dried (Stago). Reconstitute with 5 ml distilled water. Invert gently to mix. Stable for one month at 2°-8°C.

2. Thrombin: 53 nkat unit/vial, freeze-dried (Kabi Vitrim, Sweden) Reconstitute with 3 ml 2% PEG. Invert gently to mix. Stable for 7 days at 2°-8°C; 1 month at -20°C.

3. Dilution Buffer: Tris 50 mmol/l, EDTA 7.5 mmol/l, heparin 3000 IU/l and 1% PEG, pH 8.4. Stable for one month at 2°-8°C.

4. UCRP: (Thromboscreen, Pacific Hemostasis) Assayed pooled normal human plasma; freeze dried. Reconstitute with 1 ml water, wait 15 minutes, then swirl gently to mix before use.

5. 2% PEG: 2 ml Sigma Polyethylene Glycol (P-3265) in 100 ml water.

2.6.3 Quality Control/Calibration

Universal Coagulation Reference Plasma (UCRP) is used for both calibration and quality control. The UCRP calibration lot number must be different from the UCRP quality control lot number.

2.6.4 Equipment and supplies

Stopwatch
12X75 mm test tubes (glass or polyethylene)
Flow microliter plates
0.5-10 µl Eppendorf pipette with tips
10-100 µl Eppendorf pipette with tips
Multichannel pipette and tips
Flow reagent reservoirs
MR 5000/7000 ELISA reader
Microplate mixer
1 ml volumetric pipette

2.6.5 Procedure

Thrombin Reservoir Treatment

To minimize thrombin binding to the reservoir, fill new reservoir with 5% BSA. Allow to stand overnight then discard albumin and rinse well.

2.6.5.1 Sample Collection and Preparation

Blood should be collected by flawless venipuncture in 3.8% sodium citrate anticoagulant solution, in the proportion of 9 parts blood to 1 part anticoagulant solution as described in ARIC Manual 7. Invert tube gently 8 times and place in melting ice bath. Centrifuge at 4°C for 10 minutes at

3,000 x g. Separate plasma immediately and store frozen at -70°C. Plasma is thawed rapidly at 37°C immediately prior to use. After thawing, perform analysis within 2 hours.

2.6.5.2 Set-up

1. Set up assay as shown on sample worksheet. Each run should have at least 1 standard curve in duplicate. Samples and controls are run at 1:40 dilution in duplicate with at least 1 control on each column.

2. The AT-III assay is influenced by many factors including temperature, pH, substrate activity, and heparin concentration. To optimize the timing of the assay, a "window" test is performed just prior to the addition of thrombin (Step 2 of Assay) for each individual tray run. To perform the window test:

   a) Turn on the MR5000/7000
   b) Place a microtiter plate on the reader
   c) Press "MR5000"
   d) Press "Start"
   e) Enter pre-set test #, (5 = AT-III, dual wavelength, test wavelength 410nm, ref. wavelength 450nm).
   f) Add 80 µl of dilution buffer and 40 µl of thrombin to well A1 of the plate.
   g) Add 40 µl of substrate to well A1 and press "Start". The reader will automatically read the OD's at 10 second intervals for 2'40". At the end of that time a printout will be generated and the time required to reach 750nm can be recorded. This time determines the optimum incubation required in the assay, before the addition of acetic acid to stop the reaction.

3. Using dilution buffer, prepare 1:40 dilution of each sample and control in 12 X 75 mm test tubes. Pipet 25 µl of sample into 975 ml of dilution buffer for a 1:40 dilution. Samples diluted 1:40 are read directly from the calibration curve.

4. Using the UCRP standard make the following dilutions:

	DILUTION	UCRP	BUFFER
A	1:20	50 µl	950 µl
B	1:30	50 µl	1.450 µl
C	1:40	500 µl (A)	500 µl
D	1:60	500 µl (B)	500 µl
E	1:80	500 µl (C)	500 µl
F	1:160	500 µl (E)	500 µl

Values for the calibration curve must be calculated with each new lot number according to the value at 1:40.

Example: If UCRP possesses 118% AT-III activity:

Dilution	AT-III(%)	
1:20	236.0	40/20 x 118% = 236.0%
1:30	157.3	40/30 x 118% = 157.3%
1:40	118.0	40/40 x 118% = 118.0%
1:60	78.7	40/60 x 118% = 78.7%
1:80	59.0	40/80 x 118% = 59.0%
1:160	29.5	40/160 x 118% = 29.5%

5. Vortex all dilutions to mix well.

2.6.5.3 Assay

1. Pipette 80 µl of each diluted standard, control or sample into appropriate well of microplate.

2. Reconstitute substrate and thrombin.

3. Perform window test.

4. Pour thrombin, substrate and acetic acid into the appropriately labeled reservoirs. Shield substrate from light. Place tips on 2 multipettes. Place plate on mixer.

5. Start timer.

6. At precisely timed intervals, add 40 µl thrombin into each column of microliter plate except the blank well.

7. Add 40 µl substrate at the same precisely timed intervals used in step #6, beginning exactly 7 minutes after the addition of thrombin to column #1.

8. Stop the reaction by adding 40 µl of glacial acetic acid to each column of wells at the same precisely timed intervals used in steps #6 and #7, beginning seconds after the addition of substrate to column #1. "X" = the number of seconds obtained during the window test (see Set Up step 2).

9. Be sure to leave thrombin in the reagent reservoir as briefly as possible, as binding to the reservoir does occur.

10. Read microwell plate on MR5000/7000.

    a) Turn on MR5000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test #, (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
    e) Press "Enter"
    f) Enter plate ID # by pressing the key immediately below the cursor, when the ID is complete, press enter to accept it.
    g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.6.6 Back-up Procedure

The laboratory has access to another microelisa reader in the event the reader fails.

2.6.7 Computation of Results

AT-III results should be calculated using the semi-log function of the linear regression program. O.D. are plotted on the abscissa (x-axis) and the corresponding % AT-III values are plotted on the ordinate (y-axis). Patient and control values diluted 1:40 are read directly from the calibration curve. Repeat any unknown values that do not fall within the calibration range at an appropriate dilution. Repeat any unknowns with O.D. duplicates more than 10% apart. Repeat all unknowns in a column if the column control falls outside

established limits.

2.7 Protein C Antigen

2.7.1 Principle

Protein C is a vitamin K dependent plasma protein. The activated form exhibits a potent and highly specific anticoagulant activity by selectively inactivating Factors Va and VIIIa, in the presence of calcium ions and phospholipids. This quantitative enzyme immunoassay employs the "sandwich" technique. Microliter wells are coated with anti-Protein C immunoglobulins. Diluted plasma samples and standards are added, and Protein C antigenic determinants bind to the antibody. The amount bound is indicated by an immuno-enzyme conjugate, which binds to other Protein C antigenic determinants. The bound enzyme peroxidase is then evidenced by its activity on ABTS substrate. The reaction is stopped with 5% SDS and Optical Density readings are taken at 410 nm. The plasma sample readings are compared to the standard curve, and Protein C is reported in micrograms per ml.

2.7.2 Reagent Preparation

1. Pre-Coated Plates: 2 Plates, each of 96 microwells precoated with specific rabbit anti-Protein C F(ab')2 fragments, hermetically sealed in aluminum pouches. Each 96-well plate can be broken into 6 strips of 16 wells (2 rows of 8).

2. Anti-Protein C peroxidase (Stago): Immunoenzyme conjugate, in which specific anti-Protein C immunoglobulins obtained in the rabbit are labeled with peroxidase. Add 20 ml dilution buffer to the vial, invert gently to reconstitute.

3. Dilution Buffer (Stago): Each vial contains 20 ml of bovine serum albumin and Tween 20, concentrated 10 fold. Reconstitute up to 200 ml with distilled water in a graduated cylinder. Store in a clear plastic bottle. Stable for 15 days at 2°-8°C. Label and date the bottle.

4. Washing Solution (Stago): Each vial contains saline and Tween 20. Reconstitute vial in a graduated cylinder up to the 1000 ml mark. Store in a clean plastic bottle at 2°-8°C. The solution is stable for 15 days at 2°-8°C. (Note: if crystals are observed in the dilution buffer or wash solution incubate for 10-30 minutes at 37°C until crystals are dissolved).

5. ABTS peroxidase substrate (Kirkegaard and Perry): ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]. The solution is ready for use as received. The substrate should be clear and free of particulate material. Discard any remaining solution. Store the solution at 2°-8°C.

6. Sodium dodecyl sulfate (SDS) (Sigma): Dissolve 50 g of SDS in 1000 ml of water. Store the solution at room temperature. Discard the solution if it becomes turbid.

7. Universal Coagulation Reference Plasma (UCRP): Reconstitute UCRP with 1 ml distilled water using a class A volumetric pipet. Swirl gently until all the dry cake is under water. Do not invert. Let stand 15 minutes at room temperature.

2.7.3 Quality Control/Calibration Material

Universal Coagulation Reference Plasma (UCRP) is used for both quality control and calibration control material. The quality control lot number must be different from the calibration curve lot number.

2.7.4 Equipment and Supplies

Multichannel pipette and pipette tips
Reagent reservoirs
200 µl Eppendorf fixed pipet
100 - 1000 µl pipet
Culturetek Vaccupette, with 50 ml syringe attached
Microplate Mixer
MR 5000/7000 Elisa Reader
Stopwatch
Serological pipet
1 ml volumetric pipet

2.7.5 Procedure

2.7.5.1 Sample Collection

Blood is collected in sodium citrate, centrifuged at 2°-8°C and the plasma is separated for the red cells as described in ARIC Manual 7. The plasma is frozen rapidly and stored at -70°C until shipping to the CHL. Rapid thawing (37°C) is recommended. After thawing, perform the test within 8 hours.

