ATHEROSCLEROSIS RISK IN COMMUNITIES STUDY

Manual 9

Hemostasis Determinations

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

ARIC PROTOCOL

Manual 9

Hemostasis Determinations

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Version 1.0: April 28, 1987

FOREWORD

This manual entitled, Hemostasis Determinations, is one of a series of protocols and manuals of operation for the Atherosclerosis Risk in Communities (ARIC) Study. The complexity of the ARIC Study requires that a sizeable number of procedures be described, thus this rather extensive set of materials has been organized into the set of manuals listed below. Manual 1 provides the background, organization, and general objectives of the ARIC Study. Manuals 2 and 3 describe the operation of the Cohort and Surveillance Components of the study. Detailed Manuals of Operation for specific procedures, including reading centers and central laboratories, make up Manuals 4 through 11. Manual 12 on Quality Assurance and Quality Control contains a general description of the study's approach to quality assurance as well as specific protocols for each of the study procedures.

The version status of each manual is printed on the title sheet. The first edition of each manual is Version 1.0. Subsequent modifications of Version 1 (pages updated, pages added, or pages deleted) are indicated as Versions 1.1, 1.2, and so on, and are described in detail in the Revision Log located immediately after the title page. When revisions are substantial enough to require a new printing of the manual, the version number will be updated (e.g., Version 2.0) on the title page.

ARIC Study Protocols and Manuals of Operation

MANUAL	TITLE
1	General Description and Study Management
2	Cohort Component Procedures
3	Surveillance Component Procedures
4	Pulmonary Function Assessment
5	Electrocardiography
6	Ultrasound Assessment
7	Blood Collection and Processing
8	Lipid and Lipoprotein Determinations
9	Hemostasis Determinations
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11	Sitting Blood Pressure and Postural Changes in Blood Pressure and Heart Rate
12	Quality Assurance

Central Hemostasis Laboratory

1. 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 (1) atherosclerosis measured by ultrasound and (2) 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 following hemostatic factors, listed in Table 1, are used to identify 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.

This manual begins with a description of the theoretical rationale for (Chapter 1) and the step-by-step procedures for running the hemostasis assays (Chapter 2) at the ARIC Central Hemostasis Laboratory. Instructions for the drawing, processing, and short-term storage of blood at the ARIC field centers are covered in Manual 7, Blood Collection and Processing. The long-term storage of ARIC serum samples and its associated data management are outlined in Chapters 1 and 3, respectively. Quality control for assay procedures, for field center blood collection, temporary storage and shipping, and the quality assurance for the stability of long-term storage of samples is described in Chapter 4. Procedures for machine and equipment maintenance are described in Chapter 5.

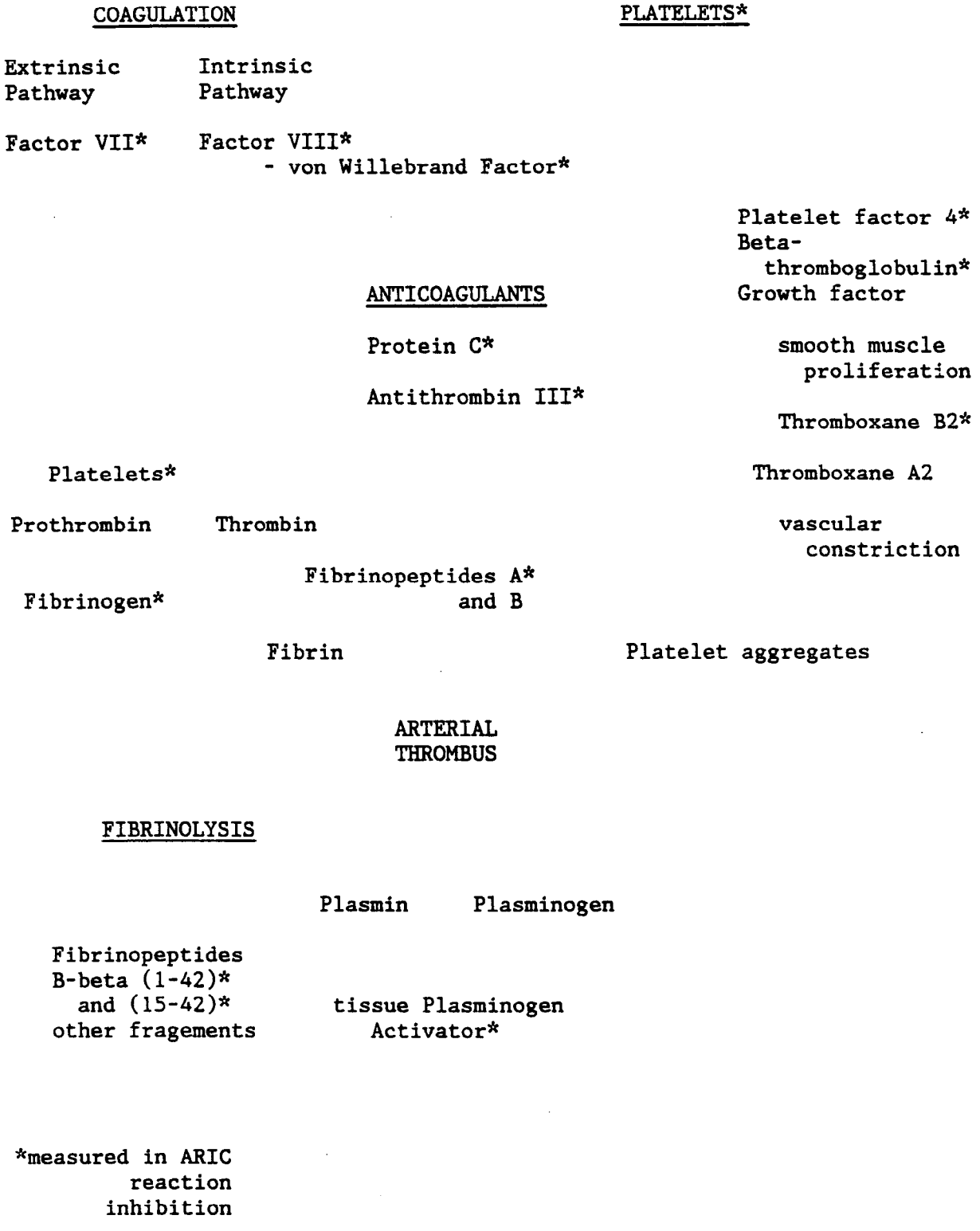
1.2 Rationale

There is compelling evidence that hemostatic factors play a pivotal role in the pathogenesis of human coronary heart disease 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; and (4) products of the 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 (and Protein S) and antithrombin III (AT-III). Once the endothelium integrity is disrupted, there is a rapid activation of platelets and coagulation factor XII triggered by the subendothelial tissues. A chain of chemical and cellular reactions ensues, leading to the formation of platelet-fibrin thrombi (see Figure 1). Thrombus formation on the atherosclerotic 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

TABLE 1. Hemostasis Measurements and Methods

Population	Measurements	Methods
All Participants	Coagulation factors:	
	Fibrinogen	coagulation test
	Factor VII	coagulation test
	Factor VIII	coagulation test
	von Willebrand factor antigen	enzyme-linked immunosorbent assay
	Coagulation inhibitors:	
	Antithrombin III	thrombin inactivation
	Protein C	enzyme-linked immunosorbent assay
	General screen:	
	Activated partial thromboplastin time	coagulation test
Cases and Controls	Coagulation activation:	
	Fibrinopeptide A	radioimmunoassay
	Platelet activation:	
	Beta-thromboglobulin	radioimmunoassay
	Platelet factor 4	radioimmunoassay
	Serum thromboxane B2	radioimmunoassay
	Fibrinolytic activity:	
	Tissue plasminogen activator antigen	enzyme-linked immunosorbent assay
	Fibrinopeptides B-beta-15-42 and B-beta-1-42	enzyme-linked immunosorbent assays



*measured in ARIC reaction inhibition

Figure 1. Components of Arterial Thrombosis Measured in ARIC

liberated from the membrane phospholipid and rapidly converted into several biologically active compounds (3). Thromboxane A_2 (TXA_2), 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 TXA_2, 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, β-thromboglobulin (βTG) and platelet factor 4 (PF-4). These proteins are useful markers for studying platelet activation <u>in vivo</u> (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 two pathways: (a) activation of Factor XII by subendothelial tissues ("intrinsic" pathway) and (b) 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 digestive products of fibrinogen. Fibrinopeptide A (FPA) and fibrinopeptide B are such markers useful for early detection of fibrin formation (9,10).

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 (11-14). AT-III, on the other hand, is a specific antagonist of thrombin and Factor Xa and hence limits thrombus formation (15). Prostacyclin, produced by the vascular endothelium, inhibits platelet aggregation and dissolves platelet aggregates (16). The fibrinolytic system provides the ultimate defense mechanism, lysing fibrin clots by generation of a powerful proteolytic enzyme, plasmin (17,18). Plasmin digests fibrin and fibrinogen into several discrete fragments (i.e., fragments X, Y, D, and E). These fibrin degradation products may occur in many disease states and therefore are nonspecific. Fragments of fibrin, primarily the fragments digested from the fibrinogen Bβ chain, e.g., Bβ (15-42) and (1-42), appear to be highly specific as an indicator of plasmin digestion of mural thrombi (19).

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 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 the ARIC Study is particularly important for the study of hemostasis. The Northwick Park Heart Study (a prospective study) reported by Meade et al from England, included 1,510 white men aged 40-64 at recruitment (20). Factors V, VII, VIII:C, fibrinogen, AT-III, fibrinolytic activity, platelet adhesiveness, platelet count, α_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 (21). 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 (MI) 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 among the cohort population in diversified American communities.

Selection of other components of the hemostatic system is based on three major criteria: (1) that the factors play a pivotal role in thrombosis, atherosclerosis and/or vasospasm, (2) that assays for these factors have a proven accuracy and precision, and (3) that these assays require small amounts of blood and can be performed on frozen samples. The specific rationale for selecting each of the following factors is briefly described below.

VIII:VWF. Von Willebrand factor is essential for platelet adhesion to damaged vessel wall (22). Its physiologic and pathophysiologic role in hemostasis and thrombosis is therefore important. In addition, VIII:VWF is complexed 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. It has been recently observed that abnormally large VIII:VWF molecules may induce intravascular platelet aggregation and cause microvascular thrombotic disorders such as thrombotic thrombocytopenic purpura (23). Measuring VIII:VWF multimers might be considered in the future.

AT-III, Protein C. Both factors are naturally occurring anticoagulants. Defects in these factors have been shown to 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, 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). Protein S assay will be developed for future measurements.

In vivo coagulation activation may be determined in several ways: (1) measuring activated coagulation factors, i.e., Xa and thrombin, (2) measuring fibrin monomers, and (3) measuring early digestive products of fibrinogen such as FPA or FPB. Fibrinopeptide A appears to fulfill the criteria. Its measurement by RIA 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 (28-29); (2) circulating platelet aggregates (CPA) by the technique of Wu and Hoak (30); (3) measurement of released products such as βTG and PF-4 (31-34); and (4) measurement of platelet TXB_2 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 βTG and PF-4 levels (36), CPA measurement is impractical for population studies. On the other hand, βTG and PF-4 can be reliably measured by RIA (34,37) and their levels have been shown by case-control studies to be elevated in CHD (38,39). Thromboxane A_2 is generated in platelets when they are activated. TXA_2 is incriminated as a factor in unstable angina and other CHD events (40).[2] The blood level of TXB_2, the stable metabolite of TXA_2, is significantly elevated in subjects with unstable angina. Measuring plasma levels of TXB_2 is not reliable because the level is near the lower limit of detection by the RIA technique. However, serum TXB_2 level, which can be accurately measured, reflects the ability of platelets to produce TXA_2 (41). Serum TXB_2 measurement will be evaluated along with βTG and PF-4 as a sensitive index of platelet activity.

Reduced fibrinolytic activity is associated with postoperative venous thrombosis and recurrent venous thrombosis (42). The importance of reduced fibrinolytic activity in CHD is suggested by a case-control study in post-myocardial infarction patients. Three measurements appear to fulfill the criteria for inclusion in this prospective study. First, tPA appears to be released by vascular endothelium when the vascular system is under stress. tPA has a high affinity for fibrin, where it converts plasminogen into plasmin which in turn digests fibrin into fragments. Of the fragments, fibrinopeptide Bβ(1-42) and (15-42) appear to provide a sensitive index for detecting specific fibrinolysis in vivo (19). Activated Protein C also plays an important role in regulating fibrinolytic activity. By measuring the two regulators of fibrinolysis (tPA and Protein C) and the specific

early products of fibrinolysis, ARIC will gain insight into the role of defective fibrinolysis in CHD events.

Lupus-type anticoagulants are a group of immunoglobulin antibodies which inhibit the phospholipid component of the testing reagents for PTT and PT (43,44). Retrospective studies suggest that lupus-type anticoagulants are associated with a higher incidence of thrombosis (45,46). These studies should be interpreted with caution because these patients had underlying diseases such as SLE, cancer, etc., which are known to cause thromboembolism. Activated partial thromboplastin time (aPTT) is useful as a screening test for this type of anticoagulant. Once a prolonged aPTT is observed, mixing experiments as well as thromboplastin dilution (or inhibition) test should be carried out to establish the presence of lupus-type anticoagulant. Moreover, aPTT is an inexpensive general screening test for the intrinsic pathway and some studies suggest that a shortened aPTT may represent a risk factor for hypercoagulability.

In summary, a number of factors are to be measured in the ARIC Central Hemostasis Laboratory. The selection of these factors was based on theoretical considerations, prospective and retrospective studies, and feasibility and reliability of available assays. The factors are grouped as follows:

1) coagulation factors: fibrinogen, VII, VIII:C, VWF:Ag
2) coagulation activation: fibrinopeptide A (FPA)
3) platelet activation: βTG, PF-4, serum TXB_2
4) fibrinolytic activity: tPA, FPBβ(1-42) and (15-42)
5) inhibitors: natural: AT-III, Protein C
   acquired: lupus-type anticoagulant
6) general screening: aPTT

Fibrinogen, VII, VIII:C, VWF:Ag, AT-III, Protein C, and aPTT will be measured in each participant while FPA, βTG, PF-4 TXB_2, tPA, and Bβ(1-42) will be measured for cases and controls only. Additional plasma samples will be obtained and stored to be used for future measurements of other new and potentially important hemostatic and thrombotic indices.

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 (CHL). 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 Protocol Manual 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, because 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. The other factors are more stable and are not as affected by changes in temperature as the coagulation factors listed above. Nevertheless, the storage and shipping conditions must be standardized. Plasma samples in appropriate aliquots from ARIC participants are freshly frozen and temporarily stored in plastic bags at -70°C in the field center laboratories for a short period of time, i.e., less than three days, and then sent to the Central Hemostasis Laboratory. There they are carefully inspected to ensure that the samples have remained frozen and that the identification of samples is correct. The coagulation tests are done within 48 hours following receipt of the samples. In the interim, the samples are kept at -70°C. Hence, all the samples for coagulation activity measurements are assayed within one week of blood drawing. The factors should remain stable under the prescribed conditions. For a more detailed description, see Manual 7, Blood Collection and Processing.

1.5 Sample Storage at the Central Hemostasis Laboratory

After bags are received at the Central Hemostasis Laboratory, the condition of the samples and of the contents are carefully inspected and logged in. The two smaller bags are removed. Bag A is placed into a freezer (-70°C) for short-term storage. Factors I, VIII:C, VWF:Ag, VII, and aPTT are performed within one week. Protein C Ag and AT-III activity are performed in less than two weeks. Bag B, containing the samples for case-control studies, is stored in long-term freezers (-70°C). For the entire period of study, two 20 ft^3 freezers are needed for short-term storage and eight freezers for long-term storage. The freezers are designated for long (L) and short (S) term storage and the bags placed accordingly. The bags are placed in boxes that can hold up to ten bags. The boxes are then placed in a metal rack, holding nine boxes each. For the purpose of rapid retrieval, each freezer is assigned a Roman numeral (I,II,..). Each horizontal row of boxes is assigned a letter (A,B,..), and the boxes in each holder are given an Arabic number (1,2,..). Each bag has an ID number and the tubes in the bags are color, as well as, number coded. For example, a number of L-II-B-3 indicates that the samples are located in the long-term freezer number II, horizontal row B and the third box of the holder. This system substantially reduces the time needed for placing and removing the boxes into and from racks at the field center, and transferring the tubes into racks at the Central Laboratory and reduces the risk of partial thawing.

All the freezers are equipped with alarm and back-up systems. The alarm is connected to a central computer system in the Medical School Building. The security personnel monitor the computer 24 hours daily. The names, telephone numbers, and beeper numbers of responsible persons of the Central Hemostasis Laboratory (Dr. Wu and Ms. Papp) 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. Moreover, the system is further guarded by emergency generator power and liquid CO_2 tanks.

Freezing of the samples at -70°C ensures stability of the hemostatic factors. However, Factors I, VIII:C, VWF:Ag, and V are unstable even at -70°C. Therefore, the aliquots used to assay these factors will be stored

for only a short time. AT-III and Protein C are more stable. These are assayed within two weeks from blood drawing.

