PEDIATRIC PULMONARY AND CARDIOVASCULAR COMPLICATIONS OF VERTICALLY TRANSMITTED HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

P^2C^2 HIV

MANUAL OF OPERATIONS

July 6, 1990

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ABBREVIATIONS

$A\text{-}aDO_2$	Alveolar arteriole oxygen difference
ABP	American Board of Pathology
ACTG	AIDS Clinical Trials Group
AIDS	Acquired immunodeficiency syndrome
BAL	Bronchoalveolar lavage
BX	Biopsy
CAP	College of American Pathologists
CBC	Complete blood count
cc	Cubic Centimeter
CCC	Clinical Coordinating Center
CD	Clusters of designation
Cm	Centimeter
CMV	Cytomegalovirus
CO_2	Carbon dioxide
COHb	Carboxyhemoglobin
CPE	Cytopathic effect
CSF	Cerebral spinal fluid
DDE	Distributed data entry
dl	Deciliter
DTPA	Diethylenetriaminepentacetate
EBV	Epstein-Barr virus
ECG	Electrocardiogram
EDD	End diastolic dimension
ELISA	Enzyme linked immunosorbent assay
EM	Electronic mail
ESD	End systolic dimension
ESR	Erythrocyte sedimentation rate
FCS	Fetal calf serum
FDA	Food and Drug Administration
FRC	Functional residual capacity
FS	Fractional shortening
Hb	Hemoglobin
HBSS	Hank's bolowed salt solution
HbO_2	Oxyhemoglobin
H&E	Hematoxylin and eosin
HI	Hydriodic acid
H_2O	Water
HR	Heart rate
Ig	Immunoglobulin
IRB	Investigational Review Board
IU	International units
IVIG	Intravenous Immunoglobulin
Kg	Kilogram
KOH	Potassium hydroxide
LDH	Lactic dehydrogenase
LED	Light emitting diode
LV	Left ventricular
MEFV	Maximum expiratory flow volume
metHb	Methemoglobin
Mg	Milligram

MHz	Megahertz
Ml	Milliliter
mm/h	Millimeter per hour
N_2	Nitrogen
$NaNCO_3$	Sodium bicarbonate
NHLBI	National Heart, Lung and Blood Institute
NIAID	National Institute of Allergy and Infectious Diseases
NICHD	National Institute of Child Health and Human Development
NIH	National Institutes of Health
O_2	Oxygen
$PaCO_2$	Partial pressure - arterial carbon dioxide
p24Ag	Protein 24 Antigen
PaO_2	Partial pressure - arterial oxygen
P^2C^2 HIV	Pediatric Pulmonary and Cardiac Complications of Vertically Transmitted Human Immunodeficiency Virus
PEFV	Partial expiratory flow volume
PI	Principal Investigator
PPD	Purified protein derivative
q3	Every 3 days
QC	Quality Control
RBC	Red blood cells
RPMI	Roswell Park Medical Institute
ROI	Regions of interest
SD	Standard deviation
SpO_2	Oxygen saturation from pulse oximetry
Tc	Technetium
VHS	Video home systems
VTI	Velocity time integral
μg	microgram
μm	micrometer

1. Manual of Operations

1.1 Introduction

The P^2C^2 HIV study Manual of Operations was developed by the Clinical Coordinating Center (CCC) with the cooperation of the National Heart, Lung and Blood Institute (NHLBI), Program Office, Steering Committee, and staff of the collaborating clinical centers.

This manual was developed to aid the clinical centers in obtaining conformity to the overall study design. In addition to listing eligibility criteria and measurement definitions, it indicates how the criteria and variables are to be determined. The manual describes in detail, subject visits, their scheduling and content. Recruitment techniques, informed consent and other issues also are addressed.

Updates and clarifications, which usually occur during the course of the study, will be made available to all participating clinical center personnel.

The Manual of Operations is supported by two other important documents in the P^2C^2 HIV study:

A. Protocol

The Protocol contains information about the study background, rational and design.

B. Forms Manual

The Forms Manual contains the study forms as well as the detailed instructions on filling out the forms and the schedule for completion of each form.