2.7.5.2 Precoated Plates

Carefully cut open one end of an aluminum pouch. Pull out the plastic plate. Break off as many strips as needed. Replace the unused strips in the pouch, not forgetting the desiccant bag. Seal the pouch with scotch tape. The unused strips may then be stored for up to 15 days at 2°-8°C. No further preparation is required.

2.7.5.3 Sample Preparation

Samples and controls are tested at a 1:120 dilution. Results obtained using a 1:120 dilution are read directly from the calibration curve. Samples containing Protein C in amounts greater than the highest standard value must be repeated at a 1:240 dilution. In this case values read from the calibration curve will be multiplied by 2.

2.7.5.4 Standard Preparation

Use UCRP to make the following dilutions:

	Standard	UCRP	Dilution Buffer
A	1:60	25 µl	1.475 ml
B	1:80	25 µl	1.975 ml
C	1:120	500 µl of "A"	500 µl
D	1:160	500 µl of "B"	500 µl
E	1:240	500 µl of "C"	500 µl
F	1:480	500 µl of "E"	500 µl

Values for the calibration curve must be calculated according to the value of Protein C at 1:120 dilution.

Example: if UCRP possess 2.94 ng/ml pf Protein C activity:

1:60	5.88	120/60 x 2.94ng/ml = 5.88 ng/ml
1:80	4.41	120/80 x 2.94ng/ml = 4.41 ng/ml
1:120	2.94	120/120 x 2.94ng/ml = 2.94 ng/ml
1:160	2.21	120/160 x 2.94ng/ml = 2.2 ng/ml
1:240	1:47	120/240 x 2.94ng/ml = 1.47 ng/ml
1:480	0.74	120/480 x 2.94ng/ml = 0.74 ng/ml

The same 1:120 dilution may be used for both Protein C and von Willebrand Factor if both assays are performed at the same time.
Double amounts when running Protein C and von Willebrand Factor assays together to assure sufficient volume.

2.7.5.5 Assay

1. Pipet 200 µl of each diluted standard, control and unknown plasma into the appropriate well of the microwell plate. Run at least one standard curve in duplicate per tray. Run diluted samples and controls in duplicate. Include at least one control per column.

2. Cover the plate with tape and incubate at room temperature for 2 hours on minimix.

3. Shortly before the end of the incubation, prepare the immunoconjugate and pour into the reagent reservoirs marked Protein C immunoconjugate. Wash the plate 5 times and drain completely.

4. Immediately add 200 µl of immunoconjugate to all wells using the 8 channel pipet. Cover and incubate for 2 hours at room temperature on minimix.

5. Before the end of the incubation time bring the ABTS to room temperature. Pour a sufficient amount of ABTS in a reservoir (approximately 20 ml) and shield from light until ready for use. Pour a sufficient amount of 5% SDS in a reservoir (approximately 10 ml) and cover until ready for use. Place a stopwatch in a handy location, as well as two 8 channel pipets. Set one pipet to deliver 200 µl and set one to deliver 100 µl.

6. At the end of the incubations, wash the plate 5 times with washing solution. Drain completely. Place plate on minimix.

7. Add 200 µl ABTS substrate at precisely timed intervals. 20 seconds are recommended.

8. Stop reaction with 100 µl of 5% SDS at the same precisely timed intervals used in step #8, beginning when the O.D. of "A" standard reaches 0.800 - 0.900 (approximately 4 minutes).

9. Allow the plate to mix for 10 minutes at room temperature before final reading.

10. Read microwell plate on MR5000/7000.

    a) Turn on MR5000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test #, (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).

e) Press "Enter"
f) Enter plate ID # by pressing the key immediately below the cursor, when the ID is complete, press enter to accept it.
g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.7.6 Back-up procedure in case of instrument failure.

The laboratory has access to another Microelisa Reader in case of instrument malfunction.

2.7.7 Computation of Results

Calculate results using a log linear regression program. Read results from 1:120 dilutions directly from the printout. Dilute samples containing elevated amounts of Protein C and multiply by the appropriate dilution factor. Results must be repeated if:

1. duplicates do not match within 10%

2. the unknown value falls outside of the calibration curve.

3. repeat all results in a column if column controls fall outside established limits.

2.8 Tissue Plasminogen Activator (tPA)

2.8.1 Principle

Tissue Plasminogen Activator is assayed by an enzyme immunoassay. A plastic microwell plate coated with mouse monoclonal anti-tPA antibody is allowed to come into contact with plasma containing the tPA to be measured. The tPA contained in the sample binds to the microwell plate by one antigenic determinant. Next, mouse monoclonal anti-tPA antibody coupled with peroxidase is added. This binds to the remaining free antigenic determinants of the tPA, forming a sandwich. The bound enzyme peroxidase is then revealed by its activity on ABTS substrate. The color change produced is a direct measure of the tPA concentration initially present in the sample.

2.8.2 Reagents/Preparation

1. Precoated Plate: Each kit contains 1 plate of 96 microwells precoated with mouse monoclonal anti-tPA F(ab')2 fragments, stabilized and hermetically sealed in an aluminum pouch. The unused strips may be stored up to 15 days at 2°-8°C if resealed in the pouch with desiccant bag.

2. Reference tPA: 1 vial contains 50ng of lyophilized, single-chain purified human tPA. Reconstitute the vial with 5 ml of dilution buffer to obtain a solution of tPA titrating exactly 10 ng/ml. This corresponds to 50 ng/ml under actual assay conditions since test plasmas are diluted 1:5.

3. Dilution Buffer: Prepare a 1:10 dilution of the buffer by transferring the contents of the vial (20 ml into a 200 ml cylinder. Fill to the 200 ml mark with distilled water. The diluted buffer is stable 15 days at 2°-8°C.

4. Washing Solution: Prepare a 1:20 dilution by transferring the contents of the vial (50 ml) into a 1 liter graduated cylinder. Fill to 1 liter.

The diluted washing solution is stable for 15 days at 2°-8°C.

5. Antibody-Enzyme Conjugate: Just before use, reconstitute the vial of anti-tPA-peroxidase conjugate with 20 ml of dilution buffer.

6. ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]): The solution is ready for use as received. Observe expiration date on vial.

7. 5% SDS (Sigma) Sodium dodecyl sulfate: Dissolve 50 grams in 1000 ml distilled water. Store the solutions at room temperature. Discard if the solution becomes turbid.

2.8.3 Quality Control Material

Universal Coagulation Reference Plasma (UCRP) is the internal quality control material used. Reconstitute with 1.0 ml water. Wait 15 minutes then invert gently to mix before use.

2.8.4 Equipment and Supplies

Titertek multichannel pipet and tips
Titertek reagent reservoirs
Eppendorf digital 10-100 μl pipette
Eppendorf 100-1000 μl pipette
Culturetik Vaccupette with 50 ml syringe attached
Microplate Mixer
MR 5000/7000 Elisa Reader
1 ml volumetric pipette
Stopwatch

2.8.5 Procedure

2.8.5.1 Sample Collection

Collect blood into 3.8% sodium citrate anticoagulant solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution as described in ARIC Manual 7. Invert tube gently 8 times and place in a melting ice bath. Spin at 4°C for 10 minutes at 3000 x g. Separate plasma immediately and store frozen at -70°C.

2.8.5.2 Precoated plates

Remove microwell strips and reseal the aluminum pouch containing the unused strips and desiccant bag with scotch tape. The unused strips thus resealed may be stored up to 15 days at 2°-8°C.

2.8.5.3 Wash

1. Fill vaccupette trough with wash buffer.
2. Decant plate completely.
3. Fill dispenser by pulling syringe to the 22 ml mark.
4. Line up wells on plate with dispenser.
5. Deliver wash solution by gently pushing syringe in. Do not allow dispenser tips to touch solution.
6. Decant and drain briefly on paper towels.

2.8.5.4 Sample Preparation

Samples are tested at 1:5 dilution. Results obtained using a 1:5 dilution are read directly form the calibration curve. Samples containing tPA in amounts

greater than the highest standard must be repeated at a 1:10 dilution. In this case the values read from the calibration curve will be multiplied by 2.

2.8.5.5 Standard Preparation

Use the reconstituted reference tPA, titrating exactly 10 ng/ml. This corresponds to 50 ng/ml in actual assay conditions since plasma is diluted 1:5.

	Standard Vol of tPa Ref. (µl)	Vol of buffer (µl)	tPA (ng/ml)
A	1,000	0	50
B	500	500	25
C	200	800	10
D	100	900	5
E	50	950	2.5
BLANK	0	1000	0

2.8.5.6 Assay

1. Pipette 200 µl of each diluted standard, control and sample in duplicate into each well of a precoated microwell plate.

2. Cover with tape and incubate 2 hours at room temperature on mixer.

3. Wash 5 times.

4. Add 200 µl of Anti-tPA-Peroxidase.

5. Cover with tape and incubate 2 hours at room temperature on mixer.

6. Wash 5 times.

7. Add 200 µl ABTS substrate at precisely timed intervals.

8. Stop reaction with 100 µl of 5% SDS at the same precisely timed intervals used in step 7 when the Optical Density of the A standard reaches 0.800-0.900.

9. Allow plate to mix for 10 minutes at room temperature before final reading.

10. Read microwell plate on MR5000/7000.

    a) Turn on MR5000/7000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test # (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
    e) Press "Enter"
    f) Enter plate ID # by pressing the key immediately below the cursor. When the ID is complete, press "Enter" to accept it.
    g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.8.6 Backup Procedure

The laboratory has access to another Microelisa reader in case of instrument malfunction.