The samples for case studies require long-term storage at -70°C. These proteins (or peptides) are stable but their variability over a prolonged period of time, i.e., a span of seven years, has not been investigated. Aliquots of pooled plasma and serum from 20 normal subjects (15 tubes for each assay) will be frozen in the beginning of the ARIC study. The fresh samples will be assayed and the frozen samples will be assayed at six-month intervals. Any obvious deviation because of long-term freezing will be detected and reported to the Steering Committee.

2. 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. $CaCl_2$ is added to initiate the reaction, and the time required for clot formation is measured in seconds.

2.1.2 Reagents

Automated aPTT Reagents (General Diagnostics, Organon Technika Co., Morris Plains, NJ): Rabbit brain phospholipids together with micronized silica in N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer. Reconstitute with 3 ml purified water. Shake vigorously, add stirring bar, and place in stir-cool well of Coag-a-Mate with 3-hole rubber stopper. Yield: 30 tests/vial (15 duplicate determinations). Stable at 2°-5°C for one week.

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

2.1.3 Quality Control Material

1) Verify Normal Citrate (VNC) (General Diagnostics): Assayed pooled normal human plasma, freeze-dried. Reconstitute with 1 ml purified water. Swirl gently and allow to stand for 30 minutes at room temperature. Expires 24 hours after reconstitution. Record expiration date on vial label.

2) Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis, Curtin Matheson Scientific Inc., Ventura, CA): Assayed pooled normal human plasma; freeze-dried. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

3) VNC is the routine Q.C. material. UCRP is used as a back-up Q.C. material.

2.1.4 Equipment and Supplies

Coag-A-Mate X-2 (General Diagnostics)
Polystyrene test tubes (12x75 mm)
Test tube racks
Melting ice bath
Rainin pipette and tips
Reagent trays
Pipetman with tips
Stirring bar

2.1.5 Sample Preparation

Blood is collected in 3.8% Na citrate, centrifuged at 2°-8°C and the plasma separated from the red cells. Plasma is frozen rapidly and stored at -70°C until shipping to CHL. Samples are thawed rapidly at 37°C to prevent denaturation of fibrinogen.

2.1.6 Procedure

1) Set up worksheet with Verify Normal Citrate and UCRP as controls in the first two work stations, followed by test plasmas. (Each test should be run in duplicate; inner and outer wells.) An additional set of controls should be run in duplicate at the end of each tray. Thaw all plasma at 37°C.

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

3) Prime lines with reagent, delivering slight amount of excess reagent into suitable receptacle. Inspect tubing for bubbles and re-prime if necessary.

4) Transfer 100 µl plasma in inner and outer cuvettes using Pipetman. (When handling cuvette trays, hold only by top ring, do not touch cuvettes!)

5) Position and seat test tray on incubation test plate, matching notch in tray with notch in 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 APPT key.

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

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

2.1.7 Back-up Procedure in Case of Instrument Failure

In case of machine problems with the Coag-A-Mate X-2, Coag-A-Mate 2001 is used as our first back-up. This instrument has a 12 test sample capacity and performs equally well. The instruments are serviced locally and substitute instruments are provided by the company as needed.

2.1.8 Back-up Procedure in Case of Reagent Problems

If the quality of reagents prepared by General Diagnostics should prove to be defective, aPTT reagents from an alternate supplier will be evaluated and, if satisfactory, will be used. Several quality products such as aPTT reagent from Dade Diagnostics or Helena Diagnostics are commonly used by many laboratories.

2.1.9 Computation of Results

The two values for each plasma are averaged and reported in seconds. Duplicates must match within 10 percent.

2.2 Fibrinogen Assay

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

Thrombin Reagent (General Diagnostics): Approximately 100 NIH units/ml Bovine Thrombin, with stabilizers and buffer. Reconstitute with 3.0 ml of purified water and mix gently. Store at 2°-8°C for not more than three days. Record expiration date on vial.

Fibrinogen Calibration Reference (Fibriquik General Diagnostics): Lyophilized human plasma, assayed for fibrinogen with stabilizers and buffer. Reconstitute with 1.0 ml of purified water, allow to stand 30 minutes, and then mix gently. Store at 2°-8°C for not more than 24 hours. Record expiration date on vial.

Veronal Buffer, pH 7.35: Dissolve 5.875 g sodium diethyl barbiturate and 7.335 g NaCl in 215 ml 0.1N HCl. Add deionized H_2O to 900 ml and adjust pH to 7.35. Adjust volume to 1.0 liter. Store at 4°C. Prepare fresh every three months.

2.2.3 Quality Control Material

1) Verify Normal Citrate (VNC) (General Diagnostics): Reconstitute with 1 ml purified water. Swirl gently and allow to stand at room temperature for 30 minutes. Expires 24 hours after reconstitution, at 2°-8°C. Record expiration date on vial label.

2) Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis): Assayed pooled normal human plasma; freeze dried. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

3) VNC is the routine Q.C. material used in the hemostasis laboratory. UCRP is used as back-up.

2.2.4 Equipment and Supplies

- Coag-A-Mate X-2
- Polystyrene test tubes (12x75 mm)
- Test tube racks
- Melting ice bath
- Rainin pipette and tips
- Reagent trays
- Pipetman with tips
- Stirring bar

2.2.5 Procedure

2.2.5.1 Sample Preparation

Blood is collected 1:10 in 3.8% Na citrate, centrifuged at 2°-8°C, and the plasma separated from red cells. The plasma is frozen rapidly and stored at -70°C until shipping to the CHL. Samples are thawed rapidly at 37°C to prevent denaturation of fibrinogen. After thawing, place samples immediately in a melting ice bath and perform analysis within two hours.

2.2.5.2 Set-up

1) Reconstitute reagents and control as per directions.

2) Set up worksheet with standards (1:7.5, 1:10, 1:20, 1:40). Controls (1:10) and test plasma (1:10). Label 12x75 mm tubes correspondingly.

3) Thaw samples at 37°C. When thawed, mix and place in melting ice bath. Prepare dilutions.

4) Pour a sufficient amount of Veronal buffer in small beaker. Using a Rainin auto pipetor on "Dilute" mode and a fresh pipet tip each time, make dilutions of a fibrinogen calibration reference as follows:

Dilution		Fibrinogen Ref. Calibration	Buffer
A	1:7.5	100 µl	650 µl
B	1:10	100 µl	900 µl
C	1:20	500 µl (B)	500 µl
D	1:40	500 µl (C)	500 µl

2.2.5.3 Analysis

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

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

3) Transfer 200 µl diluted plasma into correct cuvettes, using Pipetman.

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

2.2.6 Back-up Procedure in Case of Instrument Failure

In case of machine problems with the Coag-A-Mate X-2, a Coag-A-Mate 2001 is used as first back-up. This instrument has a 12 test sample capacity and performs equally well. The instruments are serviced locally and the company provides substitute instruments.

2.2.7 Back-up Procedure in Case of Reagent Problems

If the quality of reagents prepared by General Diagnostics should prove to be defective, then fibrinogen reagents from an alternate supplier will be evaluated, and if satisfactory, will be used. Several quality products such as fibrinogen reagent from Dade Diagnostics or Helena Diagnostics are commonly used by many laboratories.

2.2.8 Computation of Results

To calculate the fibrinogen concentration, use duplicate standard values and the linear regression function on the gamma counter. Calculate all participant samples, average, and report results in mg/dl. Results must be repeated on a new sample if:

1) duplicates do not mach within 10%.
2) the unknown value is greater than the highest standard or lower than the lowest standard. (High values are repeated at a greater dilution.)

2.3 Assay of Factor VII Activity

2.3.1 Principle

The 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 normal plasma. (In this normal plasma activity curve, a 1:20 dilution of normal plasma is considered to possess 100% Factor VII activity.) Thromboplastin and calcium are mixed with plasma, and the length of time required for clot formation is measured in seconds. This is related to percent activity by calculating from the normal activity curve.

2.3.2 Reagents

1) Thromboplastin (Thromboscreen, Pacific Hemostasis): Lyophilized extract of rabbit brain in buffered saline with stabilizers and calcium chloride. Reconstitute with distilled water according to instructions on vial label. Agitate gently until reagent is completely solubilized. Add stirring bar, and place in stir-cool well of Coag-A-Mate with 4-holed rubber stopper. Stable at 2°-8°C for one week.

2) Factor VII deficient plasma (George King Biomedical, Overland Park, KS): Store at -70°C. Observe expiration date. Thaw rapidly (37°C) and place in melting ice bath. May be refrozen and rethawed only once.

3) Veronal Buffer, pH 7.35: Dissolve 5.875 g sodium diethyl barbiturate and 7.335 g NaCl in 215 ml 0.1N HCl. Add deionized H_2O to 900 ml and adjust pH to 7.35. Adjust volume to 1.0 liter. Store at 4°C. Prepare fresh every three months.

4) Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis): Assayed pooled normal human plasma; freeze dried. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

2.3.3 Quality Control Material

Verify Normal Citrate (General Diagnostics): Reconstitute with 1 ml purified water. Swirl gently and allow to stand at room temperature for 30 minutes. Expires 24 hours after reconstitution, at 2°-8°C. Record expiration date on vial label.

2.3.4 Equipment and Supplies

- Coag-A-Mate X-2
- Polystyrene test tubes (12x75 mm)
- Test tube racks
- Melting ice bath
- Rainin pipette and tips
- Reagent trays
- Pipetman with tips
- Stirring bar

2.3.5 Procedure

2.3.5.1 Sample Preparation

Blood is collected 1:10 in 3.8% Na citrate, centrifuged at 2°-8°C and the plasma separated from red cells and platelets. Plasma is frozen rapidly and stored at -70°C until shipping to the CHL. Rapid thawing (37°C) is recommended to prevent denaturation of fibrinogen. After thawing, place samples immediately in melting ice bath and perform analysis within two hours.

2.3.5.2 Set-up

1) Set up worksheet with standards (UCRP dilutions of 1:10, 1:15, 1:20, 1:30, 1:40, 1:80), followed by VNC controls (1:20), then test plasmas (1:20), and ending with a VNC control (1:20). Label 12x75 mm tubes correspondingly. Place in rack in melting ice bath. Reconstitute control. Let stand at room temperature for 30 minutes, then place in refrigerator or ice bath.

2) Thaw Factor VII deficient plasma and all samples to be assayed at 37°C. When thawed, mix and place in melting ice bath.

3) Pour a sufficient amount of veronal buffer into small beaker. Using Rainin auto pipetor on "Dilute" mode and a fresh pipet tip each time, make serial dilutions of UCRP as follows:

	Value	Dilutions	UCRP	Veronal Buffer
A	200%	1:10	100 µl	900 µl
B	133%	1:15	100 µl	1400 µl
C	100%	1:20	500 µl (A)	500 µl
D	67%	1:30	500 µl (B)	500 µl
E	50%	1:40	500 µl (C)	500 µl
F	25%	1:80	500 µl (E)	500 µl

4) Prepare 1:20 dilutions of donor samples and controls.

2.3.5.3 Analysis

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

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

3) Using Rainin auto pipetor on "Multi" mode, transfer 100 µl Factor VII deficient plasma into each cuvette well needed for assay. (All samples must be run in duplicate: i.e., inner and outer well of #1, etc.) Transfer 100 µl diluted plasma into correct cuvettes, using Pipetman. Gently mix by rotating cuvet tray on counter top, a quarter of a turn each way. Take care not to splash any sample from cuvettes. (When handling cuvette tray, hold only by top ring, do not touch cuvettes!)

4) Position and seat 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 PT key.

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

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

2.3.6 Back-up of Procedure in Case of Instrument Failure

The Coag-A-Mate 2001 will be used as a back-up for the Coag-A-Mate X-2 with substitute instruments provided by the company as described for the aPTT assay.

2.3.7 Back-up Procedure in Case of Reagent Problems

As is the case with the Factor VIII activity assay, if the primary source, George King Biochemical, should provide inferior products, then Thromboscreen products will be used.

2.3.8 Computation of Results

To calculate Factor VII activity, use the duplicate standard values and the linear regression function on the gamma counter. Calculate all donor samples, average, and report results in percent activity. 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. (For high values: repeat at 1:40 dilution.)

2.4 Assay of Factor VIII Activity

2.4.1 Principle

This quantitative assay is based on the ability of the plasma in question to correct a Factor VIII deficient substrate. After the addition of 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 normal plasma. (In this normal plasma activity curve, a 1:20 dilution of normal plasma is considered to possess 100% Factor VIII activity.) The time required for clot formation is measured in seconds and is related to percent activity by reading off the normal activity curve.

2.4.2 Reagents

1) Automated aPTT reagent (General Diagnostics): Rabbit brain phospholipids together with micronized silica in N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer. Reconstitute with 3 ml purified H_2O. Shake vigorously, add stirring bar, and place in stir-cool well of Coag-A-Mate with 3-holed rubber stopper. Yield: 30 tests/vial (15 duplicate determinations). Stable at 2°-5°C for one week.

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

3) Factor VIII deficient plasma (George King Biomedical): Store at -70°C. Observe expiration date. Thaw rapidly at 37°C and place in melting ice bath. May be refrozen and rethawed only once.

4) Veronal Buffer, pH 7.35: Dissolve 5.875 g sodium diethyl barbiturate and 7.335 g NaCl in 215 ml 0.1N HCl. Add deionized H_2O to 900 ml and adjust pH to 7.35. Adjust volume to 1.0 liter. Store at 4°C. Prepare fresh every three months.

5) Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis): Assayed pooled normal human plasma; freeze dried. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

2.4.3 Quality Control Material

Verify Normal Citrate (General Diagnostics): Reconstitute with 1 ml purified water. Swirl gently and allow to stand for 30 minutes at room temperature. Expires 24 hours after reconstitution. Record expiration date on vial label.

2.4.4 Equipment and Supplies

- Coag-A-Mate X-2
- Polystyrene test tubes (12x75 mm)
- Test tube racks
- Melting ice bath
- Rainin pipette and tips
- Reagent trays
- Pipetman with tips
- Stirring bar

2.4.5 Procedure

2.4.5.1 Sample Preparation

Blood is collected 1:10 in 3.8% Na citrate, centrifuged at 2°-8°C and the plasma separated from red cells and platelets. Plasma is frozen rapidly at -70°C and stored at -70°C until shipped to the CHL. Plasma is thawed rapidly to prevent denaturation of fibrinogen. After thawing, place samples immediately in melting ice bath, and perform analysis within two hours.

2.4.5.2 Set-up

1) Set up worksheet with standards (UCRP 1:10, 1:15, 1:20, 1:30, 1:40, 1:80) followed by controls (1:20), then test plasmas (1:20) and ending with a VNC control (1:20). Label 12x75 mm tubes correspondingly. Place in rack in melting ice bath. Reconstitute control. Let stand at room temperature 30 minutes, then place in refrigerator or ice bath.

2) Thaw Factor VIII deficient plasma and all samples to be assayed at 37°C. When thawed, mix and place in melting ice bath.

3) Pour a sufficient amount of veronal buffer into small beaker. Using Rainin auto pipetor on "Dilute" mode and a fresh pipet tip each time, make serial dilutions of UCRP as follows:

	Value	Dilutions	UCRP	Buffer
A	200%	1:10	100 µl	900 µl
B	133%	1:15	100 µl	1400 µl
C	100%	1:20	500 µl (A)	500 µl
D	67%	1:30	500 µl (B)	500 µl
E	50%	1:40	500 µl (C)	500 µl
F	25%	1:80	500 µl (E)	500 µl

4) Prepare 1:20 dilutions of each sample and control, also using veronal buffer.

2.4.5.3 Analysis

1) Situate reagent tubing assemblies in appropriate pump positions (see chart on Coag-A-Mate for aPTTs). Insert tubing ends into reagent vials, ensuring that the pick-up tip touches bottom of vial and the stirring bar is rotating correctly.

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

3) Using Rainin auto pipetor on "Multi" mode, transfer 100 µl Factor VIII deficient plasma into each inner and outer cuvette needed for assay. Transfer 100 µl diluted plasma into correct cuvettes, using Pipetman. Gently mix by rotating back and forth on counter top, taking care not to splash any sample from cuvettes. (When handling cuvette tray, hold only by top ring, do not touch cuvettes!)

4) Position and seat 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 is performed.)

7) Lower light shield. Press aPTT key.

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

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

2.4.6 Back-up Procedure in Case of Instrument Failure

In a manner similar to the procedure for the aPTT analysis, a Coag-A-Mate 2001 will be used for primary back-up, which has a reduced capacity but performs equally well. The alternative to the smaller instrument is a substitute instrument provided by General Diagnostics.

2.4.7 Back-up Procedure in Case of Reagent Problems

George King Biomedical, Inc. is the primary source of Factor VIII deficient plasma. However, if the quality proves inferior, Thromboscreen may be used as a second commercial source of reagents.

2.4.8 Computation of Results

A calibration curve is established by using a built-in computer program based on linear regression function in Model 1272 Clinigamma Counter (LKB). The results of participants' samples are related to the calibration curve established in each batch of tests. All of the tests are done in duplicate.

2.5 Assay of Von Willebrand Factor Antigen

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 will be assayed by an ELISA technique. Von Willebrand factor is bound via one of its antigenic determinants to $F(ab^1)_2$ fragments coating the microtiter 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) Anti-VWF for coating (American Bioproducts Co., Diagnostica Stago, NJ): $F(ab^1)_2$ fragments specific for von Willebrand factor, in coating buffer, freeze-dried. Reconstitute with 20 ml distilled H_2O (add 20 ng sodium azide if preparing in advance). Coat plates as described in procedure section. May be stored up to one month at 2°-8°C. (Cover completely with masking tape, label and 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. Or: reconstitute with 2 ml distilled H_2O and aliquot in marked microvials. May be kept frozen for up to one month.