2. Clinical Coordinating Center

2.1 Role of the Clinical Coordinating Center

The Clinical Coordinating Center plays a major role in the design, implementation, and execution of the study. The Clinical Coordinating Center coordinates the writing of the Protocol, development of the Manual of Operations and database definitions for the Protocol, development of the distributed data entry system, collection of subject data from the clinical centers, storage of data in a computerized format suitable for statistical analysis, and provides periodic status reports. The CCC also is responsible for quality control and statistical analyses of the P^2C^2 HIV study data.

The CCC is represented on, and works under the direction of the Steering Committee. The CCC has the additional responsibility of organizing meetings of the Steering Committee and documenting its proceedings.

2.2 Overview of Clinical Coordinating Center Staff

The Clinical Coordinating Center staff is made up of a multidisciplinary team. The professional and clerical organization of the CCC includes: a principal investigator (director), deputy director, statistical co-investigators, medical co-investigators, consultants, cardiac technologist, pulmonary technologist, biostatistician, systems analyst, programmer, data coordinator, data manager, and secretaries (see Appendix 1).

The CCC staff meets on a monthly basis to review study progress, report on assigned work, receive work assignments, and resolve any issues related to quality control and other areas. Depending upon the need, more frequent meetings will occur between some, or all, of the members.

2.3 Clinical Coordinating Center Data Management Staff

The CCC Data Management staff consists of a study coordinator, data manager, and a secretary. They work under the supervision of the principal investigator. The Data Management section's primary responsibilities includes data review and editing, reduction, display and analysis, and generation of reports and publications.

The data management staff meet on a weekly basis with the principal investigator to discuss the daily operations of the study.

2.3.1 Study Coordinator

2.3.1.1 Job Description

The study coordinator must have prior supervisory experience; experience working with national studies, forms development, data collection and entry. He or she must also have knowledge of anatomy and physiology and medical terminology. The study coordinator must be able to interact with medical and statistical staff.

2.3.1.2 Responsibilities

The study coordinator supervises the CCC data manager and CCC secretary and oversees the daily operations of the data management section at the CCC.

During Phase I, the study coordinator is responsible for coordinating the development of the Protocol; taking minutes at the Steering Committee meetings; coordinating the writing and development of the Manual of Operations and its updates; assisting with the installation of computer systems at the clinical centers; and coordinating and participating in the training of the clinical

center Data Management personnel.

During Phase II the study coordinator will be responsible for the day-to-day activities of the office, for participating in site visits and for maintaining close liaison with participating clinical center staff by means of telephone and mail. The study coordinator also will be responsible for editing and ensuring the distribution of the monthly study reports.

2.3.2 Data Manager

2.3.2.1 Job Description

The data manager at the CCC should have a working knowledge of computers, anatomy and physiology, and medical terminology. He or she must also have experience in dealing with protocols, data collection, data entry, and forms development. The data manager must also demonstrate the ability to interact with medical and statistical staff in the CCC as well as at the clinical centers.

2.3.2.2 Responsibilities

During Phase I, the data manager's primary responsibility is coordinating the sections of the protocol and developing the study forms. The CCC data manager will actively participate in the training of clinical center data entry personnel and assist in the initial installation of the computer systems at the clinical centers.

During Phase II of the study, the data manager's primary responsibility will be to monitor the flow of data to the CCC from the clinical centers and any central facilities (e.g., Boston), to coordinate the data processing at the CCC itself and to generate regular reports reflecting the progress. Responsibilities will also include managing the query system and the error correction system. The

data manager will work closely with the biostatisticians to ensure that appropriate data are available for timely analysis. The data manager will also participate in site visits.

2.3.3 Secretary

2.3.3.1 Job Description

The CCC secretary should have a working knowledge of word processing and medical terminology. He or she should have experience in typing medical documents and interacting with the medical staff.

2.3.3.2 Responsibilities

During Phase I of the study, the secretary is responsible for typing the Protocol, the Study Directory, and the Manual of Operations. Responsibilities also include mailing these documents, seeing that all revisions in these documents are made, and maintaining a library of reference articles.

During Phase II of the study, the CCC secretary will be responsible for handling all correspondence including the date "stamping" of all materials received. He or she will also assist the CCC data manager in maintaining all study files. The secretary will also prepare the mailout of forms and other materials to the clinical centers.

2.4 Clinical Coordinating Center Statistical Support

One of the CCC's primary responsibilities includes the development of a database system and the analyses of the data. The CCC through its statistical principal investigator and co-investigators, biostatistician, systems analyst, and programmer will provide the P^2C^2 HIV study with its expertise in

biostatistics, epidemiology and computing.