2.8.7 Computation of Results

Use log-log program. O.D. are plotted on the abscissa (x--axis) and the corresponding tPA values are plotted on the ordinate (y-axis). For high levels of tPA it could be advantageous to construct the calibration curve on linear graph paper. This plot amplifies the elevated zone and allows a greater precision for these values. Patient plasmas diluted 1:5 are read directly from the calibration curve. For samples containing elevated levels of tPA prepare a 1:10 dilution and multiply results by 2.

Repeat Results if:

1. Duplicates do not match within 10%.
2. The unknown values fall outside the calibration curve.
3. Repeat all results in a column if column controls fall outside the established limits.

2.9 ß-Thromboglobulin (ß-ßTG)

2.9.1 Principle

Plasma ßTG is measured by an enzyme immunoassay. A plastic microwell plate coated with specific rabbit anti-ßTG is incubated with sample containing the ßTG to be measured. The ßTG contained in the plasma binds to the plastic plate by one specific antigenic determinant. Next, rabbit anti-ßTG antibody coupled with peroxidase is added and this binds to the remaining free antigenic determinants of the ßTG forming a sandwich. The bound enzyme peroxidase is then revealed by ABTS substrate. The color change that is produced is a direct measure of the ßTG concentration initially present in the plasma.

2.9.2 Reagents/Preparation

1. Precoated Plates: Each pouch contains 96 microwells precoated with specific rabbit anti-ßTG F(ab')2 fragments.

2. Dilution Buffer: Prepare a 1:10 dilution by transferring contents of the vial into a 200 ml graduated cylinder. Fill to the 200 ml mark with distilled water. The diluted buffer is stable for 15 days at 2°-8°C. 1 vial contains concentrated phosphate buffer with bovine albumin and Tween-20.

3. Washing Solution: Prepare a 1:20 dilution by transferring the contents of the vial into a 1 liter cylinder and fill to the 1000 ml mark with distilled water. The diluted wash solution is stable for 15 days at 2°-8°C. 1 vial contains a concentrated solution of phosphate sodium chloride and Tween-20.

4. Reference: Reconstitute the vial with 5ml of dilution buffer to obtain a 20 ng/ml solution of ßTG which, corresponds to 200 ng/ml in actual assay conditions since plasma samples are diluted 1:10. The reconstituted material is stable for 24 hours at 2°-8°C. 1 vial contains lyophilized, purified human ß- Thromboglobulin determined against the international standard.

5. Antibody-Enzyme Conjugate: Reconstitute the vial of anti-ßTG-peroxidase conjugate with 20 ml of dilution buffer. Reconstitute just before use. The reconstituted reagent is stable for 24 h at 2°-8°C. 1 vial contains specific rabbit anti-PF4 antibody coupled with peroxidase, lyophilized with stabilizers.

6. ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]): The solution is ready for use as received. Observe expiration date on vial.

7. 5% SDS (Sigma) Sodium dodecyl sulfate) Dissolve 50 grams in 1000 ml distilled water. Store at room temperature. Discard if solution becomes turbid.

2.9.3 Quality Control Material

Combination Anticoagulant Control Pool (CACP) is a normal pooled plasma collected in house using a combined anticoagulant that inhibits platelet activation in-vitro. Dilute control material 1:5 with dilution buffer.

2.9.4 Equipment and Supplies

Titertek multichannel pipet and tips
Titertek reagent reservoirs
Eppendorf digital 10-100 μl pipettes
Eppendorf digital 100-1000 μl pipettes
Culturetik Vaccupette with 50 ml syringe attached
Microplate Mixer
MR 5000/7000 Elisa Reader
1 ml volumetric pipette
Stopwatch

2.9.5 Procedure

2.9.5.1 Sample Collection

Collect blood into 0.7 ml of combination anticoagulant solution for each 7 ml of blood as described in ARIC Manual 7. Invert gently 8 times and place in melting ice bath. Spin at 4°C for 10 minutes at 3000 rpm. Filter plasma through 0.45 u Millipore filter and store frozen at -70°C.

2.9.5.2 Coating

Carefully cut open one end of the aluminum pouch. Pull out the plastic plate. Break off as many of the 16 well strips as needed. Replace the unused strips in the aluminum pouch with the desiccant bag. Seal the aluminum pouch with scotch tape. The unused strips may be stored up to 15 days at 2°-8°C.

2.9.5.3 Wash

1. Fill Vaccupette trough with wash buffer.
2. Decant plate completely.
3. Fill dispenser by pulling syringe to the 22 ml mark.
4. Line up wells on plate with dispenser.
5. Deliver wash solution by gently pushing syringe in. Do not allow dispenser tips to touch solution.
6. Decant and drain briefly on paper towels.

2.9.5.4 Sample Preparation

Plasma samples are tested at 1:10 dilution. Results obtained using a 1:10 dilution are read directly from the calibration curve. Samples containing ßTG in amounts greater than the highest standard must be repeated at a 1:20 dilution. In this case the values and read from the calibration curve will be multiplied by 2.

2.9.5.5 Standard Preparation

Use the reconstituted reference ßTG, titrating exactly 20 ng/ml. This corresponds to 200 ng/ml in actual assay conditions since patients' plasmas are diluted 1:10.

Standard	Reference Volume (µl)	Buffer Volume (µl)	ßTG (ng/ml)
A	1,000	0	200
B	500	500	100
C	250	750	50
D	100	900	20
E	50	950	10
BLANK	0	1000	0

2.9.5.6 Assay

1. Pipette 200 µl of each diluted standard, control and unknown plasma in duplicate into each well of a pre-coated micro well plate.

2. Cover with tape and incubate 1 h at room temperature on mixer.

3. Wash 5 times.

4. Add 200 µl of Anti-ßTG-Peroxidase.

5. Cover with tape and incubate 1 h at room temperature on mixer.

6. Wash 5 times.

7. Add 200 µl ABTS substrate at precisely timed intervals.

8. Stop reaction with 100 µl of 5% SDS at the same precisely timed intervals used in step 7 when the Optical density of "A" Standard reaches 0.800 - 0.900. (4 min)

9. Allow plate to mix for 10 minutes at room temperature before final reading.

10. Read microwell plate on MR5000/7000.

    a) Turn on MR5000/7000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test # (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
    e) Press "Enter"
    f) Enter plate ID # by pressing the key immediately below the cursor. When the ID is complete, press "Enter" to accept it.
    g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.9.6 Backup Procedure

The laboratory has access to another Microelisa reader in case of instrument malfunction.

2.9.7 Computation of Results

Use log-log program. ßTG levels are plotted on the abscissa (x-axis) and the

corresponding Optical density values are plotted on the ordinate (y-axis). For high levels of ßTG it could be advantageous to construct the calibration curve on linear graph paper. This plot amplifies the elevated zone and allows a greater precision for these values. Patient plasmas diluted 1:10 are read directly from the calibration curve. For samples containing elevated levels of ßTG prepare a 1:20 dilution and multiply results by 2.

Repeat Results if:

1. Duplicates do not match within 10%.
2. The unknown value falls outside of the calibration curve.
3. Repeat all results in a column if column controls fall outside established limits.

2.10 Platelet Factor 4 (PF4)

2.10.1 Principle

Plasma PF4 is measured by an enzyme immunoassay. A plastic microwell plate coated with specific rabbit anti-PF4 is incubated with sample containing the PF4 to be measured. The PF4 contained in the plasma binds to the plastic plate by one specific antigenic determinant. Next, rabbit anti-PF4 antibody coupled with peroxidase is added and this binds to the remaining free antigenic determinants of the PF4 forming a sandwich. The bound enzyme peroxidase is then revealed by ABTS substrate. The color change that is produced is a direct measure of the PF4 concentration initially present in the plasma.

2.10.2 Reagents/Preparation

1. Precoated Plates: Each pouch contains 96 microwells precoated with specific rabbit anti-PF4 F(ab')2 fragments.

2. Dilution Buffer: Prepare a 1:10 dilution by transferring contents of the vial into a 200 ml graduated cylinder. Fill to the 200 ml mark with distilled water. The diluted buffer is stable for 15 days at 2°-8°C. 1 vial contains concentrated phosphate buffer with bovine albumin and Tween-20.

3. Washing Solution: Prepare a 1:20 dilution by transferring the contents of the vial into a 1 liter cylinder and fill to the 1000 ml mark with distilled water. The diluted wash solution is stable for 15 days at 2°-8°C. 1 vial contains a concentrated solution of phosphate sodium chloride and Tween-20.

4. Reference PF4: Reconstitute the vial with 5 ml of dilution buffer to obtain a 20 ng/ml solution of PF4 which, corresponds to 100 ng/ml in actual assay conditions since plasma samples are diluted 1:5. The reconstituted material is stable for 24 hours at 2°-8°C. 1 vial contains lyophilized, purified human Platelet Factor 4 determined against the international standard.

5. Antibody-Enzyme Conjugate: Reconstitute the vial of anti-PF4-peroxidase conjugate with 20 ml of dilution buffer. Reconstitute just before use. The reconstituted reagent is stable for 24 h at 2°-8°C. 1 vial contains specific rabbit anti-PF4 antibody coupled with peroxidase, lyophilized with stabilizers.

6. ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]): The solution is ready for use as received. Observe expiration date on vial.

7. 5% SDS (Sigma) Sodium dodecyl sulfate. Dissolve 50 grams in 1000 ml distilled water. Store at room temperature. Discard if solution becomes turbid.

2.10.3 Quality Control Material

Combination Anticoagulant Control Pool (CACP) is a normal pooled plasma collected in house using a combined anticoagulant that inhibits platelet activation in-vitro. Dilute control material 1:5 with dilution buffer.