3) Dilution Buffer (Stago): Phosphate buffer containing bovine serum albumin and Tween 20, concentrated 10-fold, 20 ml per vial. Reconstitute up to 200 mls with distilled H_2O in graduated cylinder. Store in clear, plastic bottle. Stable 15 days at 2°-8°C. Label and date the vial.

4) Washing Solution (Stago): Saline solution containing Tween 20, concentrated 20-fold in a 50 ml vial. Reconstitute in a graduated cylinder up to the 1000 ml mark. Store in clean plastic bottle. Stable 15 days at 2°-8°C. Label and date the vial.
NOTE: If crystals are observed in dilution buffer or washing solution, incubate 10-30 minutes at 37°C until crystals are completely dissolved just before use.

5) Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis): Assayed pooled normal human plasma; freeze-dried. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

6) Peroxidase System ABTS (Kirkegaard & Perry): Solution A: ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate (6)]). Solution B: peroxide. Mix equal volumes of solutions A and B just prior to use. Discard any remaining mixed solution.

7) 5% SDS (Sigma): Sodium Dodecyl Sulfate (SDS). Dissolve 50 g in 1000 ml distilled H_2O. Store at room temperature. Discard if solution becomes turbid.

2.5.3 Quality Control Material

Verify Normal Citrate (VNC) (General Diagnostics): Assayed pooled normal human plasma; freeze-dried. Reconstitute with 1 ml distilled H_2O. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

2.5.4 Equipment and Supplies

- Titertek Multichannel Pipetor
- Titertek reagent reservoir
- Dynatech Immulon 2 Microtiter plates
- 200 µl Pipetman
- Culture-tek Vaccupette/96 plate washer with 50 ml syringe attached
- Fisher Microplate Mixer
- MR 600 ELISA Reader (Dynatech, Slater, VA)

2.5.5 Procedure

2.5.5.1 Sample Preparation

Blood is collected 1:10 in 3.8% Na Citrate, centrifuged at 2°-8°C for 10 minutes at 3000 x g, and the plasma removed from the red cells. Freeze rapidly and store at -70°C until shipment to the CHL. Stable one month. Thaw in 37°C water bath for 15 minutes prior to use.

2.5.5.2 Coating of Plates

Prepare enough coating solution to run two standard curves, samples in duplicate at a 1:120 dilution and VNC at the end of each row. Each well requires 200 µl coating solution.

1) Pour coating antibody into Titertek reagent reservoir marked "VWF coating."

2) Using Titertek 8-channel multipipette, add 200 µl antibody to each well.

3) Cover with masking tape, and incubate overnight at room temperature. Do not incubate on Minimix. If preparing plates in advance, plates may be coated and stored up to one month at 2°-8°C. Allow to reach room temperature before use.

2.5.5.3 Preparation of Sample Dilutions

1) Thaw all samples at 37°C for 15 minutes.
2) Vortex to mix samples.
3) Label tubes for 1:120 dilutions. Prepare dilutions with dilution buffer as follows: 10 µl plasma plus 1:19 ml dilution buffer. Vortex. Double amounts when preparing UCRP dilutions.

2.5.5.4 Preparation of Calibration Curve

To prepare an anti-VWF calibration curve, make the following dilutions with UCRP and dilution buffer, using Rainin pipette on "Dilute" mode:

	Value	Dilutions	UCRP	Buffer
A	200%	1:60	25 µl	1.475 ml
B	150%	1:80	25 µl	1.975 ml
C	100%	1:120	500 µl (A)	500 µl
D	75%	1:160	500 µl (B)	500 µl
E	50%	1:240	500 µl (C)	500 µl
F	25%	1:480	500 µl (E)	500 µl

2.5.5.5 Assay

Perform this and subsequent washings as follows:

1) After plates have been coated overnight, decant plate completely and drain briefly on paper towels. Fill tray of Vaccupette/96 with washing buffer. Fill dispenser by pulling plunger of syringe to 22 ml.

Align dispenser over wells of plate. Dispense washing buffer by gently pushing plunger in, taking care that tips do not touch surface of fluid in wells. Decant plate completely. Repeat four times.

2) Working quickly enough to prevent plate from drying out, and using 200 µl Pipetman, deliver 200 µl of well-mixed standard, control, or plasma specimens to correct well. See attached worksheet for example set-up. (BL-blank should contain 200 µl dilution buffer only.)

3) Cover with masking tape and, taking care to keep level, gently place plate on Minimix and turn on. Incubate for 3 hours.

4) Shortly before the end of the incubation period, prepare immunoconjugate as described in the reagent preparation section. Pour into Titertek reagent reservoir marked for VWF-immunoconjugate. Wash plate five times as described above. Drain completely.

5) Immediately add 200 µl immunoconjugate to each well using 8-channel micropipetor. Cover and place on Minimix, incubate 2 hours.

6) Just prior to the end of incubation, mix equal parts of ABTS solutions A+B. Prepare a sufficient amount to fill wells with 200 µl. Pour into reservoir marked ABTS and shield from light. Into the reservoir marked SDS, pour a sufficient amount of SDS to fill each well with 100 µl. Place stopwatch in handy location, as well as 8-channel pipette and pipette tips.

7) At the end of incubation, wash plates five times with washing solution. Drain completely.

8) Using 8-channel multipipette, dispense 200 µl ABTS into Row 1 while simultaneously starting stopwatch. If it is too difficult to simultaneously start the watch and add ABTS, the stopwatch can be started first, then ABTS can be added to Row 1 at a chosen time point. Do not let tips touch surface of liquid. Refill pipet tips, and at precise time intervals dispense ABTS into Row 2. Continue in a like fashion until Rows 1-6 have been filled.

9) Change pipette tips. At 4 minutes from time 0, add 100 µl 5% SDS to Row 1. At same time interval as above, add SDS to Row 2. Continue as before until wells 1-6 have been filled. Alternatively, after dispensing ABTS into Rows 1-6, transfer the microtiter plate to the ELISA reader, which is on and adjusted to read 410 nm. When highest standard (well 1 B) reads approximately 0.800, remove the plate and proceed immediately with the addition of SDS as above.

10) Using identical incubation times, repeat steps 8 and 9 with the remainder of the rows.

11) After dispensing SDS into the final row, allow plate to mix on Minimix for 10 minutes. Read O.D. on Microelisa Reader, setting "Blank" and "Plate Movement" switches to AUTO. Set O.D. at 410 nm, and set reference O.D. at 450 nm.

2.5.6 Back-up Procedure in Case of Instrument Failure

The department has access to another Microelisa Reader in case of instrument failure.

2.5.7 Back-up Procedure in Case of a Reagent Problem

A rocket immunodiffusion assay has been performed routinely in our laboratory. This procedure will be used for a back-up should the ELISA method fail.

2.5.8 Computation of Results

Use "spline" function. Report results as %. Samples whose values differ more than 10% should be repeated the following day. Repeat any sample lower than the lowest standard or higher than the highest standard.

2.6 Assay of Antithrombin III Activity

2.6.1 Principle

Antithrombin III (AT-III) is the primary plasma protein responsible for the inactivation of thrombin, together with α2-macroglobulins and α1 proteolytic inhibitor. AT-III complexes with heparin and inactivates thrombin by binding with thrombin, becoming irreversibly associated. It also acts as an inhibitor to factor Xa, 1Xa, X1a, and X11a. 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 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

1) Substrate: CBS 34.47: 2 AcOH.H-D-CHG-But-Arg-pNA. 1.8 µmole/vial, 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 Vitrium, 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/1, EDTA 7.5 mmol/1, heparin 3000 IU/1 and 1% PEG, pH 8.4. Stable one month at 2°-8°C.

4) UCRP: (Thromboscreen, Pacific Hemostasis): Assayed pooled normal human plasma; freeze-dried. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

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

2.6.3 Quality Control Material

Verify Normal Citrate (VNC) (General Diagnostics): Prepared from pooled normal human plasma, freeze-dried and standardized against a pool of fresh plasma. Reconstitute with 1.0 ml distilled water. Agitate gently until solution is dissolved. Stable one hour at 2°-8°C.

2.6.4 Equipment and Supplies

- Stopwatch
- 12x75 mm test tubes (glass or polyethylene)
- Flow microtiter plates
- Pipetman 200 µl, 1 ml with pipette tips
- Flow multichannel pipette and tips
- Flow reagent reservoirs
- MR 600 ELISA reader

2.6.5 Procedure

2.6.5.1 Sample Collection and Preparation

Blood should be collected by flawless venipuncture 1:10 in sodium citrate, centrifuged at 2°-8°C, and plasma separated from red cells. Plasma is frozen rapidly and stored at -70°C until shipping to the CHL. Plasma is thawed rapidly at 37°C. After thawing, perform analysis within two hours.

2.6.5.2 Set-up

1) Set up assay as shown on sample worksheet. Each run should have one standard curve and three rows of participant samples. Run up to four rows total at a time. Run VNC control at the end of each row at 1:40 dilution.

2) The AT-III assay is influenced by many factors including temperature, pH, substrate activity, thrombin activity, and heparin concentration. To optimize the timing of the assay, a "window" test is performed daily prior to dilution of the samples and standards. To perform the window test: 80 µl of dilution buffer and 40 µl of thrombin are added to a well on a microtiter plate. The plate is placed on the ELISA reader, then 40 µl of substrate is added while simultaneously starting a stopwatch. Tap the plate gently to mix the substrate and thrombin. The absorbance at 410 nm should be monitored continuously and the time required to reach .800 is recorded. This time ±15 sec determines the optimum incubation required in the assay, before addition of acetic acid to stop the reaction.

2.6.5.3 Specimen Preparation

1) Using dilution buffer, prepare 1:40 dilutions of each sample and VNC in 12x75 mm tubes. Use 25 µl sample and .975 ml dilution buffer for a 1:40 dilution.

2) In this assay a 1:40 dilution of UCRP or plasma is considered to possess 100% activity. Use UCRP as the standard for calculation of

sample values from the calibration curve. Using UCRP, make the following dilutions:

	Value	Dilutions	UCRP	Buffer
A	200%	1:20	50 µl	.950 µl
B	133%	1:30	50 µl	1.450 ml
C	100%	1:40	500 µl (A)	500 µl
D	67%	1:60	500 µl (B)	500 µl
E	50%	1:80	500 µl (C)	500 µl
F	33%	1:160	500 µl (E)	500 µl

3) Vortex all dilutions to mix well.

2.6.5.4 Assay

1) Pipette 80 µl each standard, control, or unknown plasma into appropriate well for first four rows of microplate.

2) Pour thrombin into reagent reservoir marked "thrombin". Place plate on mixer. Using a multipipette, simultaneously pipette 40 µl thrombin into each well of first row of microplate while starting stopwatch.

3) Exactly 30 seconds later, add 40 µl thrombin into second row of wells. Continue the same for Rows 3 and 4.

4) Pour thrombin reagent immediately back into original bottle. Pour substrate into reagent reservoir marked "substrate". Pour acetic acid into appropriately labeled reagent reservoir.

5) Exactly 7 minutes after the addition of thrombin to Row 1, add 40 µl substrate into Row 1 using new pipette tips. Continue to add to subsequent rows at 30-second intervals.

6) Exactly "x seconds" (use time determined by window test) after adding substrate to Row 1, add 40 µl glacial acetic acid to Row 1. Continue with addition of acetic acid to Rows 2-4 at 30-second intervals.

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

8) When all unknowns have been assayed, read on MR600 Microelisa Reader at 410 nm with reference set on 450 nm.

2.6.6 Back-up Procedure in Case of Instrument Failure

The department 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 spline function on the LKB gamma counter.

2.7 Assay of 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 phospholipid. This quantitative enzyme immunoassay employs the "sandwich" technique. Microtiter 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 immunoenzyme conjugate, which binds to other Protein C antigenic determinants. The activity is then evidenced by ABTS substrate, oxidized in the presence of perhydrol. The reaction is stopped with 5% SDS, and O.D. readings are taken at 410 nm. Plasma sample readings are compared to the standard curve, and Protein C is reported in µg/ml.

2.7.2 Reagent Preparation

1) $F(ab^1)_2$ Anti-Protein C (coating) (Stago): $F(ab^1)_2$ fragments specific to Protein C are freeze-dried in the presence of coating buffer. Reconstitute vial with 20 ml distilled H_2O (add 20 ng sodium azide if preparing plates in advance). Coat plates as described in procedure section. May be stored up to one month at 2°-8°C. (Cover completely with masking tape, label, date and store flat.)

2) Anti-Protein C Peroxidase (Stago): Immunoenzyme conjugate, in which specific anti-Protein C immunoglobulins obtained in the rabbit are labeled with peroxidase. Freeze-dried. Preparation if using same day: add 20 ml dilution buffer to vial. Invert gently to reconstitute. Stable 4 hours 2°-8°C. Write expiration time on vial. Preparing in advance: Reconstitute with 2 ml distilled H_2O and aliquot in marked microvials. May be kept for up to one month frozen at -30°C. Before use, thaw at 37°C and dilute 1:10 in dilution buffer.

3) Dilution Buffer (Stago): Phosphate buffer containing bovine serum albumin and Tween 20, concentrated 10-fold, 20 ml per vial. Reconstitute up to 200 ml with distilled H_2O in graduated cylinder. Store in clean, plastic bottle. Stable 15 days at 2°-8°C. Label and date.

4) Washing Solution (Stago): Saline solution containing Tween 20, concentrated 20 fold in a 50 ml vial. Reconstitute in graduated cylinder up to 1000 ml mark. Store in clean, plastic bottle. Stable 15 days at 2°-8°C. Label and date. (Note: If crystals are observed in dilution buffer or washing solution, incubate 10-30 minutes at 37°C until crystals are completely dissolved just before use.)

5) Protein C Standard (American Diagnostica, Greenwich, CT): American Diagnostica Product 39. Human Protein C. Dilute with purified water as directed.

6) Peroxidase Substrate System ABTS (Kirkegaard & Perry): Solution A: ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate (6)]). Solution

B: peroxide. Mix equal volumes of solution A and B just prior to use. Discard any remaining mixed solution.

7) 5% SDS (Sigma): Sodium Dodecyl Sulfate (SDS). Dissolve 50 grams in 1000 ml distilled H_2O. Store at room temperature. Discard if solution becomes turbid.

2.7.3 Quality Control Material

1) Normal Pooled Plasma (NPP): Collected from 20 normal donors. Pool, aliquot in 250 µl amounts, and store at -70°C. Stable 18 months.

2) Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis): Assayed pooled normal human plasma; freeze-dried. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable one hour at room temperature or 2°-8°C.

2.7.4 Equipment and Supplies

- Titertek multichannel pipette and pipette tips
- Titertek reagent reservoirs
- Dynatech Immulon 2 Microtiter Plates
- Pipetman; 1000 µl and 200 µl
- Culturetek Vaccupette, with 50 ml syringe attached
- Fisher Microplate Mixer
- MR 600 ELISA Reader
- Serologic pipette
- Stopwatch

2.7.5 Procedure

2.7.5.1 Sample Preparation

Blood is collected 1:10 in Na citrate, centrifuged at 2°-8°C, and plasma separated from red cells. 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.

2.7.5.2 Coating of Microtiter Plates

Prepare enough coating solution to run two standard curves, samples in duplicate at a 1:120 dilution and alternating Normal Pooled Plasma (NPP) and UCRP at the bottom of each row at a 1:120 dilution. Each well will be filled with 200 µl coating solution.

1) Pour coating antibody into Titertek reagent reservoir marked "Protein C, coating ab."

2) Using Titertek 8-channel pipet, add 200 µl antibody to each well.

3) Cover, using masking tape, and incubate overnight at room temperature. Do not incubate on Minimix. If preparing plates in advance, plates may be coated and stored up to one month at 2°-8°C. Allow to reach to room temperature before use.

2.7.5.3 Preparation of Standards, Controls, and Participant Samples

1) Thaw samples and controls at 37°C.

2) Vortex to mix samples.

3) Label tubes for 1:120 dilutions. Prepare dilutions with dilution buffer as follows: 10 µl plasma plus 1:19 ml dilution buffer. Vortex. Double these amounts when preparing the VNC dilution.

4) The same 1:120 sample dilution may be used for both Protein C's and von Willebrands Factor if both assays are performed the same day (within one hour).

2.7.5.4 Preparation of Calibration Curve

To prepare a Protein C standard curve, the Protein C reference standard is diluted to obtain a concentration of 200 ng/ml. This solution is then used to prepare subsequent serial dilutions for the calibration curve.

	Value	Dilutions	Standard	Buffer
A	200 ng/ml	1:4200	5 µl (reference)	21 ml
B	100 ng/ml	1:2 (A)	500 µl (A)	500 µl
C	50 ng/ml	1:2 (B)	500 µl (B)	500 µl
D	25 ng/ml	1:2 (C)	500 µl (C)	500 µl
E	12.5 ng/ml	1:2 (D)	500 µl (D)	500 µl
F	6.25 ng/ml	1:2 (E)	500 µl (E)	500 µl

2.7.5.5 Assay

Perform this and subsequent washings as follows:

1) Decant plate completely. Fill Vaccupette trough with washing buffer. Fill dispenser by pulling syringe to the 22 ml mark. Line up wells on plate with dispenser. Deliver washing solution by gently pushing syringe in. Do not allow dispenser tips to touch solution. Decant and drain briefly into paper towels. Repeat four times. Make sure plates are empty, but do not allow to dry out.