The statistical principal investigator and co-investigators have the responsibility for the design of the study, analysis and reporting of the study data.

The biostatistician is responsible for the conduct of specific analyses developed jointly by the CCC investigators. He or she will also assist in drafting the study forms and reports.

The systems analyst provides systems design support to establish the distributed data entry (DDE) system and other systems such as the electronic mail system.

The programmer is responsible for implementing and maintaining the database management system for the CCC. The programmer will also maintain all associated software for the project.

2.5 Training and Certification of Data Entry Personnel

All clinical center personnel responsible for data collection and entry, will be certified at the Clinical Coordinating Center to guarantee uniformity of procedures. These include clinical center nurse coordinators and data managers. Having more than one individual certified at each clinical center is crucial for the continuity of data submission. Only certified personnel may complete study forms, enter or transmit data, and respond to queries.

2.5.1 Training Sessions

2.5.1.1 Initial Training Session

The initial training session will be held at the Cleveland Clinic Foundation which serves as the CCC. The initial training session will provide

the opportunity for establishing good liaisons with personnel responsible for day-to-day activities of each clinical center. The CCC Data Management staff will have the opportunity to interact with nurse coordinators and data managers from the clinical centers and form a personal basis for communication.

Attendance at the three day training session is required. Attendees will be certified in four aspects of their positions: forms completion, data entry, data transmission, and query data reports. Training will also include a detailed review of the Protocol and the Manual of Operations.

2.5.1.2 Retraining Sessions

Data Management personnel from each clinical center who demonstrates, during the course of the study, expertise in the use of the data management systems will be used as a resource for assistance in training personnel at their clinical center. They will train persons not able to attend the initial training session; assist in training those personnel who are less than proficient; and assist in training new personnel.

2.5.1.3 Continuing Education

Throughout the data collection period, the CCC staff will be available on a daily basis, by phone, to respond to ad hoc questions from the clinical center personnel. Conference calls can also be arranged by the National Institutes of Health (NIH) between the clinical centers and the CCC when needed.

A monthly report will also be prepared by the CCC to keep all clinical center data management personnel informed of various aspects of the study (see Section 2.9.2).

2.5.2 Data Management Certification

At the completion of the training sessions, the CCC will certify personnel who are able to complete the data collection forms and enter the information in the distributed data entry system in the manner prescribed. In order to meet certification, clinical center personnel must demonstrate proficiency in forms completion, data entry, setting the microcomputer for data transmission, and responding to query data reports. Records documenting the data management staff who were trained and the results of their training will be maintained at the CCC.

2.5.2.1 Forms Completion Certification

In order to meet the "forms completion" certification criterion, individuals must successfully complete a forms completion quiz developed by the CCC.

2.5.2.2 Data Entry Certification

In order to meet the "data entry" certification criterion, individuals must successfully enter a form which was generated by the CCC staff. They may use only the instructions in the Manual of Operations to complete the data entry. The data file created by this entry must match a file created by the CCC staff for at least 90% of the data fields.

2.5.2.3 Data Transmission Certification

In order to meet the "data transmission" certification criterion, individuals must successfully complete the procedures necessary to initialize the transmission files and leave the microcomputer in a configuration so that

automatic transfer of data can be completed when initiated by the CCC. They must also demonstrate proficiency in executing the software which will print the "electronic mail" messages for distribution to the clinical center staff.

2.5.2.4 Query Data Report Certification

In order to meet the "query data report" certification (error correction) criterion, individuals must successfully respond to a series of queries created by the CCC staff. The responses are to be entered into the microcomputer and placed in a file which has been initiated for transmission to the CCC.

2.6 Installation of Computer Systems

Personnel from the CCC will visit each of the clinical centers prior to the recruitment of patients to install each microcomputer, modem and printer. Prior to the visit, each clinical center must have a dedicated phone line installed at the site where the microcomputer is to be located. After the installation is complete, the CCC personnel will test all systems. Trial runs will be made to ensure that data can be entered and transmitted to and from the CCC.

2.7 Confidentiality and Security of Patient Information

Confidentiality of data collected at the CCC, from the clinical centers, is a major priority of the CCC. Confidentiality statements will be signed by all employees handling the data (see Appendix 2). This statement outlines the employee's responsibility to protect patient data and the penalty for violation of this trust.