2.10.4 Equipment and Supplies

Titertek multichannel pipet and tips
Titertek reagent reservoirs
Dynatech Immulon 2 Microliter Plates
Eppendorf digital 10-100 µl pipette
Eppendorf digital 100-1000 µl pipette
Culturetik Vaccupette with 50 ml syringe attached
Microplate Mixer
MR 5000/7000 Elisa Reader
1 ml volumetric pipette
Stopwatch

2.10.5 Procedure

2.10.5.1 Sample Collection

Collect blood into 0.7 ml of combination anticoagulant solution for each 7 ml of blood as described in ARIC Manual 7. Invert gently 8 times and place in melting ice bath. Spin at 4°C for 10 minutes at 3000 rpm. Filter plasma through 0.45 u Millipore filter and store frozen at -70°C.

2.10.5.2 PreCoated Plate

Carefully cut open one end of the aluminum pouch. Pull out the plastic plate. Break off as many of the 16 well strips as needed. Replace the unused strips in the aluminum pouch with the desiccant bag. Seal the aluminum pouch with scotch tape. The unused strips may be stored up to 15 days at 2°-8°C. The plate is ready for use.

2.10.5.3 Wash

1. Fill Vaccupette trough with wash buffer.
2. Decant plate completely.
3. Fill dispenser by pulling syringe to the 22 ml mark.
4. Line up wells on plate with dispenser.
5. Deliver wash solution by gently pushing syringe in. Do not allow dispenser tips to touch solution.
6. Decant and drain briefly on paper towels.

2.10.5.4 Sample Preparation

Plasma samples are tested at 1:5 dilution. Results obtained using a 1:5 dilution are read directly from the calibration curve. Samples containing PF4 in amounts greater than the highest standard must be repeated at a 1:10 dilution. In this case the values and read from the calibration curve will be multiplied by 2.

2.10.5.5 Standard Preparation

Use the reconstituted reference PF4, titrating exactly 20 ng/ml. This corresponds to 100 ng/ml in actual assay conditions since patients' plasmas are diluted 1:5.

Standard	Reference Volume (µl)	Buffer Volume (µl)	PF4 (ng/ml)
A	1,000	0	100
B	500	500	50
C	250	750	25
D	100	900	10
E	50	950	5
F	25	975	2.5
BLANK	0	1000	0

2.10.5.6 Assay

1. Pipette 200 µl of each diluted standard, control and unknown plasma in duplicate into each well of a pre-coated micro well plate.

2. Cover with tape and incubate 1 h at room temperature on mixer.
3. Wash 5 times.

4. Add 200 µl of Anti-PF4-Peroxidase.

5. Cover with tape and incubate 1 h at room temperature on mixer.

6. Wash 5 times.

7. Add 200 µl ABTS substrate at precisely timed intervals.

8. Stop reaction with 100 µl of 5% SDS at the same precisely timed intervals used in step 7 when the Optical density of "A" Standard reaches 0.800 - 0.900.

9. Allow plate to mix for 10 minutes at room temperature before final reading.

10. Read microwell plate on MR5000/7000.

    a) Turn on MR5000/7000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test # (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
    e) Press "Enter"
    f) Enter plate ID # by pressing the key immediately below the cursor. When the ID is complete, press "Enter" to accept it.
    g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.10.6 Backup Procedure

The laboratory has access to another Microelisa reader in case of instrument malfunction.

2.10.7 Computation of Results

Use log-log program. PF4 levels are plotted on the abscissa (x-axis) and the

corresponding Optical density values are plotted on the ordinate (y-axis). For high levels of PF4 it could be advantageous to construct the calibration curve on linear graph paper. This plot amplifies the elevated zone and allows a greater precision for these values. Patient plasmas diluted 1:5 are read directly from the calibration curve. For samples containing elevated levels of PF4 prepare a 1:10 dilution and multiply results by 2.

Repeat Results if:

1. Duplicates do not match within 10%.
2. The unknown value falls outside of the calibration curve.
3. Repeat all results in a column if column controls fall outside established limits.

2.11 Fibrinopeptide A (FPA)

2.11.1 Principle

Plasma FPA is measured by enzyme immunoassay. Interfering fibrinogen in test samples is adsorbed by bentonite treatment. Reference and test samples are then incubated with a consistent quantity of excess anti-FPA rabbit antibodies. An aliquot of this mixture is then incubated in microwells previously coated with synthetic FPA. The quantity of anti-FPA bound on the solid support is inversely proportional to FPA concentration in sample. The bound antibodies are revealed by goat anti rabbit antibodies coupled with peroxidase. The enzyme activity is measured by its activity on ABTS chromogenic substrate.

2.11.2 Reagents/Preparation

1. Coating Solution: Reconstitute with 20 ml distilled water. Solution is stable for 72 h at 2°-8°C.

2. Dilution buffer: make a 1:10 dilution with distilled water. Pour contents of vial into cylinder and fill to the 20 ml mark. The solution is stable for 15 days at 2°-8°C.

3. Washing solution: Make a 1:20 dilution with distilled water. Pour contents of vial into cylinder and fill to the 1000 ml mark. The solution is stable for 15 days at 2°-8°C.

4. Anti-FPA rabbit serum: Reconstitute with 10 ml of dilution buffer. Solution is stable 24 hours at 2°-8°C.

5. FPA Reference: Reconstitute with 0.5 ml distilled water. Working Standard: Dilute Reference 1:100 in dilution buffer. Mix 100 μl of FPA Reference in 9.9 ml dilution buffer. This working standard contains 25 ng/ml of FPA. Reconstituted reference solution is stable 24 hours at 2°-8°C.

6. Immuno-conjugate (anti IgG peroxidase): Reconstitute with 20 ml dilution buffer. Solution is stable 24 hours at 2°-8°C.

7. ABTS (Kirkegaard & Perry) (2.2'-azino-di [3-ethyl- benzthiazoline sulfonate]): Solution is ready for use as received. Observe expiration date on bottle.

8. 5% SDS (Sigma) Sodium dodecyl sulfate. Dissolve 50 g in 100 ml distilled water. Store at room temperature. Discard if turbid.

2.11.3 Quality Control Material

CACP Combination Anticoagulant Control Pool (CACP) is a normal pooled plasma collected in-house using a combined anticoagulant that inhibits platelet activation "in-vitro". No dilution is required for the control material.

2.11.4 Equipment

Titertek Multichannel Pipet
Titertek reagent reservoir
Dynatech Immulon 2 Microtiter plates
Eppendorf digital 10-100 µl pipette
Eppendorf digital 100-1000 µl pipette
Culture-tek Vacupette/96 washer with 50 ml syringe attached.
MR 5000/7000 ELISA Reader.

2.11.5 Procedure

2.11.5.1 Sample Collection

Specimens should be collected by flawless venipuncture into combination anticoagulant as described in ARIC manual 7. Invert gently 8 times and place in a melting ice bath. Spin at 3000 x g for 10 minutes at 2°-8°C. Separate plasma from cells. Filter plasma through a 0.45 micron filter to remove any remaining platelets or cellular debris. Samples are frozen rapidly and stored at -70°C.

2.11.5.2 Coat Plates

1. Add 200 µl of FPA coating to each well of microplate.
2. Incubate covered night at room temperature on a level surface.

2.11.5.3 Wash

1. Fill Vaccupette trough with wash buffer.
2. Decant plate completely.
3. Fill dispenser by pulling syringe to the 22 ml mark.
4. Line up wells on plate with dispenser.
5. Deliver wash solution by gently pushing syringe in. Do not allow dispenser tips to touch solution.
6. Decant and drain briefly into paper towels.

2.11.5.4 Sample Preparation

1. Vigorously shake bentonite solution till homogenous.
2. Add 0.5 ml bentonite to 1.0 ml plasma.
3. Mix by inversion for 10 minutes.
4. Spin 15 minutes at 3000 rpm.
5. Add 0.4 ml of bentonite to 0.8 ml of supernatant
6. Repeat steps 3 and 4.
7. Pipet 0.5 ml of supernatant into a micro sample tube.
8. Add 25 µl Tween 20.

2.11.5.5 Standard Preparation

The working Standard (25 ng/ml) is used to prepare the standard curve in dilution buffer.

Standard	Qty of Std	Qty of buffer	FPA(ng/ml)
A	1 ml Working Std	-	25.0
B	1 ml Working Std	1 ml	12.5
C	1 ml (B)	1 ml	6.25
D	1 ml (C)	1 ml	3.12
E	1 ml (D)	1 ml	1.56
F	1 ml (E)	1 ml	0.78

* Discard 1 ml of dilution F

2.11.5.6 Assay

1. Pipet 500 µl of each sample and control (after bentonite treatment) and 500 µl of each standard dilution into micro sample tubes.
2. Add 50 µl anti-FPA rabbit serum to each micro tube.
3. Shake and incubate 1 hour at 37°C or overnight at 2°-8°C.
4. Wash plate 5 times.
5. Add 200 µl of each sample control or standard containing anti-FPA, in duplicate to the appropriate well of the micro well plate.
6. Cover with masking tape.
7. Incubate 1 hour at room temperature on mixer.
8. Wash 5 times.
9. Add 200 µl Anti IgG-peroxidase solution to each well.
10. Cover and incubate 1 hour at room temperature on mixer.
11. Wash 5 times.
12. Add 200 µl ABTS substrate at precisely timed intervals.
13. Stop reaction with 5% SDS at the same precisely timed intervals used in step 12 when the Optical Density (O.D.) of "F" Standard reaches 0.700.
14. Allow plate to mix for 10 minutes at room temperature before final reading.
15. Read microwell plate on MR5000/7000.

    a) Turn on MR5000/7000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test # (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
    e) Press "Enter"
    f) Enter plate ID # by pressing the key immediately below the cursor. When the ID is complete, press "Enter" to accept it.

g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.11.6 Back-up Procedure

The laboratory has access to another Microelisa reader in case of instrument malfunction.