2) Working quickly enough to prevent plate from drying out, use the Pipetman and deliver 200 µl of well-mixed standard, control, or plasma specimens to correct well. See attached sample worksheet for set-up. (BL is Blank, which should contain 200 µl dilution buffer.)

3) Cover with masking tape, gently place on Fisher-Minimix, turn Minimix on, and allow to incubate for two hours.

4) Shortly before the end of incubation, prepare immunoconjugate as described in reagent preparation section. Pour into Titertek reagent well-marked Protein C-immunoconjugate. Wash plate five times as described above. Drain completely.

5) Immediately add 200 µl immunoconjugate using 8-channel pipettor. Place on Minimix and incubate two hours.

6) Just before end of incubation time, mix equal parts of ABTS solutions A+B. Prepare a sufficient amount to fill wells with 200 µl. Pour into reagent well marked "ABTS" and shield from light until use. Pour sufficient amount of SDS to fill each well with 100 µl into reagent reservoir marked "SDS". Place stopwatch in handy location, as well as 8-channel pipet and tips.

7) At end of incubation, wash plates five times with washing solution. Drain completely.

8) Using 8-channel multipipette, dispense 200 µl ABTS into Row 1 while simultaneously starting stopwatch. If it is too difficult to simultaneously start the watch and add ABTS, the stopwatch can be started first, then ABTS can be added to Row 1 at a chosen time point. Do not let tips touch surface of liquid. Refill pipet tips, and at precise time intervals dispense ABTS into Row 2. Continue in a like fashion until Rows 1-6 have been filled.

9) Change pipette tips. At 4 minutes from time 0, add 100 µl 5% SDS to Row 1. At same time interval as above, add SDS to Row 2. Continue as before until wells 1-6 have been filled. Alternatively, after dispensing ABTS into Rows 1-6, transfer the microtiter plate to the ELISA reader, which is on and adjusted to read 410 nm. When highest standard (well 1 B) reads approximately 0.800, remove the plate and proceed immediately with the addition of SDS as above.

10) Using identical incubation times, repeat steps 8 and 9 with the remainder of the rows.

11) After dispensing to the final row, allow plate to mix on Minimix approximately 10 minutes. Read O.D. on Microelisa Reader, setting "Blank" and "Plate Movement" switches to AUTO. Set O.D. at 410 nm, and set reference O.D. at 450 nm.

2.7.6 Back-up Procedure in Case of Instrument Failure

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

2.7.7 Computation of Results

To calculate µg/ml Protein C, use both sets of standards points and the "spline" function on the gamma counter. Since all samples are assayed at a 1:120 dilution, the calculation of their final values can be simplified by multiplication of the standard values by 120 during computation of the calibration curve. Thus the 200 ng/ml standard becomes equivalent to a sample value of 24 µg/nl, 100 ng/ml = 12 µg/ml, 50 ng/ml = 6 µg/ml, 25 ng/ml = 3 µg/ml, 12.5 ng/ml = 1.5 µg/ml, and 6.25 µg/ml = 0.75 µg/ml. By using this modification, the sample values can be read directly from the computer printout. 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 high values at a dilution.)

2.8 Assay of Tissue Plasminogen Activator Antigen

2.8.1 Principle

Tissue plasminogen activator (tPA) is a serine protease with a molecular weight of 60,000-70,000 daltons. Plasminogen is converted to the active enzyme plasmin by the action of tPA. tPA's effect is greatly enhanced by its affinity to fibrin. This quantitative ELISA assay employs the double-antibody sandwich technique. Anti-tPA is coated on microtiter wells, then allowed to bind to tPA in the plasma sample. The plate is washed, and peroxidase-conjugated anti-tPA is then bound to the tPA. The amount of immunoconjugate is evidenced by the oxidation of the ABTS substrate. The quantity of product formed is compared to a standard curve, and results are reported in ng/ml.

2.8.2 Reagent Preparation

1) 0.1 M $NaHCO_3$, unadjusted: Add 8.4 g $NaHCO_3$ to a 1000 ml graduated cylinder, q.s. to 1 liter using distilled H_2O. Mix with stirring bar on stirring plate. Store at 2°-8°C. Discard if solution becomes turbid. Store in plastic bottle. Bring to room temperature before use.

2) 0.1 M $NaHCO_3$, pH 9.2: Adjust 100 ml of 0.1 M $NaHCO_3$ to pH 9.2 using NaOH. Store at 2°-8°C in plastic bottle. Discard if solution becomes turbid. Bring to room temperature before use.

3) Dulbecco's PBS Stock (0.1M): Dissolve the following in 1 liter distilled water:

2.0 g	KH_2PO_4
2.0 g	KCl
80 g	NaCl
21.6 g	Na_2HPO_4 $7H_2O$ or 11.44 g Na_2HPO_4

Pour into plastic bottle and store at 2°-8°C. Bring to room temperature before use. Discard if turbid.

4) Washing Buffer and Dilution Buffer: Dilute 100 ml Dulbecco's PBS Stock in 900 ml distilled water. Add 0.5 ml Tween-20 and 1 g Ovalbumin pH to 7.4. Store at 2°-8°C. Bring to room temperature before use. Discard if turbid.

5) Coating: 3 mg goat antihuman tPA immunoglobulin. (American Diagnostica). Reconstitute contents in 1.5 ml 0.1 M $NaHCO_3$ and store in 35 µl aliquots in microtubes at -20°C or colder. Label "coating".

6) Conjugate: Peroxidase-conjugated anti-tPA IgG. (American Diagnostica). Reconstitute the contents in 250 µl 0.1 M $NaHCO_3$, pH 9.2. After dissolution, add 250 µl glycerol. Mix well. Store in dark at -20°C in tightly capped glass tube. Label "conjugate".

7) Potassium thiocyanate (KSCN): Reconstitute according to the vial label to give 1.0 M solution. Store at 2°-8°C.

8) Standard: 2 µg highly purified human tPA in 125 µg BSA. Reconstitute contents in 125 µl 1.0 M KSCN. Then add 3.0 ml PBS-Tween-BSA. Store in 75 µl portions in tightly capped microtubes at -20°C or colder. Label "standard".

9) ABTS Peroxidase Substrate System (Kirkegaard & Perry): Solution A: ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulfonate(6)]). Solution B: peroxide. Mix one part solution A with one part solution B immediately prior to use. Discard any remaining mixed solution.

10) 5% SDS (Sigma): 5 grams sodium docedyl sulfate in 100 ml distilled water. Store in plastic bottle.

2.8.3 Quality Control Material

1) Normal Pooled Plasma (NPP): Collected from 20 normal donors. Pool, aliquot in 250 µl amounts, and store at -70°C. Stable 18 months.

2) Universal Coagulation Reference Plasma (UCRP) (Thromboscreen, Pacific Hemostasis). Assayed pooled normal plasma; lyophilized. Reconstitute with 1 ml distilled water. Invert gently to mix. Stable 1 hour at room temperature or 2 hours at 2°-8°C.

2.8.4 Equipment and Supplies

- Titertek multichannel pipette and tips
- Titertek reagent reservoirs
- Dynatech Immulon 2 Microtiter Plates
- 200 µl Pipetman
- Rainin Auto Pipette, 1 ml
- Fisher Microplate Mixer
- MR600 ELISA Reader

2.8.5 Procedure

2.8.5.1 Sample Preparation

1) Blood is collected 1:10 in Na citrate, centrifuged at 2°-8°C for 10 minutes at 4200 rpm and plasma separated from red cells. Plasma is frozen rapidly and stored at -70°C until shipping to CHL. Thaw rapidly at 37°C.

2) Using dilution buffer prepare 1:4 dilutions.

2.8.5.2 Preparation of tPA Standards

1) Thaw one tube of 75 µl tPA standard at 37°C. Dilute to 1.0 ml with dilution buffer. This "working" standard contains 48 ng/ml tPA.

2) Using Rainin auto pipette and fresh pipette tips each time, prepare following dilutions from the working standard:

	Value	Dilutions	Standard	Buffer
A	12 ng/ml	1:4	250 µl ("working")	750 µl
B	8 ng/ml	1:6	200 µl ("working")	1.0 ml
C	4 ng/ml	1:12	500 µl (B)	500 µl
D	2 ng/ml	1:24	500 µl (C)	500 µl
E	1 ng/ml	1:48	500 µl (D)	500 µl
F	0.5 ng/ml	1:96	500 µl (E)	500 µl

2.8.5.3 Assay

1) Set up samples, standards, and control as in sample worksheet. Standard curve should be run twice, samples should be run twice, and an NPP control should be run at the bottom of every row.

2) Dilute 30 µl "coating" to 24 ml with 0.1 M $NaHCO_3$ (unadjusted). Pour into titertek reagent well appropriately marked.

3) Using 8-channel multipipette, add 200 µl to each well. Incubate for three hours at room temperature, covered. (Alternatively, allow to incubate at 2°-8°C overnight.)

4) Discard the solution and wash plate four times. These and all subsequent washings should be performed as follows: Fill the Culture Tek Vaccupette chamber with wash buffer. Pull back syringe to 22 ml mark. Empty microelisa plate by inverting over sink. Keeping inverted, blot on paper towel until empty. Place upright on counter, and line up Vaccupette over it. Dispense contents by smoothly pushing in plunger. Repeat.

5) Add 200 µl of well-mixed sample, control, or standard to appropriate wells. Incubate plate overnight, well covered, on Fisher Minimix.

6) Add 12 µl conjugate to 24 ml dilution buffer. Pour into appropriate titertek reservoir.

7) Wash plate as above four times. Add 200 µl conjugate to each well. Incubate the plate (covered) for three hours on Fisher Minimix.

8) Shortly before end of incubation, mix equal portions of ABTS solutions A and B. Pour into appropriate reagent well and shield from light until use. Pour a sufficient amount of SDS to fill each well with 200 µl into reagent well marked SDS. Place stopwatch, 8-channel pipette, and pipette tips in convenient location.

9) At end of incubation, wash plates four times as above.

10) Start stopwatch. Add 200 µl ABTS to first row of wells. At timed intervals, dispense 200 µl to following rows.

11) Change pipette tips and reset 8-channel pipette for 100 µl. Place plate on ELISA reader, moving plate occasionally to evenly disperse

color. When highest standard (well 1 B) reads at .800-1.200, remove plate and go quickly to next step.

12) At same time intervals as above, dispense 100 µl 5% SDS to rows.

13) After dispensing to the final row, allow plate to mix in Minimix approximately 10 minutes. Read O.D. on Microelisa Reader, setting "Blank" and "Plate Movement" switches to AUTO. Set O.D. at 410 nm, and set reference at 450 nm.

2.8.6 Back-up Procedure in Case of Instrument Failure

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

2.8.7 Computation of Results

Use "spline" function on LKB gamma counter. Report all results in ng/ml. Repeat any results that differ by more than 10%, or that have values falling greater than the highest standard or lower than the lowest standard.

2.9 Assay of β-Thromboglobulin

2.9.1 Principle

Low affinity platelet Factor 4 is a glycoprotein present in α granules of platelets. It is secreted and converted to β-thromboglobulin (βTG) in the circulation by plasmin or other proteases. βTG present in the circulation is considered to be a marker for the platelet release reaction. This radioimmunoassay depends on competition between βTG present in plasma and ^{125}I labeled βTG for sites on a βTG specific antibody. The antibody-bound ^{125}I βTG is separated from "free" βTG by ammonium sulfate precipitation. After centrifugation and decanting of the supernatant, the precipitated radioactive βTG is measured in a gamma counter. The amount bound will be inversely proportional to the unlabeled βTG present, and concentrations will be compared to a standard curve and reported in ng/ml.

2.9.2 Reagent Preparation (Amersham βTG Kit, Amersham, Arlington Hts. IL)

1) ^{125}I βTG (human, freeze-dried): Contains up to 2 µCi ^{125}I in 10 ml solution after reconstitution. Reconstitute the freeze-dried ^{125}I βTG reagent with 10 ml distilled H_2O. Invert gently to mix. Store at 2°-4°C for up to one week after reconstitution. Bring reagent to room temperature before use.

2) Anti-βTG (rabbit, freeze-dried): Contains antiserum sufficient to bind at least 40% of 0.5 ng of βTG in a 10 ml solution after reconstitution. Reconstitute with 10 ml distilled H_2O. Invert gently to mix. Store at 2°-8°C for up to one week after reconstitution. Bring reagent to room temperature before use.

3) Ammonium Sulfate Solution: Contains greater than 25 ml of 3.3 M solution of ammonium sulfate. Use at room temperature.

4) βTG (human) standards in Buffer (freeze-dried): After reconstitution each vial contains 0.5 ml of solution with βTG concentrations of 10, 20, 50, 100, and 250 ng/ml. Exact values are stated on vial. Reconstitute each vial with 500 µl freshly distilled water. Invert gently to dissolve. Using saline, prepare further dilutions of 1:2 with the 10 standard and 3:4 with the 20 standard to provide a 5 ng/ml and 15 ng/ml standard, respectively. Expires one week after reconstitution. Standards must be at room temperature before use.

2.9.3 Quality Control Material

Combination Anticoagulant Control Pool (CACP): Collected from flawless venipunctures on 20 normal subjects into the combination anticoagulant. Plasma pooled and aliquoted into 250 µl amounts. Stable indefinitely at -70°C.

2.9.4 Equipment and Supplies

- Pipetman: 1 ml and 200 µl and pipet tips
- Assay tubes: 12x75 mm polystyrene tubes
- Vortex mixer
- Centrifuge
- Tube racks
- Decantation racks
- Absorbant towels
- Gamma scintillation counter
- Eppendorf Pipette

2.9.5 Procedure

2.9.5.1 Sample Preparation

Samples must be collected by flawless venipuncture into combination anticoagulant. They must be inverted gently and placed in a melting ice bath. Samples are spun at 4°C for 10 minutes at 3000 xg then filtered through a 0.45 micron filter to remove any remaining platelets or cell debris. Freeze and store at -70°C until shipping to the CHL.

2.9.5.2 Assay

1) See sample worksheet for set up. Thaw all samples at 37°C for 15 minutes. Vortex to mix. Take test components out of refrigerator and allow to equilibrate to room temperature.

2) Label polystyrene assay tubes and place in rack with samples in front of them.

3) Using Pipetman and new pipette tips for each sample, pipette duplicate 50 µl aliquots of standards, controls, and plasma specimens into assay tubes. Pipette distilled water into the total, blank, and reference tubes.

4) Using Eppendorf pipette, pipette 200 µl of βTG ^{125}I solution into each assay tube. Dispose of pipette tip in radioactive waste can.

5) Using Eppendorf pipette, pipette 200 µl anti-βTG solution into each assay tube and vortex to mix them. Total tube and blank tube do not receive any antibody.

6) Cover with parafilm and incubate for one hour at room temperature.

7) At the end of incubation time, pipette 500 µl of ammonium sulfate using Eppendorf pipette, excluding total tubes. Immediately vortex all tubes to assure homogeneous mixtures.

8) Centrifuge the tubes for 10-15 minutes at 1000-1500 xg at room temperature.

9) Remove the tubes from the centrifuge, place them in decantation racks, and smoothly pour off supernatants into sink specified for radioactive wastes. Do not decant total counts tubes.

10) Keeping tubes inverted, drain briefly on paper towels until noticeable drops of liquid are absorbed into towels.

11) Count the precipitates in gamma counter.

2.9.6 Back-up Procedure in Case of Instrument Failure

The department has access to another gamma counter in case of an instrument malfunction.

2.9.7 Computation of Results

Results are computed by linear regression using the LKB gamma counter (1272 Clinigamma). Results are reported as ng/ml. Results must be repeated if:

1) duplicates do not match within 10%.
2) the unknown is greater than the highest standard or lower than the lowest standard. (Repeat samples that are too high at a greater dilution.)

2.10 Assay for Platelet Factor 4

2.10.1 Principle

Platelet Factor 4 (PF-4) is a glycoprotein present in α granules and released from platelets following platelet aggregation. It possesses antiheparin activity and is a potent agent for triggering platelet aggregation.

In this radioimmunoassay, PF-4 in test plasma competes with labeled ^{125}I-PF-4 for antibody sites on anti-PF-4. The bound and free fractions are then separated by precipitation with saturated ammonium sulfate, and the precipitate is counted to determine the amount of ^{125}I-PF-4 bound to antiserum. This amount will be inversely equal to the amount of nonlabeled PF-4 present in the unknown sample. Results are reported in ng/ml.

2.10.2 Reagent Preparation (Abbott PF-4 Kit, Abbott, North Chicago, IL)

1) ^{125}I Platelet Factor 4 (human) in dilution buffer with stabilizers. Radioactivity: 0.35 µCi or less/ml. Preservative: 0.02% sodium azide. Ready to use. Allow to reach room temperature and invert gently before use. Observe expiration date. Store at 2°-8°C.

2) Platelet Factor 4 Antiserum (Goat) in dilution buffer: Preservative 0.02% sodium azide. Ready for use. Must be room temperature before use. Invert gently to mix; observe expiration date on vial. Store at 2°-8°C.