Data collected by the CCC will be housed in a secure area within the

Cleveland Clinic Foundation which will be kept locked during off duty hours. All patient information will be kept in locked files and will be accessible to authorized study personnel only. All computerized files will be protected by means of a password system.

All disk files, backed up weekly at the CCC, will be stored in a locked cabinet. Disk files backed up monthly, will be stored in a vault in a remote location. This vault is especially secure and climate controlled to protect and preserve magnetically stored information.

All correspondence (e.g., letters, forms, tapes, reports, etc.) containing patient information, mailed from/to the clinical centers and CCC, will be marked "CONFIDENTIAL". Only study personnel will be allowed to handle such correspondence. All individuals requesting information via telephone will be required to provide proper identification to verify entitlement to receive such information.

Any destruction of correspondence containing patient data will be accomplished by incineration or shredding. Data stored on electronic media will be bulk erased.

2.8 Quality Control of Patient Data

A major activity of the Clinical Coordinating Center will be quality control of data received by the clinical centers. Several methods have been developed to ensure that the highest quality of data possible is being collected and provided to the CCC (see Chapter 4).

Initially, all data will be screened on the microcomputers at the clinical centers for range checks and valid codes. The clinical center personnel will

enter all data twice (verification) to provide their own quality control checks.

Once data have been uploaded to the CCC, extensive computer edits and verification of data will be performed. Five percent of each clinical center's forms will also be randomly selected, key entered in the CCC and used to verify the original transmitted data. This step will identify systematic discrepancies.

The clinical centers will be informed of data inconsistencies via "queries" by electronic mail. Follow-up procedures will be performed to assure that all inconsistencies are resolved. All corrections will be made on the appropriate form and corrected on the paper form at the CCC and the clinical center. No corrections will be allowed over the phone so that all changes are properly documented.

When necessary, an audit trail will also be performed to identify changes that have been made in the data file and when these changes occurred.

2.9 Reports

Monthly reports will be provided by the Clinical Coordinating Center to the clinical centers and the National Institutes of Health during the course of the study. Other reports will be generated as necessary. Any publication of any study information must have the approval of the Publications Committee as outlined in Chapter 13 of the Protocol.

2.9.1 Follow-up Schedule

Follow-up appointment schedules for the routine screening tests for each subject will be computer generated at the CCC at study entry and annually thereafter. The appointment schedule will give the estimated date for each visit

and procedures to be completed as well as a time interval during which the visit must occur. This schedule will also identify which forms are to be completed at that visit and the date by which they should be entered into the (DDE) system and the paper forms mailed to the CCC. The CCC will keep logs of the appointment schedules for each clinical center as the schedules are sent out.

2.9.2 Monthly Report of Clinical Center Activities

Investigators and study personnel at the clinical centers and the NIH will be provided a brief monthly summary of the progress of the study. This monthly report will be prepared by the CCC. The report will include announcements of meetings, number of subjects entered and follow-up status by each clinical center, and announcements of additions and clarifications regarding study forms, procedures and other items.

2.9.3 Study Summary Report

A final report will be prepared by the CCC for the NHLBI at the conclusion of the study. This report will include a complete description of all study activities and an in-depth analyses of all data in accordance with the statistical design and objectives of the study.

2.10 Site Visits

Quality control (QC) will be monitored by a Quality Control subcommittee of the Steering Committee comprised of one representative from each clinical center and one representative from the CCC and the National Heart, Lung and Blood Institute. Members of this committee will have diverse backgrounds (e.g.,

pulmonary, cardiology, infectious diseases, immunology, data management, biostatistics, etc). This committee will establish QC criteria and direct the clinical center audits. They will review site visit reports and make recommendations to the executive committee.

The first site visit will occur six months after the initiation of the study. Thereafter, site visits will occur once a year.

The site visits will be coordinated with the clinical centers by a CCC site visit team member. The site visit team will consist of two members of the CCC staff and two physicians. The physicians will include representatives from cardiology, pulmonology and may be investigators from clinical centers. After the first site visit, the site visit team may vary depending upon need. The visit will be scheduled with the principal investigator (PI) at least one month in advance with a final agenda at least two weeks prior to the visit. Representatives of the NHLBI will be invited to observe site visits. The nurse coordinator and data manager will receive copies of all correspondence associated with the site visit. Such site visits can enhance the liaison between clinical center personnel, CCC personnel and the National Institutes of Health.