2.11.7 Computation of Results

Use a semi log program. Enter FPA levels on the abscissa and the corresponding O.D. on the ordinate. Bentonite treatment introduces a 1:2 dilution. Therefore, all results must be multiplied by 2. Values obtained that are higher than the highest standard concentration must be diluted 1:4. The results of the 1:4 dilution are multiplied by 8.

Repeat Results if:

1. Duplicates do not match within 10%.
2. The unknown values fall outside the calibration curve.
3. Repeat all results in a column if column controls fall outside established limits.

2.12 D-dimer

2.12.1 Principle

A plastic microwell support coated with specific mouse monoclonal anti-D-dimer antibody is allowed to come into contact with plasma containing the D-dimer to be measured. The D-dimer contained in the plasma binds to the plastic support by one specific antigenic determinant. Next, rabbit anti-D-dimer antibody coupled with peroxidase is added and this binds to the remaining free antigenic determinants of the D-dimer, forming a sandwich. The bound enzyme peroxidase is then revealed by ABTS substrate. The color change that is produced is a direct measure of the D-dimer concentration initially present in the plasma.

2.12.2 Reagents/Preparation

1. Precoated Plate: Plate of 96 microwells precoated with mouse monoclonal anti-D-Dimer F(ab')2 fragments, stabilized and hermetically sealed in an aluminum pouch. The 96 well plate consists of 6 strips, each of 16 wells (2x8-well columns).

2. Dilution Buffer: Prepare a 1:10 dilution by transferring the contents of the vial into a 200 ml graduated cylinder. Then fill to the 1000 ml mark. The diluted wash solution is stable for 15 days at 2°-8°C.

3. Washing Solution: Prepare a 1:20 dilution by transferring the contents of the vial into a 1 liter cylinder. Then fill to the 1000 ml mark. The diluted buffer is stable for 15 days at 2°-8°C.

4. Reference D-dimer: Reconstitute the vial with 5 ml of dilution buffer to obtain a 50 ng/ml solution of D-dimer which corresponds to 1,000 ng/ml in actual assay conditions since unknown plasma is diluted 1:20. The reconstituted material is stable for 8 h at room temperature and 12 h at 2°-8°C.

5. Anti-D-Peroxidase Conjugate: rabbit anti-fragment D antibody coupled with peroxidase, lyophilized with stabilizers. Just before use,

reconstitute with 20 ml of dilution buffer. Stable for 24 hrs at 2°-8°C.

6. ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]): The solution is ready for use as received. Observe expiration date on vial.

7. 5% SDS (Sigma) Sodium Dodecyl Sulfate. Dissolve 50 grams in 1000 ml distilled water. Store at room temperature. Discard if solution becomes turbid.

2.12.3 Quality Control Material

Universal Coagulation Reference Plasma (UCRP) is the internal quality control material used. Reconstitute the vial with 1 ml distilled water. Wait 15 minutes then swirl gently to mix before use.

2.12.4 Equipment and Supplies

Titertek multichannel pipet and tips
Titertek reagent reservoirs
Dynatech Immulon 2 Microtiter Plates
Eppendorf digital 10-100 µl pipette
Eppendorf digital 100-1000 µl pipette
Culturetik Vaccupette with 50 ml syringe attached
Microplate Mixer
MR 5000/7000 Elisa Reader
1 ml volumetric pipette
Stopwatch

2.12.5 Procedure

2.12.5.1 Sample Collection

Collect blood into sodium citrate anticoagulant solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution as described in ARIC manual 7. Invert tube gently 8 times and place in melting ice bath. Spin for 10 minutes at 3000 x g. Separate plasma immediately and store frozen at -70°C.

2.12.5.2 PreCoated Plate

Carefully cut open one end of the aluminum pouch. Pull out the plastic plate. Break off as many of the 16 well strips as needed. Replace the unused strips in the aluminum pouch with the desiccant bag. Seal the aluminum pouch with scotch tape. The unused strips may be stored up to 15 days at 2°-8°C. The plate is ready for use.

2.12.5.3 Wash

1. Fill Vaccupette trough with wash buffer.
2. Decant plate completely.
3. Fill dispenser by pulling syringe to the 2 ml mark.
4. Line up wells on plate with dispenser.
5. Deliver wash solution by gently pushing syringe in. Do not allow dispenser tips to touch solution.
6. Decant and drain briefly on paper towels.

2.12.5.4 Sample Preparation

Patients' plasmas are tested at 1:20 dilution. Results obtained using a 1:20 dilution are read directly from the calibration curve. Samples containing D-dimer in amounts greater than the highest standard must be repeated at a 1:40 dilution. In this case the values read from the calibration curve will be multiplied by 2.0.

2.12.5.5 Standard Preparation

Reconstitute with 1 ml of distilled water. Use Dilution Buffer to prepare a 1:20 dilution of the D-Di Reference by adding 100 µl of reference to 1,900 µl of Dilution Buffer. This dilution represents the starting solution. The starting solution corresponds to a D-dimer level of 1000 ng/ml. Then use Dilution Buffer to prepare 1:2, 1:4, 1:10, and 1:20 dilutions of the starting solution.

Construct the calibration curve as follows:

Dil.	Vol of Start Solution (µl)	Vol of Buffer (µl)	D-Di (ng/ml)
1:2	500	500	500
1:4	500 of 1:2	500	250
1:10	100	900	100
1:20	500 of 1:10	500	50
BLANK	0	1000	0

2.12.5.6 Assay

1. Pipet 200 µl of each diluted standard, control and sample in duplicate into appropriate wells of the micro well plate.

2. Cover with tape and incubate 1 h at room temperature on mixer.

3. Wash 5 times.

4. Just before use, reconstitute the Anti-D-Peroxidase conjugate with 20 ml dilution buffer.

5. Add 200 µl of Anti-D-Peroxidase to all wells.

6. Cover with tape and incubate 1 h at room temperature on mixer.

7. Wash 5 times.

8. Add 200 µl ABTS substrate at precisely timed intervals.

9. Stop reaction with 100 µl of 5% SDS at the same precisely timed intervals used in step 7 when the Optical Density (O.D.) of "A" Standard reaches 0.800 - 0.900. (approximately 8 min.)

10. Allow plate to mix for 10 minutes at room temperature before final reading.

11. Read microwell plate on MR5000/7000.

    a) Turn on MR5000/7000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test # (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).

e) Press "Enter"
f) Enter plate ID # by pressing the key immediately below the cursor. When the ID is complete, press "Enter" to accept it.
g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.12.6 Backup Procedure

The laboratory has access to another Microelisa reader in case of instrument malfunction.

2.12.7 Computation of Results

Use log-log program. D-dimer levels are plotted on the abscissa (x-axis) and the corresponding O.D. values are plotted on the ordinate (y-axis). For high levels of D-dimer it could be advantageous to construct the calibration curve on linear graph paper. This plot amplifies the elevated zone and allows a greater precision for these values. Patient plasmas diluted 1:20 are read directly from the calibration curve. For samples containing elevated levels of D-dimer prepare a 1:40 dilution and multiply results by 2.0.

Results must be repeated if:

1. Duplicates do not match within 10%.

2. The unknown value falls outside the calibration curve.

3. Repeat all results in a column if column controls fall outside the established limits.

2.13 Plasminogen Activator Inhibitor-1 (PAI-1)

2.13.1 Principle

Plasma PAI-1 is measured by enzyme immunoassay. Micro-test wells are pre-coated with mouse monoclonal anti-PAI-1 antibodies, which upon sample incubation binds the PAI-1 molecules present in the sample. A second monoclonal anti-PAI-1 antibody conjugated to the enzyme peroxidase, is added after the sample incubation period. The antibody conjugate binds to another free antigenic determinant on the PAI-1 molecules immobilized in the well by the coat antibody forming a "sandwich". All unbound material is then washed away and a peroxidase enzyme substrate is added. The substrate yields a yellow color upon reaction with the peroxidase conjugated second antibody. The amount is directly proportional to the amount of PAI-1 in the original sample.

The assay detects active and latent inactive forms of PAI-1, though tPA/PAI-1 and uPA/PAI-1 complexes are recovered with low efficiency. The detection level is below 1 ng/ml PAI-1 in the undiluted sample.

Reagents/Preparation

1) Pre-coated Plate: Each kit contains 1 plate of 96 microwells, pre-coated with anti-PAI-1 mouse monoclonal IgG. Store at 2°-6°C and use prior to the expiration date on the label.

2) PBS-EDTA-Tween 20 buffer substrate (PET). Dissolve the vial contents in the PET-buffer vial in 1 L of water (use a magnetic stirrer for 10 minutes). The PET-buffer is used for washing of the plate, dilution of the PAI-1 standard calibrator, dilution of test samples (when

necessary), and in preparation of the conjugate-antibody solution.

3) 1 vial of peroxidase conjugated anti-PAI-1 mouse monoclonal IgG. Dissolve the contents of the vial in 100 µl PET-buffer with gentle agitation for 3 minutes. 80 µl conjugate is diluted to 6 ml with PET-buffer. Mix well.

4) 1 (one) tablet of substrate; OPD.

5) Stop Solution: 4.5 mol/L H_2SO_4: To 15 ml distilled water add 5 ml of concentrated H_2SO_4 (95-97%). Mix.

2.13.3 Quality Control Material

Universal Coagulation Reference Plasma (UCRP) is the internal quality control material. Using a class A volumetric pipet, add 1.0 ml of water, swirl gently until all the cake is under water. Do not invert. Let stand for 15 minutes.