3) Platelet Factor 4 (human) Standards: 10, 30, 50, and 100 ng/ml in dilution buffer with stabilizers. Preservative 0.02% sodium azide. Using dilution buffer, make further 1:2 dilutions of the 10 and 30 ng/ml standards to provide 5 and 15 ng/ml standards, respectively. Invert to mix gently before use. Use at room temperature. Store at 2°-8°C.

4) Dilution Buffer: 0.01 M Tris buffer with 0.15 M BSA. Preservative: 0.02% sodium azide. Ready for use. Use at room temperature. Store at 2°-8°C.

5) Ammonium Sulfate: 73% saturated in distilled water. Use as it is. Bring to room temperature and invert gently to mix. Store at 2°-8°C.

2.10.3 Quality Control Material

Combination Anticoagulant Control Pool (CACP): Collected from flawless venipunctures on 20 normal subjects into the combination anticoagulant. Plasma pooled and aliquoted into 250 µl amounts. Stable indefinitely at -70°C.

2.10.4 Equipment and Supplies

- Pipetman: 200 µl and 1 ml capabilities and disposable tips
- Assay tubes: 12x75 mm polystyrene
- Vortex mixer
- Test tube racks
- Decantation racks
- Eppendorf pipette
- Absorbant towels
- LKB gamma counter

2.10.5 Procedure

2.10.5.1 Sample Preparation

Specimens should be collected by flawless venipuncture into combination anticoagulant. Invert gently to ensure mixing and place in melting ice bath. Spin at 3000 xg for 10 minutes at 2°-8°C, separate plasma from red 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 in microvials until shipping to the CHL.

2.10.5.2 Assay

See sample worksheet for assay set-up. Thaw samples at 37°C for 15 minutes. Vortex to mix. Take reagents out of refrigerator and allow to reach room temperature before testing.

1) Label duplicate sets of tubes according to protocol.

2) Pipette 50 µl of dilution buffer into blank tubes.

3) Pipette 50 µl of standards and unknown specimens into appropriate tubes. (Dilution buffer should be used for reference sample.)

4) Pipette 250 µl ^{125}I-PF-4 reagent solution into all tubes. Cap the total tubes and set aside.

5) Pipette 250 µl dilution buffer into blank tubes. Pipette 250 µl PF-4 antiserum into all other tubes.

6) Mix all tubes well using vortex. Return reagents to refrigerator (except ammonium sulfate).

7) Cover tubes with parafilm and incubate for two hours at room temperature.

8) Pipette 1 ml ammonium sulfate to all tubes using Eppendorf pipette. Vortex tubes for 3-5 seconds, allow to stand at room temperature at least 10 minutes, but no longer than 60 minutes, before centrifugation.

9) Centrifuge all tubes at 1000-1500 xg for 20 minutes.

10) After centrifugation, carefully transfer all tubes to decantation rack. Smoothly in one continuous motion, decant into radiation-approved sink. Leaving tubes inverted, drain briefly onto several layers of absorbant towels until noticeable drops of liquid have been absorbed.

11) Count precipitates in gamma counter.

2.10.6 Back-up Procedure in Case of Instrument Failure

The department has access to another gamma counter in case of an instrument malfunction.

2.10.7 Computation of Results

Calculate results according to protocol found in βTG procedure. Report results as ng/ml. Results must be repeated on new samples 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 high values at dilution.)

2.11 Assay of Fibrinopeptide A

2.11.1 Principle

When thrombin is activated and in turn hydrolyzes fibrinogen, fibrinopeptide A (FPA) is the first peptide to be cleaved from the molecule. Four arginyl-glycyl bonds are cleaved from the N terminal ends of fibrinogen to release 2 FPA molecules and 2 FPB molecules. In this quantitative assay, unlabeled FPA in the plasma sample and radioactively labeled FPA compete for antibody binding sites. Following incubation, free and bound FPA are separated by the polyethylene glycol-second antibody solution. A standard curve is prepared by plotting the percent B/Bo of labeled FPA bound by each standard versus its concentration. Participant plasma samples are compared to the curve, and results are reported in ng/ml.

2.11.2 Reagent Preparation (Mallinkrodt FPA kit, Mallinkrodt, St. Louis MO)

1) Fibrinopeptide A ^{125}I Reaction Solution: Contains <10 μCi desaminotyrosyl-fibrinopeptide A ^{125}I in 11 ml buffered, stabilized, aqueous solution containing cyclohexamide and chloramphenicol as preservatives. Ready for use. Allow to come to room temperature before use. May be used up to the expiration date on label. Store at 2°-8°C.

2) Fibrinopeptide A Standards: Standards have FPA concentrations of 0, 1.0, 2.0, 5.0, 10.0, 20.0 and 40.0 ng/ml in a stabilized buffered protein solution containing cyclohexamide and chloramphenicol as preservatives. Volume of the zero standard is 5 ml. Volume of the other standards is 3 ml. Standards are stable up to the expiration date on vial. Store at 2°-8°C. Bring to room temperature before use.

3) Fibrinopeptide A Antiserum (rabbit): This solution contains antiserum in a dilution greater than 1:100 in a buffered aqueous solution containing cyclohexamide and chloramphenicol as preservatives. Volume: 11 ml. Ready for use. Stable up to expiration date on vial. Store at 2°-8°C. Bring to room temperature before use.

4) Polyethylene Glycol(PEG)-Second antibody: Contains 6.5% PEG w/v, silica gel, and anti-rabbit precipitating antibody in a dilution greater than 1:10 in a buffered, aqueous solution containing normal human serum as carrier protein and thimerosal as the preservative. Volume: 110 ml. Ready to use. Observe expiration date on bottle. May be used at 2°-8°C.

5) Bentonite Slurry: Suspension of bentonite in a stabilized buffered protein solution containing cyclohexamide and chloramphenicol as preservatives. Volume: 50 ml. Ready to use. Mix on stirring plate, allow to mix while pipeting. Use at room temperature. Observe expiration date on label.

6) Nonspecific binding buffer solution: This is a stabilized, buffered protein solution containing cyclohexamide and chloramphenicol as preservatives. Volume: 5 ml. Allow to reach room temperature before use. Observe expiration date.

2.11.3 Quality Control Material

1) Fibrinopeptide A (FPA) Controls (A and B): One vial each. Contain fibrinopeptide A in a stabilized buffered protein solution containing cyclohexamide and chloramphenicol as preservatives. Control A contains a normal level of FPA, control B contains an elevated level of FPA. Volume: 5 ml. Bring to room temperature before use. Observe expiration date on vial.

2) Combination Anticoagulant Control Pool (CACP): Collected from flawless venipunctures on 20 normal subjects into the combination anticoagulant. Plasma pooled and aliquoted into 250 µl amounts. Stable indefinitely at -70°C.

2.11.4 Equipment and Supplies

- Pipetman: 200 µl capability
- Assay tubes: 12x75 mm polystyrene tubes
- Vortex mixer
- Stirring plate with stirring bar
- Centrifuge
- Pasteur pipettes
- Tube racks
- Decantation racks
- Eppendorf pipette
- Absorbent towels
- LKB gamma counter

2.11.5 Procedure

2.11.5.1 Sample Preparation

Specimens should be collected by flawless venipuncture into combination anticoagulant. They must be inverted gently to ensure total mixing and placed in a melting ice bath. They are then spun at 3000 xg for 10 minutes at 2°-8°C, and plasma separated from red cells. Plasma is frozen rapidly and stored at -70°C freezer in microtubes until shipping to the CHL. See attached worksheet for sample set-up. Thaw samples at 37°C for 15 minutes. Vortex to mix. Take test components out of refrigerator and allow to equilibrate to room temperature.

2.11.5.2 Test Plasma Treatment

1) Label 12x75 mm polystyrene tubes for each participant sample. These will be used for fibrinogen precipitation.

2) Pour bentonite slurry into clean beaker. Set on stirring plate with stirring bar.

3) Using Pipetman, dispense 500 µl plasma into polystyrene tubes.

4) Using Eppendorf pipette and allowing slurry to continue stirring, add 1 ml bentonite slurry to each tube.

5) Vortex and centrifuge at 1000 xg for 10 minutes.

6) Using Pasteur pipette, carefully aspirate supernatant and transfer to second labeled tube, taking care not to disturb precipitate. Recentrifuge the supernatant and transfer the upper 2/3 to another clean tube. Caution: Do not treat standards or controls. These are ready to use.

2.11.5.3 Assay

Note: Invert reagents gently to assure homogeneity before adding to test system.

1) Label 12x75 mm assay tubes (polystyrene) according to protocol sheet. Add 100 µl of each FPA standard, control, or plasma sample to appropriate tubes. Add 200 µl of 0.0 ng/ml standard to "Blank" tubes. "Total" tubes remain empty.

2) Dispense 100 µl of FPA ^{125}I solution to all tubes using an Eppendorf pipette. Dispose of pipette tip in appropriate container. Vortex all tubes.

3) Add 100 µl of FPA antiserum to all tubes except "Blank" tubes. Add 100 µl of nonspecific binding buffer solution to "Blank" tubes.

4) Gently vortex all tubes for several seconds to assure thorough mixing. Return all reagents to refrigerator. Cover tubes with parafilm.

5) Incubate tubes at room temperature for 40-120 minutes.

6) Cap total tubes and set them aside. They are ready for counting and need no further processing. Thoroughly mix the PEG-second antibody solution, without causing foaming. Add 1 ml to each tube except total tubes. Vortex vigorously for several seconds.

7) Centrifuge tubes at 1000 xg for 20 minutes. They may be centrifuged at 2°-8°C (preferred) or at room temperature.

8) After centrifugation, carefully place all tubes in decanting racks. Smoothly decant into approved sink. Leaving tubes inverted, drain briefly onto several layers of absorbent paper towels. Press gently on tube bottom to ensure absorption of noticeable liquid.

9) Count precipitates in gamma counter.

2.11.6 Back-up Procedure in Case of Instrument Failure

The department has access to another gamma counter in case of instrument malfunction.

2.11.7 Computation of Results

See "Data Reduction" section in βTG procedure. Report results in ng/ml. 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 high values at dilution.)

Note: In view of the recent unexpected decision by Mallinkrodt to terminate this product, this leaves us no alternative but to set up an Elisa assay, a product of Stago Company in France. This work is being done and the validity of this assay will be disclosed. Once the validity is proved, the procedure will be revised. Since successful transition is anticipated, we have listed this product in Table 1.

2.12 Assay of Fibrinopeptide Bβ15-42 and Bβ1-42

2.12.1 Principle

Activated thrombin attacks fibrinogen and converts it to fibrin I, cleaving from it two fibrinopeptide A molecules. Plasmin and thrombin then act on fibrin I polymers, releasing fibrinopeptide Bβ1-42 and producing fibrin II, respectively. When plasminogen is converted to plasmin, plasmin acts on fibrin II to release Bβ15-42. An assay for Bβ15-42 is therefore a good monitor for both thrombin and plasmin activation. In this quantitative assay, Bβ15-42 peptide in plasma competes with fibrin-coated plates for anti-Bβ15-42. Antibody bound to the plate is quantitated with anti-IgG coupled to horseradish peroxidase. The amount of peroxidase is evidenced by ABTS peroxidase substrate, and amount of color is inversely proportional to the amount of Bβ15-42 in the sample. Amounts are compared to known values from a standard curve and reported in ng/ml.

2.12.2 Reagents

1) Fibrin Monomer (coating) (New York Blood Center, NYBC): 1 mg, lyophilized. Reconstitute with 200 µl of 10% acetic acid. Store at 2°-8°C. To use, dilute 20 µl in 10 ml of coating buffer. Observe expiration date.

2) Monoclonal Antibody to Bβ15-42 Peptide (NYBC): Reconstitute vial with 0.5 ml distilled water. Store at 2°-8°C. Observe expiration date. Dilute stock solution 1:100 in dilution buffer for use. 50 µl are needed for each well.

3) Bβ15-42 Peptide Standard (NYBC): Reconstitute vial with 1.0 ml distilled water. Store 2°-8°C. Observe expiration date.

4) Coating Buffer: Dissolve 1.59 g sodium carbonate and 2.95 g sodium bicarbonate in 1 liter distilled water. Adjust pH to 9.6 with HCl or NaOH. Store at 2°-8°C, discard if turbid.

5) Dulbecco's PBS Stock (0.1 M): Dissolve the following in 1 liter distilled water:

2.0 g KH_2PO_4
2.0 g KCl
80 g NaCl
21.6 g $Na_2HPO_4.7H_2O$ or 11.44 g Na_2HPO_4

Pour into plastic bottle and store at 2°-8°C. Bring to room temperature before use. Discard if turbid.

6) Washing Buffer (Tween-PBS): Dilute 100 ml Dulbecco's PBS stock with 900 ml distilled water. Add 0.5 ml Tween-20. Store at 2°-8°C. Bring to room temperature before use. Discard if turbid.

7) 1% BSA: Dissolve 0.1 g Bovine Serum Albumin in 10 ml Tween-PBS. Use that day.

8) Dilution Buffer (NYBC): Dilute to 200 ml with distilled water. Store at 2°-8°C. Do not use if turbid.

9) HRP-Conjugate (NYBC): Horseradish Peroxidase conjugated to Anti-Mouse IgG. Dilute stock solution 1:200 in Tween-PBS (add 50 µl to 10 ml). 100 µl are needed per well. Store at 2°-8°C. Observe expiration date.

10) Working Color Reagent (ABTS system) (Kirkegaard & Perry): Mix one part solution A (ABTS) with one part solution B (H_2O_2) immediately prior to use. 100 µl are needed per well. Discard any remaining mixed solution.

11) 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

Normal Bβ pool (NBβC): Collected from 20 normal donors. Collect blood in combination anticoagulant, and spin at 4°C for 10 minutes at 3000 xg. Pool all plasma and mix with an equal volume of 95% ethanol (ice cold). Allow mixture to sit for 30 minutes on ice. Centrifuge 15 minutes at 3000 xg and remove supernatant with Pasteur pipette. Supernatant is then lyophilized to remove ethanol. Resuspend in dilution buffer to the original plasma volume and freeze at -70°C in 200 µl aliquots.

2.12.4 Equipment and Supplies

- Titertek multichannel pipette and tips
- Titertek reagent reservoirs
- Dynatek Immulon 2 Microtiter Plates
- 200 µl Pipetman and tips
- Rainin Autopipetor, 1 ml
- Culture-tek Vaccupette/96 Plate Washer with 50 ml syringe attached

2.12.5 Procedure

2.12.5.1 Sample Collection

Specimens should be collected by flawless venipuncture into combination anticoagulant. Separate plasma by centrifuging 3000 xg for 10 minutes. Filter plasma through Millex filter, then add 500 µl of filtered plasma plus 500 µl of saline into Centricon filter. Centrifuge for 30 minutes at 3000 xg. Transfer filtrate into microtainer tubes. Plasma is frozen rapidly and stored at -70°C until shipping to the CHL.

2.12.5.2 Coating of Plates

Dilute 20 µl of fibrin monomer in 10 ml coating buffer and apply 100 µl to each well. Allow plate to stand three hours at room temperature or overnight at 2°-8°C.

2.12.5.3 Assay

Prepare worksheet as illustrated by the sample worksheet.

1) After incubation, wash plates four times with Tween-PBS. Perform this and subsequent washings as follows: Fill tray of Vacupette/96 with Tween-PBS. Decant microtiter plate completely and drain on paper towels. Fill Vacupette dispenser by pulling back plunger to 22 ml mark. Align dispenser over microtiter plate. Dispense buffer by gently pushing plunger in, taking care that tips do not touch surface of fluid in wells. Decant plate completely. Repeat. (Note: Decant completely but do not allow to dry out between washings.)

2) Working quickly enough to prevent plate from drying out and using 8-channel pipette, deliver 100 µl per well of 1% BSA. Let stand 15 minutes at room temperature on Fisher Minimix.

3) During incubation, using Eppendorf pipette, prepare the following standards: 10 pmoles/ml, 5 pmoles/ml, 2.5 pmoles/ml, 1.25 pmoles/ml, 0.625 pmoles/ml, 0.312 pmoles/ml. Pipette 100 µl of each into 12x75 mm polystyrene tubes.

4) Pipette 100 µl of each unknown sample into 12x75 mm polystyrene tubes.

5) Using Eppendorf pipette, add 100 µl diluted antibody solution to each control, standard, and unknown.

6) Wash plate four times with Tween-PBS as above. Drain completely.

7) Not allowing plate to dry out, pipette 85 µl of each standard, control, and unknown into designated wells.

8) The well labeled "Blank" should contain 85 µl dilution buffer. Cover with tape.

9) Incubate plate on Fisher Minimix for 30 minutes.

10) Wash four times with Tween-PBS as above.

11) Add 100 µl diluted HRP-Conjugate to each well. Place on mixer for 45 minutes.

12) Wash four times as above.

13) Add 100 µl ABTS to Row 1 while simultaneously starting stopwatch. Exactly 15 seconds later, dispense 100 µl to Row 2. Continue through the last row.

14) Change pipette tips. At 5 minutes from time 0, add 100 µl 5% SDS to Row 1. Exactly 15 seconds later, add 100 µl to Row 2. Continue through the rest of the rows.