2.13.4 Equipment and Supplies

Titertek multichannel pipet and tips
Titertek reagent reservoirs
Eppendorf digital 10-100 µl pipette
Eppendorf digital 100-100 µl pipette
Microplate Mixer
MR 5000/7000 Elisa Reader
Class A 1 ml volumetric pipette
Stopwatch
Distilled water
Concentrated Sulfuric Acid
Fresh 30% Hydrogen Peroxide (of good quality)

2.13.5 Procedure

2.13.5.1 Sample Collection

Collect blood into 3.8% sodium citrate anticoagulate solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution as described in ARIC Manual 7. Invert tube gently 8 times and place in a melting ice bath. Spin at 4°C for 10 minutes at 3000 x g. Separate plasma from cells immediately and store at -70°C.

2.13.5.2 Prefilling the micro-test plate

Remove microwell strips from the aluminum pouch and empty the plate by tapping it against absorbing material. To remove the preservative (0.1% sodium azide), fill the wells completely with PET-buffer, then discard. Ensure that the plate is completely emptied. Add 100 µl PET-buffer to all wells. The plate is now ready for use.

2.13.5.3 Wash

1) Fill vaccupette trough with PET-buffer.

2) Decant plate completely.

3) Fill the dispenser by pulling the syringe to the 22 ml mark.

4) Line up the wells with the dispenser.

5) Deliver PET-buffer by gently pushing the syringe in. Do not allow the dispenser tips to touch the solution.

6) Wait 3 minutes.

7) Decant and drain briefly on paper towels.

8) Do not let plate dry out.

2.13.5.4 Sample Preparation

Thaw samples rapidly at 37°C. Samples are tested undiluted. Results must be repeated if the value obtained is higher than the highest reference value. Dilute the sample. Then multiply results by the appropriate dilution factor.

2.13.5.5 Standard Preparation

1) Add 0.5 ml water to the PAI-1 standard plasma vial (gently agitate for 3 minutes). The standard plasma can be stored if necessary, as aliquots in small tightly capped tubes at -20°C or colder.

2) Add 100 µl PET-buffer to 7 12x75 test tubes. Make a serial two-fold dilution in the following manner:

Standard	wkg. std/dil (µl)	vol. buffer (µl)	PAI-1 (ng/ml)
A	100 (wkg.std)	100	24
B	100 of "A"	100	12
C	100 of "B"	100	6
D	100 of "C"	100	3
E	100 of "D"	100	1.5
F	100 of "E"	100	0.75
G		100	0

2.13.5.6 Assay

1. Tear off the sealing foil and empty the plate by hitting it against paper towels. This removes the preserving agent (0.05% sodium azide). Fill the wells with PET-buffer, then discard, making sure the plate is completely drained. Add 100 µl of PET-buffer to all wells with an eight channel pipette. The plate is now ready for use.

2. Pipette 10 µl of each standard, control, and sample in duplicate into the proper wells.

3. Cover and incubate at room temperature for two hours on the minimixer.

4. Add 50 µl of conjugate solution to all wells, without emptying the plate.

5. Incubate the plate on the minimix for 1 hour at room temperature.

6. Wash the plate 4 times.

7. Add 200 µl of the substrate at precisely timed intervals to all wells.

8. Incubate the plate for 20 minutes on the minimum at room temperature.

9. Stop the reaction by adding 100 ml of 4.5 mol/L at the same timed

intervals as step #7.

10. Allow the plate to mix for 10 minutes at room temperature before reading.

11. Read microwell plate on MR5000/7000.

    a) Turn on MR5000/7000.
    b) Press "MR5000".
    c) Press "Start".
    d) Enter pre-set test # (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
    e) Press "Enter"
    f) Enter plate ID # by pressing the key immediately below the cursor. When the ID is complete, press "Enter" to accept it.
    g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.13.6 Back-up Procedure

The laboratory has access to another Micro Elisa Reader in case of instrument malfunction.

2.13.7 Computation of Results

Plot the optical density (O.D.) against the amount of PAI-1 in the calibration samples. The amount of PAI-1 in the unknown is determined by comparing the sample O.D. with the calibration curve.

Repeat Results if:

1) Duplicates do not match within 10%.

2) The unknown values fall outside the calibration curve.

3) Repeat all results in a column if column controls fall outside established limits.

2.14 Protein S (Total and Free)

2.14.1 Principle

A plastic microwell support coated with specific rabbit anti-protein S antibody is allowed to come into contact with plasma containing the protein S to be measured. The protein S contained in the plasma binds to the plastic support by one specific antigenic determinant. Next, rabbit anti-protein S antibody coupled with peroxidase is added and this binds to the remaining free antigenic determinants of the protein S, forming a substrate. The color change that is produced is a direct measure of the protein S concentration initially present in the plasma.

2.14.2 Reagents/Preparation

1. Precoated plate: Each kit contains 1 plate of 96 microwells precoated with specific rabbit anti-protein S F(ab)' fragments, stabilized and hermetically sealed in an aluminum pouch.

2. Dilution Buffer: Each vial contains 20 ml of 10 times concentrated solution of phosphate buffer with bovine albumin and Tween-20. Prepare

a 1:10 dilution by transferring the contents of the vial into a 200 ml graduated cylinder. Fill to 200 ml. The diluted buffer is stable for 15 days at 2°-8°C.

3. Washing Solution: Each vial contains 50 ml of 20 times concentrated solution of phosphate, sodium chloride and Tween-20. Prepare a 1:20 dilution by transferring the contents of the vial into a 1 liter cylinder and fill to the 1000 ml mark. The diluted wash solution is stable for 15 days at 2°-8°C.

4. Anti-Protein S-Peroxidase Conjugate: Each vial contains specific rabbit anti-protein S antibody coupled with peroxidase, lyophilized with stabilizers. Reconstitute the vial of anti-protein S peroxidase conjugate with 20 ml of dilution buffer. Reconstitute just before use. The reconstituted reagent is stable for 24 hours at 2°-8°C.

5. ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate]): The solution is ready for use as received. Observe expiration date on vial.

6. 5% SDS (Sigma) Sodium Dodecyl Sulfate. Dissolve 50 grams in 1000 ml distilled water. Store at room temperature. Discard if solution becomes turbid.

7. PEG: Solution of polyethylene glycol 6000 at 25%. The solution is ready for use.

2.14.3 Quality Control Material/Calibration Material

Universal Coagulation Reference Plasma (UCRP) Reconstitute with 1 ml distilled water. Wait 15 minutes then swirl gently to mix before use.

Calibration material is Stago Proteins Control Plasma. Reconstitute with 0.5 ml of distilled water. Swirl gently until complete dissolution. Let stand at room temperature for 10 minutes. Stable for 4 hours at 20 C, 12 hrs at 2°-8°C, and 1 month at -20 C.

2.14.4 Equipment and Supplies

Titertek multichannel pipet and tips
Titertek reagent reservoirs
Eppendorf digital 10-100 µl pipette
Eppendorf digital 100-1000 µl pipette
Culturetik Vaccupette with 50 ml syringe attached
Microplate Mixer
MR 5000/7000 Elisa Reader
1 ml volumetric pipette
Stopwatch
Ice (for Free Protein S Assay)

2.14.5 Procedure

2.14.5.1 Sample Collection

Collect blood into 3.8% trisodium citrate anticoagulant solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution. Invert tube gently 8 times and place in melting ice bath. Spin at 4°C for 10 minutes at 3000 X g. Separate plasma immediately and store frozen at -70 C.

2.14.5.2 Coating

Remove microwell strips and reseal the aluminum pouch containing the unused strips and desiccant bag with scotch tape. The unused strips thus resealed may be stored up to 15 days at 2°-8°C.

2.14.5.3 Wash

1. Fill Vaccupette trough with wash buffer.

2. Decant plate completely.

3. Fill dispenser by pulling syringe to the 22 ml mark.

4. Line up wells on plate with dispenser.

5. Deliver wash solution by gently pushing syringe in. Do not allow dispenser tips to touch solution.

6. Decant and drain briefly on paper towels.

2.14.5.4 Sample Preparation

Thaw samples rapidly at 37°C. Patients' plasmas are tested at 1:100 dilution. Results obtained using a 1:100 dilution are read directly from the calibration curve. Samples containing Protein S in amounts greater than the highest standard must be repeated at a 1:200 dilution. In this case the values read from the calibration curve will be multiplied by 2. For samples containing low levels of Protein S it is preferable to test the plasma at 1:50 or 1:20 dilution. In these cases, the values obtained from the calibration curve are divided by 2 or 5 respectively.

2.14.5.5 Standard Preparation

Use dilution buffer to prepare a 1:100 dilution of the Protein S Control Plasma by adding 20 µl of the reconstituted control plasma to 1980 ml of dilution buffer. This dilution represents the starting solution. By definition this dilution represents the 100% point of the value indication on the Control Plasma Assay Value insert. (This value will change from lot number to lot number.)

Use Dilution Buffer to prepare 1:2, 1:4. 1:10, and 1:20 dilutions of the starting solution.

1:2 = 50% of the starting solution
1:4 = 25% of the starting solution
1:10 = 10% of the starting solution
1:50 = 5% of the starting solution

Prepare the calibration curve as follows:

Dilution	Working Sol. (µl)	Dil Buffer (µl)	Prot S (%)
1:2	1000	1000	50
1:4	1000(1:2)	1000	25
1:10	100	900	10
1:20	500(1:10)	500	5

2.14.5.6 Assay

1. Pipette 200 µl of each standard, control and patient in duplicate into

each well of a precoated microwell plate.

2. Cover with tape and incubate 2 h at room temperature on mixer.

3. Wash 5 times.

4. Add 200 µl of Peroxidase conjugate.

5. Cover with tape and incubate 2 h at room temperature on mixer.

6. Wash 5 times.

7. Add 200 µl ABTS substrate at precisely times intervals.

8. Stop reaction with 100 µl of 5% SDS at the same precisely timed intervals used in step 7 when the O.D. of A Standard reaches 0.800 - 0.900. (4 MIN.)

9. Allow plate to mix for 10 minutes at room temperature before final reading.

11. Read microwell plate on MR5000/7000.

a) Turn on MR5000/7000.
b) Press "MR5000".
c) Press "Start".
d) Enter pre-set test # (1 = ABTS, dual wavelength, test filter 410nm, ref. filter 490nm).
e) Press "Enter"
f) Enter plate ID # by pressing the key immediately below the cursor. When the ID is complete, press "Enter" to accept it.
g) The plate will then be read and a printout will be generated with the OD's to be used in the calculation of results.

2.14.6 Backup Procedure

This department has access to another Microelisa reader in case of instrument malfunction.

2.14.7 Computation of Results

Use log-log program. Protein S levels are plotted on the abscissa (x-axis) and the corresponding O.D. values are plotted on the ordinate (y-axis). In some cases, it could be advantageous to use a straight line regression program. This plot amplifies the normal zone and allows a greater precision for these values. The log log program is recommended for low protein S values. Patient plasmas diluted 1:100 are read directly from the calibrated curve. For samples containing elevated levels of Protein S prepare a 1:200 dilution and multiply results by 2. Test samples with low levels of Protein S at 1:50 or 1:20 dilution. The results obtained must be divided by 2 or 5 respectively.

2.14.8 Results must be repeated if:

1. Duplicates do not match within 10%.

2. The unknown value falls outside the calibration curve.

3. Repeat all results in a column if column controls fall outside established limits.

2.14.9 Alternative Protocol for Free Protein S Assay

Treat all patient samples, Standards, and Controls as follows:

1. Pipet 300 µl of citrate plasma into a plastic tube
2. Add 50 µl of 25% PEG
3. Shake well
4. Incubate for 30 minutes in a melting ice bath
5. Centrifuge 10 minutes at 3,000 rpm

The C4b-BP bound protein S is precipitated whereas the free protein S remains in the plasma supernatant. Perform the assay of free protein S as in ASSAY PROCEDURE.

3.0 DATA MANAGEMENT

The Central Hemostasis Laboratory is semi-automated for sample logging, sample retrieval, data storage, and data transmittal. The general concept is discussed below.

3.1 Computer Network at the Central Hemostasis Laboratory

The basic network provides a communication environment linking blood analysis devices through control stations (IBM PC's) to a primary data storage and processing facility (IBM PC/AT). All IBM processing units are connected in such a fashion to enable them to share programs, data and hardware such as disk drives, printers, and other external processing units.

The laboratory's file server is an IBM PC/AT base unit with 20 MB and 30 MB hard disks. The file server also has an AST Advantage memory multifunction board and 1.5 MB extended RAM.

A Maynard Maynstream tape backup drive with Maynard software and interface card provides protection against local data loss. The laboratory supervisor backs up the system three times per week.

Three work stations are connected to the file server via IBM PCN network interface cards and PCN modulator units. Two of the work stations are linked to two separate Coag-A-Mate instruments via serial ports. This allows either raw data, generally time in seconds, or calculated results to be transferred directly to the data base.

3.2 Software for Data Management

This system uses non-dedicated Novell Netware version 2.0a.

3.3 Data Transmission

Thirteen aliquots of plasma and serum per subject are sent in weekly batches from each Field Center to the Hemostasis Lab. Donor Information Forms and an inventory record on paper accompanies each batch of specimens. Specimen analyzes are performed on a gamma counter, a Coag-A-Mate analyzer and an ELISA reader: software written for Coag-A-Mate permits transmittal of results directly onto an IBM PC-XT. Other results are entered manually by tech. Results are sent monthly form the Central Hemostasis Laboratory to the Coordinating Center on floppy disks for transfer into the main study database.

The Central Hemostasis Laboratory notifies Field Centers promptly by electronic mail when participant results are in clinically critical ranges ("ARIC alert values").

Inventory records listing participant ID numbers for blood specimens are sent weekly from the Field Centers to the Coordinating Center. Data backup at the Field Centers includes electronic copies of the inventory records of specimens sent but does not include extra blood specimens. The Coordinating Center sends a floppy disk weekly to each Field Center containing relevant results from all central agencies in order to update the local databases.

4.0 QUALITY CONTROL

4.1 Quality Control For Assay Procedures

4.1.1 Introduction

The Hemostasis Laboratory has adhered to rigid internal quality control and external assurance programs provided by the college of American Pathologists (CAP). A high degree of precision and accuracy has been achieved and established. Data obtained from controls are analyzed daily and monthly. The mean and S.D. of results from the Central Hemostasis Laboratory are comparable with those from other laboratories using the same reagents and instruments. The coefficient of variation (C.V.) of PT is always <4% and that of PTT is <5%.

The internal quality control for measuring coagulation activities is of particular importance because of variabilities of reagents and instruments. Since water ions may cause variation in clot information, the Central Hemostasis Laboratory has a built-in distilled water supply which is routinely used for buffer preparation and making dilutions. All instruments have maintenance schedules that are rigidly adhered to.

Several types of quality control material are used for ARIC analysis, including freshly prepared, fresh frozen, and lyophilized controls. Because there are advantages and disadvantages of each type of preparation, several of the tests include both lyophilized and fresh-frozen controls. Commercial lyophilized controls have been proved by external survey programs to be clearly the best for quality control. The commercial lyophilized controls used for ARIC analyses include VNC and UCRP. They are more stable than regular plasma samples and therefore an analysis of precision on such controls tends to underestimate the variability of testing subjects' plasma samples.

Hence, normal in-house pools are run to duplicate the conditions of subjects' plasma. The in-house pools used for ARIC analyses include CACP and NPP.

All tests performed on the Coag-A-Mate (PTT, fibrinogen, Factor VII, and Factor VIII) are performed in duplicate. Duplicates must match within 10% or be repeated. Controls are run each day and mean values, standard deviations, and coefficients of variation have been established. A chart monitors intra- and inter- day variations. As an additional control on Factor VII and Factor VIII, the deficient plasmas have been determined at the factory to have less than 1% activity and contain no inhibitors.

The quality control for AT-III chromogenic assay and all Elisa assays will be performed by running UCRP on each column. As the addition of reagents to each row is critically timed, each UCRP value must fall within two standard deviations of the cumulative mean or the entire column must be repeated.

4.1.2 Standards

Standards which are used for constructing a calibration curve in each assay are listed in Table 1. When changing standards or lot numbers of standards, the new standard material is run simultaneously with the established standard material for at least 20 runs. The standards for each assay are briefly described below.

4.1.2.1 Fibrinogen

Standard fibrinogen preparation (Fibriquick) is a supplied by General Diagnostic.

4.1.2.2 Factors VII:C, VIII:C, VWF: Ag, Protein C: Ag and AT-III activity

Universal Coagulation Reference Plasma (UCRP) is purchased from Pacific Hemostasis.

4.1.2.3 PF-4, TG, FPA, D-dimer and Protein S

Reference samples are made and supplied by the manufacturers with their kits.

4.1.3 Quality Control Material

Quality control materials for each assay and sources are listed in Table 2. One control material is run several times in duplicate per run. This will allow assessment of intra assay variability. The control material used for each assay is described below.

4.1.3.1 aPTT, Fibrinogen, Factor VII:C, Factor VIII:C, VWF, AT-III and Protein C

Universal Coagulation Reference Plasma (UCRP) Curtin Matheson is the internal quality control material. Duplicates of each control are included at the beginning and the end of each coagulation tray and on each column of ELISA trays.

4.1.3.2 PF-4 and TG and FPA

Combination anticoagulant control pool (CACP) is prepared from at least 20 normal subjects in our laboratory. Venous blood is drawn into a tube containing 1:10 volume of a combined anticoagulant (aprotinin, chloromethyl ketone peptide, EDTA and isobutyl methylxanthine). Following mixing, the sample is centrifuged at 3000 x g for 10 min. The plasma is filtered through 45 1 filters to remove cellular debris. Then the plasma is aliquoted and stored at -70°C. This control is included in each run for RIA assays and on each column for ELISA assays.

4.1.3.3 Control Limits

To initially establish limits for a new control material or lot number, the control material is run at least twenty times in duplicate on separate runs. Acceptable values will fall within two standard deviations of the mean though all values within 3 standard deviations of the mean will be included in quality control statistics.

A cumulative mean ±2SD ending on the last day of the previous month will be used for control limits. This target range will be recalculated monthly. A coagulation run will be repeated if the mean exceeds 2 standard deviation of the cumulative mean. ELISA column controls must fall within 2 standard deviation of the cumulative mean or all sample results on that column must be

repeated. A control shift is defined as five or more consecutive runs in which the daily mean for a control is above or below the overall mean, while a trend is considered to be seven consecutive runs in which a daily mean is either increasing or decreasing. Various conditions may result in shifts or trends, such as deterioration of control material, a defective lot of reagents, or a malfunction in a piece of equipment. The supervisor is notified in the case of an outlier, shift, or trend.

Reagent lot numbers are recorded on all assay worksheets as this information will aid in troubleshooting.

4.1.3.4 Calculation of control limits for accuracy

1. The run mean (X) for each control is calculated according to the formula: $X = \frac{x}{n}$
   where X is each control value on a run and n is the number of control values determined on that run.

2. The cumulative mean (X) is calculated by the formula:
   $X = \frac{x}{n}$
   where x is each control value beginning the first day that control material or lot number was run and ending on the last day of the previous month. N is the number of control values used in this calculation. A new cumulative mean is calculated at the beginning of each month.