15) After dispensing to the final row, allow plate to mix on Minimix approximately 10 minutes. Read O.D. on Microelisa Reader, setting "Blank" and "Plate Movement" switches to AUTO. Set O.D. at 410 nm and reference at 450 nm.

2.12.6 Back-up Procedure in Case of Instrument Failure

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

2.12.7 Computation of Results

Use "spline" function in LKB gamma counter. Report all results in pmoles/ml. Repeat results that do not duplicate within 10%, or that have values greater than the highest standard or less than the lowest standard. The samples greater than the highest standard are repeated at dilution. (Note: Fibrinopeptide Bβ(1-42) will be done in an identical manner except monoclonal antibodies against FBβ(1-42) will be used.)

2.13 Assay of Thromboxane B_2

2.13.1 Principle

Thromboxane A_2, when released from platelets, constricts blood vessels and is a potent stimulus for platelet aggregation. However, TXA_2 has a half-life of only 30 seconds in the peripheral system and is therefore impossible to monitor. The breakdown product, TXB_2, is significantly more stable and is therefore a suitable monitor for TXA_2 release.

In this quantitative assay, TXB_2 and ^{3}H-labeled TXB_2 compete for antibody sites on anti-TXB_2. After equilibration has been reached, charcoal is added to precipitate the free TXB_2. The tubes are centrifuged, and the supernatant (containing bound TXB_2) is poured into scintillation fluid and counted. Results are compared to a normal curve. This radioimmunoassay has a sensitivity of approximately 20 pg/200 µl and the antibody exhibits minimal cross reactivity (<3%) with other prostaglandins.

2.13.2 Reagents

1) RIA Buffer: 0.05 M Tris-HCl, pH 7.5 + 0.1% gelatin. Prepare 1 liter, heat slightly to dissolve gelatin. Store at 2°-8°C. Stable for one month.

2) TXB_2 antibody: Anti-TXB_2, prepared and characterized in house (batch 1965-2-5-85), should be diluted 1:1000. Generally antiserum is diluted 1:10 with RIA buffer and store at -20°C as a stock solution. For each run, calculate the amount of antiserum needed: (100 µl x number of tubes = number of ml of RIA buffer). Add 5 µl anti-TXB_2 per ml of buffer.

3) ^3H-TXB_2 antigen (New England Nuclear): For each run, calculate amount needed: (100 µl x number of tubes = number of ml RIA buffer). Add 1 µl ^3H-TXB_2 per ml buffer.

4) Charcoal: Dissolve 1 gm bovine γ-globulin in 100 ml RIA buffer. When IgG is dissolved, add 3 gm of Norit A charcoal. Stir for a minimum of one hour before use. Store at 2°-8°C.

5) Standards: Prepare a 1 mg/ml solution of TXB_2 (add 1 ml distilled water to 1 mg TXB_2). Add 5 µl/5 ml RIA buffer. This solution is 1000 pg/µl (Solution A). Dilute as follows:

10	ng/ml	100 µl (A)	+	9.9 ml RIA Buffer
5	ng/ml	50 µl (A)	+	9.95 ml RIA Buffer
2.5	ng/ml	25 µl (A)	+	9.98 ml RIA Buffer
1	ng/ml	10 µl (A)	+	9.99 ml RIA Buffer
0.5	ng/ml	0.5 ml (10)	+	9.5 ml RIA Buffer
0.25	ng/ml	0.5 ml (5)	+	9.5 ml RIA Buffer
0.1	ng/ml	0.2 ml (5)	+	9.8 ml RIA Buffer
0.05	ng/ml	0.2 ml (2.5)	+	9.8 ml RIA Buffer

2.13.3 Quality Control Material

Normal Serum Pool (NSP): Collected from 20 normal subjects into clot-activated tubes and incubated at 37°C for one-half hour. Aliquot, freeze, and store at -70°C.

2.13.4 Equipment and Supplies

- Eppendorf pipette with tips
- Pipetman with tips 200 µl capacity
- 10x75 mm glass tubes
- Test-tube rack
- Cardboard cut to enable inversion of tubes in test-tube rack
- Stirring plate and stirring bars
- Scintillation vials with caps
- Beta scintillation counter
- Hydrofluor scintillation fluid
- Luckham caps

2.13.5 Procedure

2.13.5.1 Sample Collection

Samples must be collected from a free-flowing venipuncture into a 7 ml clot-activated tube, mixed gently and immediately placed in a 37°C heat block. After one-half hour incubation, tube should be spun at 3000 xg for 10 minutes. Serum should be removed from the clot and stored in microtubes at -70°C.

2.13.5.2 Sample Preparation

1) Samples should be thawed at 37°C and mixed by vortexing.

2) Label 10x75 mm tubes for each plasma sample and control. Each sample will need a tube to prepare a 1:200 dilution.

3) Using RIA buffer, prepare 1:200 dilutions on each sample. Mix by vortexing.

2.13.5.3 Assay

1) Label duplicate 10x75 mm tubes for totals, blanks, reference, standards, and plasma samples.

2) Using a Pipetman, pipette 200 µl of each standard, control, and plasma sample to appropriate tubes. Total tubes receive nothing. Blank tubes receive RIA buffer. Reference tubes receive nothing.

3) Using Eppendorf pipette, add 100 µl anti-TXB_2 to each tube. Vortex to mix. Omit total and blank tube.

4) Using Eppendorf pipette, add 100 µl ^{3}H-TXB_2 to each tube.

5) Vortex well, cover with parafilm, and allow to stand at room temperature for one hour. Bring charcoal to room temperature, stir vigorously on stirring plate.

6) After incubation, add 1 ml water to each tube. Add 200 µl charcoal to Luckham caps using Eppendorf pipette. Keep charcoal stirring while pipetting.

7) Invert and insert caps into incubation tubes without spilling. Invert the tubes five times to dispense the charcoal, using cardboard to hold tubes in rack.

8) Allow to stand at room temperature for ten minutes. Dispense 10 ml scintillation fluid (Hydrofluor) into scintillation vials. Label caps for each tube in assay.

9) Centrifuge tubes 1000 xg for ten minutes.

10) Decant supernatant (contains bound fraction), cap vial, and mix gently. Tubes are best decanted by smoothly inverting, using a slight

twisting motion, and touching rim to inside of scintillation vial to extract last drop. Do not allow tube to stay inverted long enough to allow charcoal particles to fall into vial. Most importantly, use a consistent technique.

11) Count all tubes in a scintillation counter.

2.13.6 Back-up Procedure in Case of Instrument Failure

The department has two scintillation counters.

2.13.7 Computation of Results

Linear regression is used to calculate thromboxane levels. A "least squares" regression line of a (B/Bo) vs. log concentration plot is used to fit a straight line to a standard curve. This method is especially useful when the curve is sigmoidal over the entire range of concentrations. 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.

3. DATA MANAGEMENT

The Central Hemostasis Laboratory is automated for sample logging, sample retrieval, data storage, and 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 PCs) to a primary data storage and processing facility (IBM/AT). All IBM processing units are connected in such a fashion as to enable them to share programs, data, and hardware such as disk drives, printers, and other external processing units.

Each Hemostasis analysis unit (i.e., Coag-A-Mate, gamma counter, and ELISA Reader) has a capability for asynchronous transmission of ASCII character code via standard RS-232 interface. RS-232 cabling provides physical linkage for data transmission between each laboratory device and its unique control station.

Three IBM PCs, consisting of a model 176 Processor and Display Unit, accept data as documented by UTHSCH or its supplier via an AST multifunction card. In addition, these processors provide mechanisms for data validation and transmission of finalized results to the central network processor by the responsible laboratory technician.

An IBM/AT acts as the final component of the local network. It serves as a storehouse for network programs, data, and connections to telephone lines via modem.

Network programs and associated hardware, residing within the AT, provide the mechanisms for data transmission from the server PCs to ATs centralized database and storage area. In addition, the AT houses capabilities for peripheral interface (Epson Printer) and enhanced mathematical processing.

A mainframe computer is used for back-up storage of the data. Availability of such a computer is advantageous not only for keeping a duplicate copy but also for enhancing data-management capacity.

3.2 Software for Data Management

Knowledge Man software is used. It has a large capacity for data storage and is compatible with the software of the ARIC Coordinating Center.

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 and on a diskette accompany each batch of specimens. Specimen analyses are performed on a gamma counter, a Coag-A-Mate analyzer, and an ELISA reader: software written for each machine permits transmittal of results directly onto an IBM XT. Results (approximately 20 variables per subject) are re-sent weekly from the Central Hemostasis Laboratory to the Coordinating Center on diskettes 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 back-up 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 diskette weekly to each field center containing relevant results from all central agencies in order to update the local databases.

4. 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 CAP and commercial sources. A high degree of precision and accuracy has been achieved and established. For example, the Central Hemostasis Laboratory participates in the quality control survey program sponsored by General Diagnostics. Data obtained from controls are analyzed monthly. The mean and standard deviation (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 Central Hemostasis Laboratory has also participated in the College of American Pathologists (CAP) survey program for a number of years. The survey is carried out four times a year and the coagulation factors surveyed include PT, PTT, fibrinogen, VIII:C, and VWF:Ag. The references, in this instance, are provided by CAP. The internal control, as well as interlaboratory comparisons, have been satisfactory. While analyzing the hemostatic factors for ARIC, the Central Hemostasis Laboratory continues to adhere to these programs (see Table 1).

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 formation, the Central Hemostasis Laboratory has a built-in double-deionized 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 NSP, CACP, NPP, and NBβC (see Table 2).

All tests performed on the Coag-A-Mate (aPTT, 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 interday variations. The Central Hemostasis Laboratory continues to participate in General Diagnostics and CAP quality control programs and participates in an additional program sponsored by Thrombo-screen. As an additional control on Factor VII and Factor VIII, the

deficient plasma has been determined at the factory to have less than 1% activity and no inhibitors.

The quality control for AT-III chromogenic assay will be performed by running VNC at the end of each row. As the addition of reagents to each row is critically timed, each VNC value must fall within two standard deviations of the mean or the entire row must be repeated.

General principles of quality control for RIA and ELISA assays are similar for the protein and peptide assays and hence will be discussed together. The internal standardization includes (1) specificity and dilution of antibody to be used, (2) the stability (half-life) of radiolabeled materials and the radioactive counts (RIA), (3) methods to separate bound from free ligand, (4) the maximal antigen binding (Bo) to be between 30 and 50% (RIA), (5) the uniform binding of antibody to the support system (ELISA), (6) the integrity of the immunoconjugate (ELISA), and (7) the calibration curve with antigen standards. The standard curve is particularly important to determine the quality of the RIA system. The Central Hemostasis Laboratory has computer software to transform the regular plots into logit plots. The plot should be linear as verified by the linear regression analysis and have a correlation coefficient of 0.99 or better, which all the RIA assays (PF-4, βTG, FPA, and TXB_2) have consistently displayed. The RIA assays each include a minimum of one control in duplicate at the beginning and in duplicate at the end of each run. ELISA assays, like AT-III, have a control at the end of each row. The control values for RIAs and ELISAs are charted, and any set of samples with a daily mean control value beyond the two S.D. range is repeated. All samples for each assay are run in duplicate, and any sample with a difference of 10% or higher between the two values is repeated. The standard deviation, coefficient of variance, and range are calculated each month.

4.1.2 Standards

Standards that are used for constructing a calibration curve in each assay are listed in Table 1. Please note that we have recently (December 1, 1986) changed the standards for Factor VII, VIII, VWF, and AT-III from NPP to UCRP, as the latter is a lyophilized plasma pool which is more stable than NPP. Moreover, the same batches of standards will be used for up to three years. In contrast, it is virtually impossible to prepare a batch of NPP sufficient for the entire ARIC study. The standards for each assay are briefly described below.

4.1.2.1 Fibrinogen

Standard fibrinogen preparation (Fibriquik) is supplied by General Diagnostic.

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

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

4.1.2.3 Protein C: Antigen

A purified compound is supplied by American Diagnostics. Proper dilutions are made in the laboratory during the assay.

4.1.2.4 PF-4, βTG, FPA, FPBβ(15-42) and (1-42)

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

4.1.2.5 TXB_2

Standards are prepared in our laboratory prior to each assay. Pure TXB_2 is obtained from Cayman and proper dilutions are prepared before each run.

4.1.3 Quality Control Material

Quality control materials for each assay and sources are listed in Table 2. There have been changes (December 1, 1986) from our original proposal because of the change of standards as mentioned above. We have routinely used two control materials for each of the seven assays performed on each participant (i.e., aPTT, fibrinogen, factor VII, VIII, VWF, AT-III, and protein C). Analysis of the recent results indicated that one control material is sufficient, since no differences have been noted with two control materials. Use of one control material per assay has an additional advantage of being able to perform four controls per run. This will allow a better assessment of intraassay variability. The control material used for each assay is described below.

4.1.3.1 aPTT, Fibrinogen, Factor VII:C, Factor VIII:C

Verify Normal Citrate plasma control (VNC) is obtained from General Diagnostics. With each run, duplicates of each control are included at the beginning and the end of the tray. Four values of each control are obtained daily. In case of prolonged aPTT, a Verify Abnormal Citrate is included in a separate run.

4.1.3.2 AT-III and VWF:Ag

VNC is run at the end of each row.

4.1.3.3 tPA

Normal citrate plasma pool (NPP) is prepared from 20 normal donors in our laboratory periodically and is included with each run.

4.1.3.4 Protein C:Ag

UCRP is included with each run.

4.1.3.5 PF-4 and βTG

Combination anticoagulant control pool (CACP) is prepared from 20 normal subjects in our laboratory. Venous blood is drawn into a tube containing

1/10 volume of a combined anticoagulant (trasylol, chloromethyl ketone peptide, EDTA, and isobutyl methylxanthine). Following mixing, the sample is centrifuged at 3000 xg for 10 minutes and the plasma is aliquoted and stored at -70°C. This control is included in each run.

4.1.3.6 FPA

Two control materials, A for normal and B for high values, are included in each kit. These control materials are included in each run. In addition, CACP is run in duplicate to monitor the efficiency of the precipitation of fibrinogen with bentonite slurry.

4.1.3.7 FPBβ(1-42) and (15-42)

The Bβ control material is prepared in our laboratory. The peptides are isolated by mixing the plasma sample with an equal volume of ice-cold 95% ethanol and allowing the mixture to sit for 30 minutes on ice. After centrifugation, the supernatant is lyophilized to remove the ethanol. The dried extract is suspended in dilution buffer to the original plasma volume and is included in each run of Bβ(1-42) and Bβ(15-42) assays.

4.1.3.8 TXB_2

A normal serum pool (NSP) is prepared from 20 normal subjects. Procedure of serum preparation follows the blood-processing procedure detailed in Manual 7.

4.1.4 Control Limits

To establish the limits for an acceptable value for a control material, the control is run 20 times on separate runs in duplicate. When running any assay on unknowns, if the daily mean for one or more controls is beyond its control limit (calculated as shown below), the run is repeated. In addition, the operator looks for shifts and trends in an assay and their possible causes. A 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. There is a space on each worksheet for lot numbers of reagents. Lot numbers are always recorded, as this information may be important to aid possible trouble-shooting. When changing lot numbers or in-house pools, the new control material is run simultaneously with the established control material for 20 runs. New control limits are then established.

4.1.4.1 Calculation of Control Limits for Accuracy

1) The daily mean ($\bar{x}$) for each control is calculated according to the formula:

$$\bar{x} = \frac{\Sigma x}{n}$$

where Σx is the summation of each control value x and n is the number of control values determined on that day.

2) The overall mean ($\bar{\bar{x}}$) is calculated by the formula:

$$\bar{\bar{x}} = \frac{\Sigma \bar{x}}{N}$$

where N is generally 20 days and $\Sigma \bar{x}$ is the summation of the daily means.

3) The standard deviation of the daily mean is calculated by the formula:

$$S = \frac{\Sigma(\bar{\bar{x}}-\bar{x})^2}{N-1}$$

Control limits for the daily mean control chart are $\bar{\bar{x}} \pm 2$.

4.1.4.2 Calculation of Control Limits for Precision

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

$$R = X_H - X_L$$

where X_H is the highest value and X_L the lowest.

2) The average range $\bar{R}$ is then calculated

$$\bar{R} = \frac{\Sigma R}{N}$$

where N is generally 20 days. Limits for the range are obtained by multiplication of $\bar{R}$ by the appropriate probability factor, which is 1.855 for control limits when the sample is run in quadruplicate.

4.1.4.3 Calculation of Coefficient of Variance (C.V.)

The coefficient of variance of intraday and interday variations is calculated as the standard deviation divided by the mean, and expressed as a percentage.

4.1.4.4 Repeating Analyses

Runs are repeated when:

1) The daily mean, $\bar{x}$, of a control pool exceeds the control limits.
2) R exceeds the control limits.
3) C.V. exceeds control limits.

Individual values are repeated when their duplicate values 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 two computerized quality control programs. The first of these is by General Diagnostics. Quality control values may be entered manually or automatically and are set up for all tests run on the Coag-A-Mate X2 (aPTT, fibrinogen, Factor VII, and Factor VIII). Levy-Jennings charts are prepared for each assay, as well as the mean, standard deviation, and coefficient of variation. The program enables the operator to edit results and print a hard copy of the results. In addition, it stores up to 90 results for each control of each test.