3. The standard deviation of the cumulative mean (SD) is calculated by the formula: $SD = \frac{(X-X)2}{n-1}$

   Control limits are defined as the cumulative mean ± 2 standard deviation.

4.1.3.5 Calculation of control limits for precision

The range, R, for controls is the difference between the highest and lowest value obtained in one day.

$$R = XH - XL$$

where X is the highest value and XL the lowest.

4.1.3.6 Calculation of Coefficient of Variance (CV)

The coefficient of variance of intra-day, and inter-day variations is calculated as the standard deviation divided by the mean, and expressed as a percentage.

4.1.3.7 Repeating analyses

1. Repeat a coagulation assay run if the run mean value is not within 2 standard deviation of the cumulative mean.

2. Repeat ELISA columns if the column control value is not within 2 standard deviation of the cumulative mean.

3. Repeat individual values if their duplicates differ by 10% or more.

4.1.5 Records of Control Values

To keep track of quality control values and to monitor for shifts and trends, the Central Hemostasis Laboratory uses a customized computerized quality control program. Quality control values may be entered manually or automatically for all tests run on the Coag-A-Mate X2 (aPTT, fibrinogen, Factor VII, and Factor VIII). Quality control results for all other assays are entered manually. This program calculates the mean, number, standard deviation, maximum value, minimum value, range, and coefficient of variation of a given time period. Monthly statistics and cumulative statistics are calculated the first week of every month. Monthly statistics are used to help monitor shifts and trends over time. Cumulative means are used to determine target ranges.Cohorts daily means and monthly means are also monitored for shifts and trends using a similar program.

In addition to these statistics, Levi-Jennings charts are prepared manually for each.

4.2 Quality Control for Field Center Blood Collecting, Processing, Temporary Storage & Shipping

Several approaches are taken to standardize and evaluate the blood drawing and processing at the field center laboratories. This is extremely important because any deviation from standardized phlebotomy and blood processing procedures may lead to variations in the results despite the establishment of high accuracy and precision for the assays.

4.2.1 Standardization of blood collection and processing procedures

Procedures for blood collection and processing are detailed in ARIC Manual 7. Assurance of standard performance is accomplished through on-site teaching, demonstration, practice and initial evaluation. Field center equipment maintenance and evaluation constitutes an important component of this training. The performance of field center laboratory technicians is monitored periodically by visits from the Central Hemostasis Laboratory staff and on a continuing basis by analysis of mean values of labile factors by the Coordinating center.

4.2.4 Analysis of blind samples

The central Hemostasis Laboratory receives 7% blind samples for quality control analysis. These samples are collected, processed, and labeled exactly the same way as the routine samples. The results will be sent to the Coordinating Center and periodic reports are made to the Central Hemostasis Laboratory and to the ARIC Steering Committee.

4.3 Quality Assurance for Stability of Long-Term Storage Samples

Samples are stored at -70°C for case-control studies. Some of these samples must be stored for up to 8 years. Stability of these samples is a major concern and will be systematically analyzed. The control materials (Table 4) will be prepared in the Central Hemostasis Laboratory in a large batch. The Central Hemostasis Laboratory also will include the evaluation of some reference samples with abnormally high and low values concurrently with the control materials. It is important to include these references because the decay rate of some of the proteins may be influenced by the concentrations of these proteins.

TABLE 1

Standard and Calibration References used for each assay

Assay	Standards	Source
aPTT	N/A	N/A
Fibrinogen	Fibriquick	G.D.
Factor VII:C	UCRP	P.H.
Factor VIII:C	UCRP	P.H.
vWF:Ag	UCRP	P.H.
AT-III activity	UCRP	P.H.
Protein C:Ag	UCRP	P.H.
PF-4	supplied by manufacturer	Stago
TG	"	Stago
FPA	"	Stago
tPA:Ag	"	Stago
PAI-1	"	American Diagnostics
D-dimer	"	Stago
Protein S	"	Stago

G.D.: General Diagnostics (Organon Technika)
UCRP: Universal Coagulation Reference Plasma
P.H.: Pacific Hemostasis

TABLE 2

Quality Control Materials for each assay

Assay	Controls	Source
aPTT	UCRP	P.H.
Fibrinogen	UCRP	P.H.
Factor VII:C	UCRP	P.H.
Factor VIII:C	UCRP	P.H.
vWF:Ag	UCRP	P.H.
AT-III activity	UCRP	P.H.
Protein C:Ag	UCRP	P.H.
PF-4	CACP	C.H.L.
TG	CACP	C.H.L.
FPA	CACP	C.H.L.
tPA:Ag	UCRP	P.H.
D-dimer	UCRP	P.H.
PAI-1	UCRP	P.H.
Protein S	UCRP	P.H.

G.D.: General Diagnostics
P.H.: Pacific Hemostasis
C.H.L.: Central Hemostasis Laboratory

TABLE 3

Control Materials for Evaluating Long-Term Stability

Assay	Control	Source
PF-4	CACP	C.H.L.
TG	CACP	C.H.L.
FPA	CACP	C.H.L.
tPA:Ag	NPP	C.H.L.

5.0 MACHINE AND EQUIPMENT MAINTENANCE

5.1 Pipettes

5.1.1 Eppendorf (200 µl fixed)

Maintenance: Quarterly

1. Cleaning: Clean tip with isopropyl alcohol and stylet.

2. Calibration: Pipette 200 µl distilled water into a preweighed boat 3 times, recording each value in mg.

   Acceptable Range: 194-206 mg

5.1.2 Eppendorf (0.5-10 µl, digital)

Maintenance: Quarterly

1. Cleaning: Same as above.

2. Calibration: Pipette 10 µl distilled water into a preweighed boat 3 times, recording each value in mg.

   Acceptable range: 9.7-10.3 mg

5.1.3 Eppendorf (10-100 µl, digital)

Maintenance: Quarterly

1. Cleaning: same as above.

2. Calibration:

   a. Pipette 10 µl distilled water into a preweighed boat 3 times, recording each value in mg.

      Acceptable range: 9.7-10.3 mg

   b. Pipette 100 µl of distilled water into a preweighed boat 3 times, recording each value in mg.

      Acceptable range: 97-103 mg

5.1.4 Eppendorf (100-1000 µl, digital)

Maintenance: Quarterly

1. Cleaning: same as above.

2. Calibration:

   a. Pipette 100 µl distilled water into a preweighed boat 3 times, recording each value in mg.

      Acceptable range: 97-103 mg

   b. Pipette 1000 µl distilled water into a preweighed boat 3 times, recording each value in mg.

      Acceptable range: 970-1030 mg

5.1.5 Eppendorf Repeater Pipette

Maintenance: quarterly

1. Cleaning: same as above.

2. Calibration: using a 5 ml Combitip with the dial set on "1" pipette 100 µl distilled water into a preweighed boat 3 times, recording each value in mg.

   Acceptable range: 97-103 mg.

5.1.6 8 Channel Titertek Pipettes (40-300 µl digital)

Maintenance: Quarterly

1. Cleaning: Same as above

2. Calibration:

   a. Pipette 40 µl of distilled water into a preweighed boat for each channel 3 times, recording the values for each channel in mg.

      Acceptable range: 38.8-41.2 mg

   b. Pipette 100 µl of distilled water into a preweighed boat for each channel 3 times, recording the values for each channel in mg.

      Acceptable range: 97-103

   c. Pipette 200 µL distilled water into a preweighed boat for each channel 3 times, recording the values for each channel in mg.

      Acceptable range: 194-206 mg

5.2 MR 5000/7000 Microelisa Reader

5.2.1 Every 3 months, run a Dynatech Calibrate Test Plate.

Perform a four corner optical alignment test, a three point linearity check, and a positive filter identification check.

5.2.2 Weekly maintenance

The detector window should be cleaned with soft lens tissue.

5.3 Coag-A-Mate X-2

See Appendix C for detailed instructions of maintenance for the Coag-A-Mate X-2.

5.3.1 Pump tubing

Must be rinsed daily, washed weekly, and replaced as needed.

5.3.2 Pump rotor

Must be washed monthly, with isopropyl alcohol.

5.3.3 Pump volume

A tolerance verification check must be performed weekly, as well as a calibration check.

5.3.4 Temperature

Must be verified every six months.

5.4 LKB Clinigamma Gamma Counter

5.4.1 Weekly Maintenance

Cleaning: with damp cloth, wipe conveyor area to clean any dust, spills, etc.

Background: count 10 empty tubes 5 minutes each. Average background values and enter on chart. If any upward drift is noticed, notify supervisor as the elevator tip may be contaminated and need to be changed.

5.4.2 Every 3 months

Follow the instructions provided by the manufacturer.

5.5 Kelvinator Model UC-927CR Upright Ultracold Freezer and Harris Model DLT-21V-85D-U-A Upright Ultracold Freezer

In case of a failure in one or more freezers, several steps have been taken to insure the integrity of the freezer systems:

1. There is a 7-day temperature chart that records the temperature twenty-four hours a day.

2. Every morning, a technologist will record the temperature on a temperature log and check the 24-hour chart to ensure that the temperature did not rise over -65°C.

3. There is an alarm system implemented with audio, visual, and remote signals, so that if the freezer malfunctions during the night security personnel can phone the supervisor to implement the emergency freezer system.

4. In case of a power failure, the lab freezers are attached to a university emergency generator which is activated immediately. Recheck the 24-hour chart to ensure that the temperature did not rise over -65°C.

5.6 Refrigerator

Most reagents are stored at 2°-8°C, so quality assurance may be maintained by monitoring and recording the temperature daily on a chart. If the refrigerator reaches 10° or higher, there is a back-up refrigerator that may be used for storing reagents.

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