The second computerized quality control program used is Computrak by Curtin Matheson Scientific. Like the General Diagnostics program, it provides Levy-Jennings charts, printing the mean, standard deviation, and coefficient of variation for each test.

Every assay run has a file for each control. The program provides a month-to-date report, which is a listing of raw data and summary statistics for the beginning of the month through the present day, and a continuous monthly report which shows 12-month as well as year-to-date statistics. Shifts and trends are readily apparent using this program.

Each assay is evaluated for intraassay and interassay variation. The coefficients of variation are calculated using a minimum of four control samples within each run for each test. This is entered automatically using the computerized quality control program.

In addition to the computerized monitoring of controls as discussed above, linear regressions are performed cumulatively on a three-month basis using the "Mini Tab" statistical analysis program. The slope of any assay's control value should always be near zero. This will keep a tight control on monitoring the stability of an assay over the eight-year time span.

4.1.6 External Quality Control

Another facet of the quality control program is an external survey of quality control (Table 3). The Central Hemostasis Laboratory participates in two programs that compare the more common of the assays with results obtained with the same reagents, equipment, and control material used in other laboratories. The General Diagnostics program is used for aPTT, fibrinogen, and Factor VIII to screen Verify Normal Citrate. The Curtin-Matheson Scientific program is used for aPTT and fibrinogen for the Universal Coagulation Reference Plasma, with the possibility of the addition of the Factor VII assay soon. The quality of coagulation laboratory procedures is assured by an external CAP program, which surveys aPTT, fibrinogen, Factor VII activity, Factor VIII activity, and VWF:Ag antigen four times a year.

4.1.7 Quality Control Data from the Central Hemostasis Laboratory

New control data have been established in the Central Hemostasis Laboratory using the new blood collection and processing techniques that are used in the ARIC field centers, the new equipment, and the combined anticoagulant

for the βTG, PF-4, FPA, and FPBβ peptide assays. The intraday and interday data are listed in Appendix A. These data provide evidence of the validity of these new techniques and reagents for studying hemostatic factors in the ARIC study.

4.2 Quality Control for Field Center Blood Collecting, Processing, Temporary Storage, and 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 [e.g., PF-4, βTG, FPA and FPBβ(1-42)] for each month of samples collected at each ARIC field center (each month is an independent representative sample) by each technician.

4.2.2 Pretesting the Feasibility of the Unified Phlebotomy System

Blood samples were collected and processed from 21 healthy donors by newly trained technicians according to the procedures detailed in Manual 7. The samples were stored temporarily in the laboratory at Hermann Hospital for one week. They were then packed in dry ice and left at room temperature for 48 hours prior to "shipping" to the Central Hemostasis Laboratory at the University of Texas Medical School. The samples were evaluated visually and stored at -70°C for 1-2 weeks. The assays were performed and data analyzed. The pretesting data are summarized in Appendix B. The pretesting values are generally within the acceptable range of the reference values established in our laboratory and compared well with the values reported, with the following exceptions:

1) Both βTG and PF-4 values are lower than those reported in the literature. Apparently the blood-processing technique and combination anticoagulants used in this study reduce <u>in vitro</u> platelet activation.

2) FPBβ(15-42) and tPA values are highly variable among the donors. Some variability may be induced by different exercise regimens. This hypothesis must be tested. Individual values are attached.

The pretesting tests ran smoothly. There were few logistical problems. It is concluded that the blood drawing and processing system proposed is suitable for the field centers' studies.

4.2.3 Pilot Study at the Field Centers

A pilot study has been performed at the four field centers to determine the reproducibility of local collection and processing of samples for hemostatic factors analysis. After the personnel had completed training, blood samples were obtained from normal men and women, with ages in the same range as for the ARIC study. Blood was collected and processed according to the procedures detailed in Manual 7. The samples were stored at -70°C for a few days and sent weekly to the Central Hemostasis Laboratory. All assays were performed in duplicate. The mean value, S.D., and C.V. of each assay for each center were being analyzed and compared with those concurrently obtained on normal volunteers at the Central Laboratory. If significant variations are encountered in terms of (mean values) or precision (S.D. and C.V. of duplicate determinations), a thorough review of the procedures will be undertaken and the results will be reported to the Steering Committee. Special attention will be devoted to the labile factors (PF-4, βTG, Bβ(1-42) and FPA), which may reflect the adequacy of the venipuncture technique, and to Factor VIII, which may reflect the adequacy of storage and shipping conditions.

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 testing 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 eight 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. They will be aliquoted, stored at -70°C, and assayed at six-month intervals. 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. Standards and Calibration References Used for Each Assay

Assay	Standards[1]	Sources[1]
aPTT	N/A	N/A
Fibrinogen	Fibriquik	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	supplied by manufacturer	American Diagnostica
PF-4	supplied by manufacturer	Abbott
βTG	"	Amersham
FPA	"	Mallinkrodt
FPBβ(1-42)	"	N.Y. Blood Center
FPBβ(15-42)	"	N.Y. Blood Center
tPA:Ag	"	Stago
TXB_2	"	Cayman

[1]The abbreviations are:

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[1]	Sources[1]
aPTT	VNC	G.D.
Fibrinogen	VNC	G.D.
Factor VII:C	VNC	G.D.
Factor VIII:C	VNC	G.D.
VWF:Ag	VNC	G.D.
AT-III activity	VNC	G.D.
Protein C:Ag	UCRP	P.H.
		T.S.
PF-4	CACP	C.H.L.
βTG	CACP	C.H.L.
FPA	A&B (kit)	Stago
	CACP	C.H.L.
FPBβ(1-42)	NBβC	C.H.L.
FPBβ(15-42)	NBβC	C.H.L.
tPA:Ag	NPP	C.H.L.
TXB_2	NSP	C.H.L.

[1]The abbreviations are:

VNC: Verify Normal Citrate Plasma
UCRP: Universal Coagulation Reference Plasma
CACP: Normal Combined-Anticoagulant Plasma Pool
NBβC: Normal Bβ Peptides Control
NSP: Normal Serum Pool
NPP: Normal Pooled Plasma
G.D.: General Diagnostics
P.H.: Pacific Hemostasis
C.H.L.: Central Hemostasis Laboratory
T.S.: Thromboscreen (Pacific Hemostasis Curtin-Matheson Scientific program)

Table 3. External Quality Assurance Program

Assay	Agency		
	CAP[1]	G.D.[1]	T.S.[1]
aPTT	X	X	X
Fibrinogen	X	X	X
Factor VII:C			X
Factor VIII:C	X	X	X
VWF:Ag	X		X
AT-III activity	X		

[1]The abbreviations are:

CAP: College of American Pathologists
G.D.: General Diagnostics
T.S.: Thromboscreen (Pacific Hemostasis Curtin-Matheson Scientific program)

Table 4. Control Materials for Evaluating Long-Term Stability

Assay	Control[1]	Source[1]
PF-4	CACP	C.H.L.
βTG	CACP	C.H.L.
FPA	CACP	C.H.L.
FPBβ(1-42)	NBβC	C.H.L.
FPBβ(15-42)	NBβC	C.H.L.
tPA:Ag	NPP	C.H.L.
TXB_2	NSP	C.H.L.

[1]The abbreviations are:

CACP: Normal Combined-Anticoagulant Plasma Pool
NBβC: Normal Bβ Peptides Control
NPP: Normal Pooled Plasma
NSP: Normal Serum Pool
C.H.L.: Central Hemostasis Laboratory

5. MACHINE AND EQUIPMENT MAINTENANCE

5.1 Pipettes

5.1.1 Pipetman (200 µl capacity): Maintenance every three months.

1) Cleaning: clean tip with isopropyl alcohol
2) Calibration: pipette 100 µl distilled water into a preweighed beaker 10 times. The permitted weight range is 99.0-101.0 mg.

5.1.2 Pipetman (1 ml capacity): Maintenance every three months.

1) Cleaning: same as for 200 µl capacity Pipetman.
2) Calibration: pipette 500 µl distilled water into a preweighed beaker 10 times. The acceptable weight range is 495-505 mg.

5.1.3 Rainin 250 Electronic Digital Pipette: Maintenance every three months.

1) Cleaning: same as above.
2) Calibration: see Pipetman, 200 µl capacity.

5.1.4 Rainin 1 ml Electronic Digital Pipette: Maintenance every three months.

1) Cleaning: same as above.
2) Calibration: see Pipetman, 1 ml capacity.

5.1.5 Eppendorf Repeater Pipette: Maintenance every three months.

1) Cleaning: same as above.
2) Calibration: using 5 ml combitip and dial set on 1 for 100 µl, pipet distilled water into preweighed beaker 10 times. The permitted weight range is 99.0-101.0 mg.

5.2 MR 600 Microelisa Reader

5.2.1 Every three 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 monthly.

5.3.2 Pump rotor: must be washed monthly.

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 five 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 three months
Follow the instructions provided by the manufacturer.

5.5 Kelvinator Model UC-927CR Upright Ultracold Freezer

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

1) There is a seven-day temperature chart that records the temperature 24 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.

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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Appendix A. Quality Control Data

The quality control data for each of seven assays performed on each participant are shown in the following pages. These figures represent updated quality control data obtained on testing samples. We have selected the work done in the most recent month (March 1987) to represent the period starting December 1986 when ARIC testing began.

The quality control data of six tests to be performed in case-control studies were obtained from our earlier preliminary data. As case-control studies have not begun, new data are not available.

Quality Control Data: aPTT
Control Material: VNC
Dates: March 1987

Intraday Values

	aPTT (seconds)										
Day	3/4	3/5	3/6	3/11	3/12	3/13	3/19	3/23	3/25	3/26	3/30
Sample 1	30.9	32.0	32.1	32.6	30.6	30.3	32.1	32.4	31.6	30.0	32.2
2	32.1	31.3	32.3	32.3	31.5	31.6	32.0	33.3	32.2	31.3	32.2
3	32.0					31.6	32.2	33.0	32.4		32.4
4								33.1			
n	3	2	2	2	2	3	3	4	3	2	3
$\bar{x}$	31.7	31.7	32.2	32.5	31.1	31.2	32.1	33.0	32.1	30.7	32.3
$\bar{s}$	0.7	-	-	-	-	0.8	0.1	0.4	0.4	-	0.1
Max	32.1	32.0	32.3	32.6	31.5	31.6	32.2	33.3	32.4	31.3	32.4
Min	30.9	31.3	32.1	32.3	30.6	3.3	32.0	32.4	31.6	30.0	32.2
R	1.2	0.7	0.2	0.3	0.9	1.3	0.2	0.9	0.8	1.3	0.2
C.V. (%)	2.1	-	-	-	-	2.6	0.3	1.2	1.2	-	0.3

Interday Values (March)

N	=	11
$\bar{\bar{x}}$	=	31.9
$\bar{\bar{s}}$	=	0.7
MAX	=	33.0
MIN	=	30.7
$\bar{R}$	=	2.3
C.V. (%)	=	2.2

Comments: Satisfactory

Quality Control Data: Fibrinogen
Control Material: VNC
Dates: March 1987

Intraday Values

Fibrinogen (mg/dl)

Day	3/4	3/6	3/9	3/10	3/12	3/13	3/17	3/18	3/19	3/25	3/26	3/30
	228	250	249	264	255	232	239	230	232	250	232	229
	230	255	259	255	241	233	231	247	237	238	229	226
	222	240	259	242	247	239		243	237	256	235	201
	228	228	270	242	235	216		236		276	232	229
				245		232						
n	4	4	4	5	4	5	2	4	3	4	4	4
$\bar{x}$	227	243	259	250	245	230	235	239	235	255	232	221
$\bar{s}$	3	9	7.5	9.7	8.5	8.6	-	7.5	2.9	15.9	2.5	13.6
Max	230	255	270	264	255	239	239	247	237	276	235	229
Min	222	228	249	242	235	216	231	230	232	238	229	201
R	18	27	21	22	20	23	8	17	5	38	6	28
C.V. (%)	1.4	4.3	2.9	3.9	3.5	3.7	-	3.2	1.2	6.2	1.1	6.1

Interday Values (March)

N	=	12
$\bar{\bar{x}}$	=	239
$\bar{\bar{s}}$	=	11.5
MAX	=	259
MIN	=	221
$\bar{R}$	=	38
C.V. (%)	=	4.8

Comments: Satisfactory

Quality Control Data: VII
Control Material: VNC
Dates: March 1987

Intraday Values

Factor VII (%)

Day	3/9	3/10	3/12	3/13	3/18	3/19	3/25	3/26	3/30
	77	69	79	81	85	84	91	78	83
	74	57	76	75	90	83	93		69
	68	65	82	77	90		83		82
		58	75	86					65
		64							
n	3	5	4	4	3	2	3	1	4
$\bar{x}$	73	63	78	80	88	84	89	78	75
$\bar{s}$	4.6	5.0	3.2	4.9	2.9	-	5.3	-	9.1
Max	77	69	82	86	90	84		-	83
Min	68	57	75	75	85	83		-	65
R	9	12	7	11	5	1		-	18
C.V. (%)	6.3	8.0	4.1	6.1	3.3	-	6.0	-	12.1

Interday Values

N	=	9
$\bar{\bar{x}}$	=	79
$\bar{\bar{s}}$	=	8.0
MAX	=	88
MIN	=	63
$\bar{R}$	=	25
C.V. (%)	=	10.2

Comments: High interday. C.V. may be due to one aberrant value of March 10, 1987.

Quality Control Data: VIII:C
Control Material: VNC
Dates: March 1987

Intraday Values

	VIII (%)										
Day	3/4	3/5	3/9	3/11	3/12	3/13	3/16	3/19	3/23	3/25	3/30
	104	107	107	104	102	106	96	98	89	103	98
	109	99	114	102	99	104	96	104	92	106	98
			111	102	98			104		107	
n	2	2	3	3	3	2	2	3	2	3	2
$\bar{x}$	107	103	111	103	100	105	96	103	90	106	98
$\bar{s}$			3.3	0.8	2.1			6	2.2	2	
Max	109	107	114	104	102	106	96	104	92	107	98
Min	104	99	107	102	98	104	96	98	89	103	98
R	5	8	7	2	4	2	0	6	3	4	0
C.V. (%)			3.0	0.8	2.1			2.8			

Interday Values

N	= 11
$\bar{\bar{x}}$	= 102
$\bar{\bar{s}}$	= 5.8
MAX	= 111
MIN	= 90
$\bar{R}$	= 21
C.V. (%)	= 5.7

Comments: Satisfactory

Quality Control Data: VWF
Control Material: VNC
Dates: March 1987

Intraday Values

	VWF (%)						
Day	3/5	3/6	3/10	3/11	3/12	3/13	3/17
	122	122	118	124	117	124	121
	124	121	117	125	119	122	122
	130		118	124	113	121	122
	122		113	120			
	120		112	123			
	120		114				
n	6	2	6	5	3	3	3
$\bar{x}$	123	122	115	123	116	122	122
$\bar{s}$	3.7		2.7	1.9	3.1	1.5	0.6
Max	130	122	118	125	119	124	122
Min	120	121	112	120	113	121	121
R	10	1	6	5	6	3	1
C.V. (%)	3.0		2.3	1.6	2.6	1.3	0.5

Interday Values

Control		VNC
N	=	7
$\bar{\bar{x}}$	=	120
$\bar{\bar{s}}$	=	3.4
MAX	=	123
MIN	=	115
$\bar{R}$	=	8
C.V. (%)	=	2.8

Comments: Satisfactory

Quality Control Data: AT-III
Control Material: VNC
Dates: March 1987

Intraday Values

AT-III (%)

Day	3/4	3/5	3/6	3/10	3/11	3/12	3/13	3/16	3/18	3/19	3/20	3/25	3/26	3/30
	117	91	113	117	120	102	118	133	134	119	132	116	103	105
	107	116	132	102	106	111	112	124	135	122	121	116	113	100
			112	120	108	108	111			122	129	115	103	
				106	105	104				110	119			
					125	100				117				
n	2	2	3	4	5	5	3	2	2	5	4	3	3	2
$\bar{x}$	112	104	119	111	113	105	114	129	135	118	125	116	106	103
s	-	-	11	8.6	9.1	4.5	3.8	-	-	4.9	6.2	0.6	5.8	-
Max	117	116	132	120	125	111	118	133	135	122	132	116	113	105
Min	107	91	112	102	105	100	111	124	134	110	119	115	103	100
R	10	25	20	18	20	11	7	9	1	12	13	1	10	5
C.V.(%)	-	-	9.5	7.8	8.1	4.3	3.3	-	-	4.2	5.0	0.5	5.5	-

Interday Values

N	=	14
$\bar{\bar{x}}$	=	115
$\bar{\bar{s}}$	=	9.6
MAX	=	129
MIN	=	103
$\bar{R}$	=	26
C.V. (%)	=	8.4

Comments: Satisfactory

Quality Control Data: Protein C
Control Material: UCRP
Dates: March 1987

Intraday Values

Protein C (µg/ml)

Day	3/5	3/6	3/10	3/11	3/12	3/16	3/18	3/19	3/20	3/24
	3.5	3.4	3.1	3.1	3.1	3.6	2.8	3.0	2.4	3.7
	3.4	3.3	3.2	2.9	2.5	3.1	2.4	2.9	2.0	3.5
		3.3	2.9				2.4	2.7	2.4	3.6
			3.0				2.7	2.5	2.3	3.2
n	2	3	4	2	2	2	4	4	4	4
$\bar{x}$	3.5	3.3	3.1	3.0	2.8	3.4	2.6	2.8	2.3	3.5
$\bar{s}$	-	0.06	0.13	-	-	-	0.21	0.22	0.19	0.22
Max	3.5	3.4	3.2	3.1	3.1	3.6	2.8	3.0	2.4	3.7
Min	3.4	3.3	2.9	2.9	2.5	3.1	2.4	2.5	2.0	3.2
R	0.1	0.1	0.3	0.2	0.6	0.5	0.4	0.5	0.4	0.5
C.V.(%)	-	1.8	4.2	-	-	-	7.9	7.9	8.2	6.2

Interday Values

N	=	10
$\bar{\bar{x}}$	=	3.03
$\bar{\bar{s}}$	=	0.41
MAX	=	3.5
MIN	=	2.3
$\bar{R}$	=	1.2
C.V. (%)	=	13.4

Comments: Needs to be improved. The reason for the high C.V. is due to (1) pipette precision at 5 µl range and (2) technician's variability (3/18-3/20) done by a different technician.

Quality Control Data: Fibrinopeptide A
Control Material: FPA Control A
Dates: 2/16/86 - 6/24/86

Interday Values

Day	2/16	3/15	3/19	3/25	4/18	5/01	6/24
Sample 1	1.0	1.1	1.1	0.9	1.6	0.6	0.9
2	1.2	1.1	1.1	0.9	0.7	0.9	1.0
3	1.1	1.3	1.0	0.9	1.2	1.6	
4			1.0	1.0	1.3	1.1	
n	3	3	4	4	4	4	2
$\bar{x}$	1.1	1.2	1.1	0.9	1.2	1.0	1.0
S.D.	0.1	0.1	0.1	0.1	0.4	0.4	0.8
Max	1.2	1.3	1.1	1.0	1.6	1.6	1.0
Min	1.0	1.1	1.0	0.9	0.7	0.6	0.9
R	0.2	0.2	0.1	0.1	0.9	0.9	0.1
C.V. (%)	9.5	10.0	5.2	5.6	31.2	40.0	8.1

Interday Values

N	=	7
$\bar{\bar{x}}$	=	1.1
S.D.	=	0.1
MAX	=	1.2
MIN	=	0.9
$\bar{R}$	=	0.3
C.V. (%)	=	9.9

Quality Control Data: Fibrinopeptide A
Control Material: FPA Control B
Dates: 2/19/86 - 6/24/86

Interday Values

Day	2/16	3/15	3/19	3/25	4/18	5/01	6/24
Sample 1	7.2	10.6	6.4	6.6	6.6	7.7	8.9
2	8.9	8.4	6.5	6.8	7.6	8.0	7.3
3	8.2	8.2	6.2	8.0	8.1	8.1	8.3
4	8.5	7.5	6.0	8.3	8.9	9.5	
n	4	4	4	4	4	4	3
$\bar{x}$	8.2	8.7	6.3	7.5	7.8	8.3	8.2
S.D.	0.7	1.3	0.2	0.9	1.0	0.8	0.8
Max	8.9	10.6	6.5	8.3	8.9	9.5	8.9
Min	7.2	7.5	6.0	6.6	6.6	7.7	7.3
R	1.7	3.1	0.5	1.7	2.3	1.8	1.6
C.V. (%)	8.9	15.4	3.5	11.3	12.3	9.7	9.9

Interday Values

N	= 7
$\bar{x}$	= 7.8
S.D.	= 0.8
MAX	= 8.7
MIN	= 6.3
$\bar{R}$	= 2.4
C.V. (%)	= 10.1

Quality Control Data: Fibrinopeptide A
Material: Combined Anticoagulant Pool
Date: 6/24/86

Intraday Values

Day	6/24
Sample 1	3.2
2	2.9
3	3.6
4	3.1
5	3.4
6	3.3
7	3.5
8	3.1
9	3.3
10	2.7
11	2.8
12	2.8
n	12
$\bar{x}$	3.1
S.D.	0.3
Max	3.6
Min	2.7
$\bar{R}$	0.9
C.V. (%)	8.7

Quality Control: βThromboglobulin
Control Material: Combined Anticoagulant Pool
Date: 6/30/86

Intraday Values

Day	6/30
Sample 1	23.8
2	27.2
3	26.1
4	24.2
5	23.4
6	23.1
7	22.7
8	26.4
9	24.5
10	24.8
11	26.6
12	24.4
n	12
$\bar{x}$	24.8
S.D.	1.48
Max	27.2
Min	22.7
$\bar{R}$	4.5
C.V. (%)	6.0

Quality Control Data: Platelet Factor 4
Control Material: Combined Anticoagulant Pool
Date: 6/26/86

Intraday Values

Day	6/26
Sample 1	6.9
2	7.9
3	7.9
4	6.1
5	6.3
6	5.7
7	5.9
8	6.3
9	8.6
10	7.3
n	10
$\bar{x}$	6.9
S.D.	1.0
Max	8.6
Min	5.7
$\bar{R}$	2.9
C.V. (%)	14.5

Quality Control Data: Thromboxane B_2
Control Material: Normal Serum Pool
Dates: 3/25/86, 5/01/86

Intraday Values

Day	3/25	5/01
Sample 1	261.0	238.0
2	226.0	
3	237.0	
4	265.0	
5	240.0	
6	240.0	
n	6	1
$\bar{x}$	244.8	238.0
S.D.	15.0	0
Max	265.0	238.0
Min	226.0	238.0
$\bar{R}$	39.0	0
C.V. (%)	6.1	0

Quality Control Data: Tissue Plasminogen Activator
Control Material: Normal Plasma Pool
Dates: 1/18/86 - 8/08/86

Intraday Values

Day	1/18	3/19	8/08
Sample 1	6.4	6.0	5.9
2	4.8	7.0	5.9
3	6.7	5.5	6.6
4		5.4	
n	3	4	3
$\bar{x}$	6.0	6.0	6.1
S.D.	1.0	0.7	0.4
Max	6.7	7.0	5.9
Min	4.8	5.4	6.6
$\bar{R}$	1.9	1.6	0.7
C.V. (%)	17.1	12.3	6.6

Interday Values

N	=	3
$\bar{\bar{x}}$	=	6.0
S.D.	=	0.1
MAX	=	6.1
MIN	=	6.0
$\bar{R}$	=	0.1
C.V.	=	1.0

Appendix B. Pretesting Data

Pretesting data aimed at determining the effects of blood collection and processing and temporary storage on various hemostatic factors. Reference values were obtained from the ARIC CHL.

Because of a high variability of FPBβ(15-42) and tPA, presumably due to exercise effects, we listed the individual values below.

		Pretesting Results		Reference	
Test	No. of Normal Donors	$\bar{x}$	S.D.	$\bar{x}$	S.D.
aPTT (sec)	21	29.8	2.4	28.4	2.2
Fibrinogen (mg%)	"	274	65	237	58
VII:C (%)	"	106	29.2	101	17.5
VIII:C (%)	"	98.2	25.2	102	29.7
VWF (%)	"	113	34.1	106	22.4
AT-III (%)	"	106	12	104	51
Protein C (ug/ml)	"	3.4	0.56	3.5	1.1
βTG (ng/ml)	"	14.4	4.5	16.9	4.5
PF-4 (ng/ml)	"	2.2	0.8	3.8	1.8
FPA (ng/ml)	"	1.2	0.47	0.76	0.38
FPBβ(15-42) (ng/ml)	"	5.3	6.7	3.16	1.52
tPA (ng/ml)	"	13.7	7.3	4.64	1.92
Serum TXB (ng/ml)	"	361	155	448	179

Fibrinopeptide Bβ(15-42) (ng/ml)

Subject		
	1	1.1
	2	1.1
	3	1.2
	4	1.2
	5	2.2
	6	2.5
	7	7.2
	8	13.2*
	9	2.4
	10	1.2
	11	1.9
	12	3.4
	13	3.2
	14	4.8
	15	10.0*
	16	31.0*
	17	3.6
	18	3.5
	19	4.4
	20	4.5
	21	8.6
	n	21
	x	5.3
	S.D.	6.7

Tissue Plasminogen Activator (ng/ml)

Subject		
	1	29
	2	7.1
	3	17.2
	4	21.4
	5	7.6
	6	11.8
	7	16.1
	8	24.2
	9	7.4
	10	9.9
	11	8.9
	12	5.2
	13	7.9
	14	9.1
	15	10.8
	16	14.8
	17	16
	18	28
	19	8.7
	n	19
	x	13.7
	S.D.	7.3

*regular exerciser.

APPENDIX C

SERVICE AND MAINTENANCE (Coag-A-Mate X2)

A. PREVENTIVE MAINTENANCE SCHEDULE

PROCEDURE	FREQUENCY
PUMP TUBING	
Rinse	Daily
Wash	Weekly
Replace	Monthly*
PUMP ROTOR	
Wash	Monthly
PUMP VOLUME	
Tolerance Verification	Weekly
Calibration	Weekly
TEMPERATURE	
Verification	Semi-Annually

*Or as necessary

B. REAGENT TUBING ASSEMBLY, Installation and/or Replacement (See Figure 11)

INSTALLATION

1. Refer to test procedure for selection and location of tubing. (See example of PT Figure 6.)
2. For each tube, insert delivery tip into appropriate hole on REAGENT INCUBATION ARM and push down until delivery tip extends below arm 1/16 inch. Gently bend tubing in direction of groove and press to seat tubing in groove. Work toward the base of the arm, taking care not to stretch the tubing.
3. Lubricate the pumping zone of new tubing as described in Section B. To install tubing in PUMP 1, grasp both collars and stretch tubing around front of pump rotor. Slide tubing into slot on either side of stator. Ensure that each tube is properly positioned in appropriate notch. Pull left collar against stator by pulling from right of pump. Use PRIME 1 to draw right collar snugly against stator. Check tubing position by pressing PRIME 1. TUBING MUST NOT BE TWISTED IN PUMP. Repeat procedure for PUMP 2.

REMOVAL

1. Remove pick up tips from reagent vials; grasp uppermost tubing in pump on either side of pump stator and gently lift to remove. Remove remaining tubes from pump in similar manner.
2. Remove tubing from groove and gently lift delivery tip from hole in REAGENT INCUBATION ARM. (Tubing need not be removed from arm between runs except for cleaning, calibration, lubrication or replacement.)

C. REAGENT TUBING ASSEMBLY, LUBRICATION AND CLEANING.

1. LUBRICATION OF TUBING

   Lightly lubricate the pumping zone section of the tubing on a daily basis. CAUTION: Do not lubricate excessively. (Use only Tubing Lubricant, Product No. 35350, supplied with COAG-A-MATE® •X2.)

   a) Remove tubing from pump. Wipe tubing and pump rollers clean with tissue moistened with isopropanol.

   b) Apply one drop of lubricant to forefinger and apply to tubing between the two "collars". DO NOT over-lubricate.

   c) Replace tubing in pump.

2. CLEANING OF TUBING

   a) Rinse Tubing After Completion of Each Test Run.

      1. Use alternate air and purified water rinses for approximately 15 seconds or for the duration of two "cancel/prime" cycles (CAM-X2).
      2. Remove all water from the tubing.

   b) Wash Tubing Weekly*

      1. Use alternate air and purified water rinses for approximately 20 seconds or for the duration of three "cancel/prime" cycles (CAM-X2).
      2. Fill tubing with purified water.
      3. Remove tubing from the instrument noting position of tubing in arm and rotor/stator.
      4. Roll tubing in the palm of the hands to loosen particles or debris which may have adhered to the inner wall.
      5. Reinstall tubing in same arm and rotor/stator position from which removed and repeat rinse procedure.
      6. Recalibrate tubing.

D. PUMP, CLEANING

1. Loosen set screw with a 0.050 inch Allen wrench and remove ROTOR.
2. Immerse ROTOR for several minutes in isopropanol. Rollers should rotate freely when dust and old lubricant are removed.
3. With an absorbent tissue, moistened with isopropanol, wipe dust and excess lubricant from PUMP stator.
4. Replace ROTOR and tighten set screw. ROTOR should clear instrument top panel by about .015 inch.
5. Check REAGENT DELIVERY VOLUME (SECTION D).

E. PUMP CALIBRATION, (VERIFICATION OF VOLUME TOLERANCE)

1. Turn power ON.
2. Position REAGENT TUBING ASSEMBLIES in PUMPS.
3. Remove REAGENT TUBING ASSEMBLIES from REAGENT INCUBATION ARM.

*Perform every other day if instrument is used for all shifts.

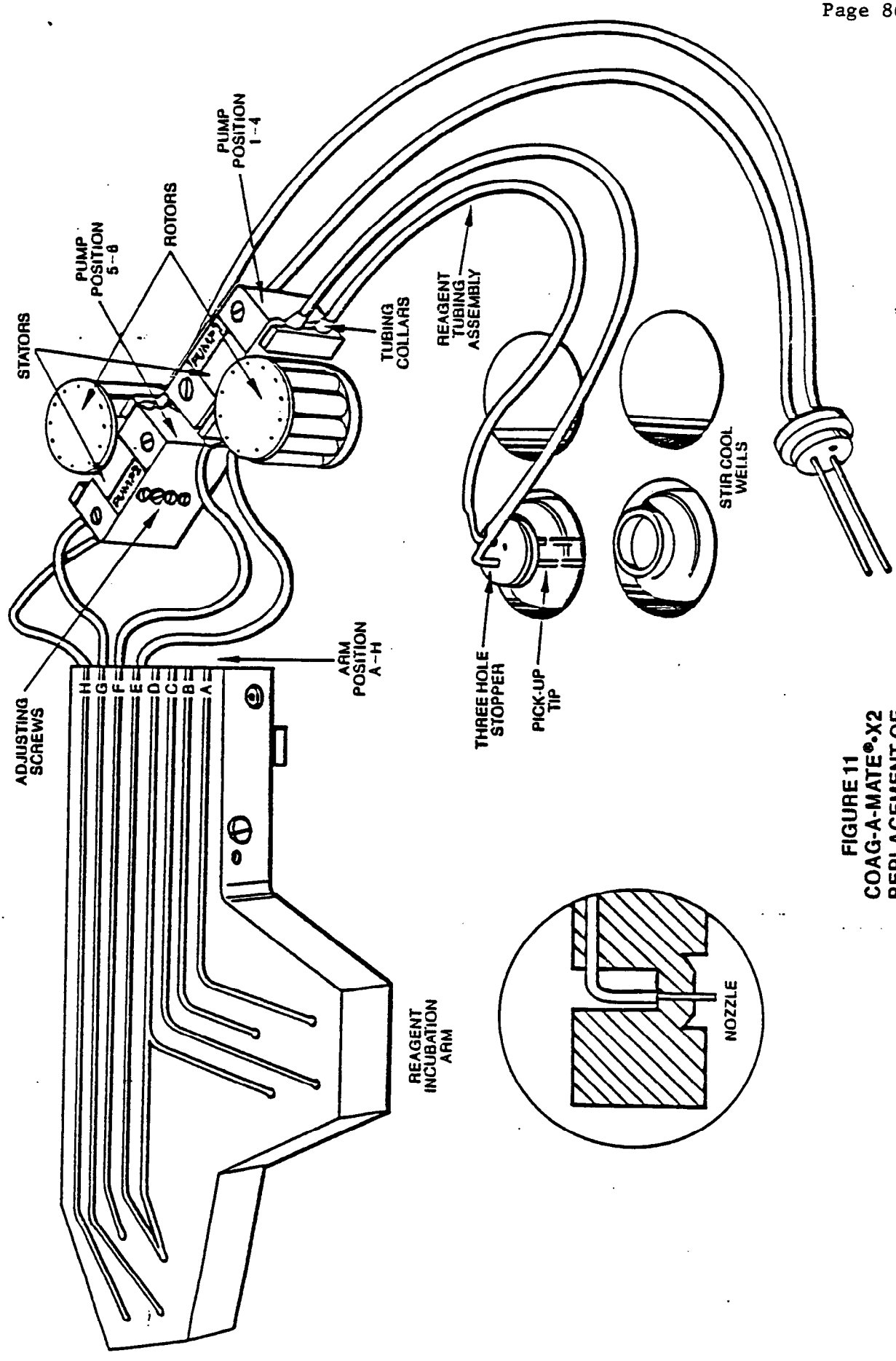

FIGURE 11
COAG-A-MATE®X2
REPLACEMENT OF
REAGENT TUBING ASSEMBLY

4. Connect dispensing tip of the tubing to a 1.0 ml serological pipette using a section of tubing for the connection.
5. Pump purified water through the tubing to 0.5 ml marking on the pipette (vertical position).
6. Turn the volume adjustment screw on the stator clockwise until the water level begins to descend.
7. Reverse the adjustment (counterclockwise) until the water level stops descending.
8. Check the volume for pump delivery at 0.1 ml setting.
9. If the volume is greater than 0.1 ml continue with pump calibration. If the volume is 0.1 ml or less, discard tubing.

Calibrate Pump Delivery Volume at 0.1 ml Setting.

10. Decrease volume delivery by turning the volume adjustment screw counterclockwise.
11. Increase volume delivery by turning the volume adjustment screw clockwise.

FIGURE 12
COAG-A-MATE®•X2
REAGENT DELIVERY
VOLUME ADJUSTMENT