The CCC will give advanced notice to the clinical center of any outstanding issues which need to be resolved prior to the site visit (e.g., missing forms, missing data, missing responses to queries).

Quality control checks will be made on all facets of each clinical center's program, such as recruitment techniques, proper informed consent, confidentiality standards, chart content, data entry, and schedule of required tests/procedures.

The site visit team will review the clinical center's recruitment techniques, which may be individualized at each center, to ensure that all means

are being explored to recruit the highest number of patients possible.

Each center will be reviewed to determine whether or not they enforce confidentiality standards. Confidentiality statements will be reviewed to confirm that all study personnel are informed of the importance of confidentiality. Security of files will be checked as well as logs recording the dispensing of patient information to authorized personnel. Proper destruction of patient documents will also be reviewed.

Patient's charts, to be reviewed, will be randomly selected by the CCC and examined for required study forms, flow sheets, lab reports, informed consents, and when applicable, death certificates/autopsy reports. An audit will be done to make sure that all paper forms in the clinical center files are also in the CCC files.

The site visit team will review data from the CCC database and compare it to data from the medical record (e.g., lab reports, pulmonary procedures, cardiology procedures, etc.) to check for consistency. Timeliness of data entry will also be monitored. The query log will also be reviewed for resolution of queries.

The patients whose charts were also selected for review will also be audited for adherence to the tests and procedures that were to be performed. They will be evaluated for completion of required tests/procedures at the required intervals. During this process the clinical centers will be checked to see if the tests/procedures are standardized as outlined in the Manual of Operations and if quality control measures are in place.

The site visit provides the opportunity to review any outstanding questions that might exist regarding individual patients or study procedures in general.

Any discrepancies found during the site visit will be resolved during the visit.

At the end of the site visit there will be an executive session of the site visit team followed by feedback to the PI. A written report, coordinated by the CCC, will be sent within one month of the visit to the Quality Control Committee. The QC committee will generate the final report to be provided to the clinical center and the NHLBI.

Indicators of inadequate performance by a clinical center, such as a high frequency of missed visits, large amounts of missing data, and a high rate of invalid data submitted might result in repeat visits.

3. Clinical Centers

3.1 Role of the Clinical Centers

The primary responsibility of each clinical center is to provide patient data in accordance with the study protocol by recruiting mothers, infants and children for the study who meet the eligibility requirements. Laboratory, pulmonary and cardiac tests must also be performed as scheduled in accordance with the protocol. The clinical center staff is responsible for following the patients enrolled in the study. Each clinical center is responsible for transmitting the specified data to the Clinical Coordinating Center in the time frame specified by the protocol.

3.2 Overview of the Clinical Center Staff

The professional and clerical organization of each clinical center will include the following: a senior clinical investigator in pediatric cardiology and pediatric pulmonology, a senior investigator or consultant in immunology/infectious diseases; associate investigators and consultants (M.D.'s and Ph.D's) in a variety of other specialties such as obstetrics, pediatrics, pathology, nuclear medicine, and radiology. The following positions may vary from center to center depending upon the skills of the staff recruited. The following positions are suggested to support the P^2C^2 HIV study: a nurse coordinator, clinical research nurse, a patient interviewer/medical record abstractor, a social worker, data manager, data entry technician, laboratory and clerical support staff (see Appendix 1; Figure 1).

The clinical center staff will meet regularly to review local study progress, report on work completed, review the P^2C^2 HIV study monthly report

generated by the CCC, and convey operational problems. Patient adherence data will be reviewed on a quarterly basis for identification of problems and to ensure that problems are solved. The record of scheduled and missed appointments, generated on a weekly basis, will also be reviewed at the staff meeting.

3.2.1 Clinical Center Principal Investigator

The principal investigator of each clinical center will assume those administrative responsibilities necessary for compliance with and completion of the contract protocol.

The responsibilities of the principal investigator are subject to intramural policies of each clinical center; however, it is the ultimate responsibility of the principal investigator that each clinical center meet its primary responsibility to the contract that include:

1. provide patient data in accordance with the study protocol;
2. laboratory tests are recorded in all subsections of the contract according to the schedule of the protocol;
3. longitudinal follow-up of the study patients is conducted in an expeditious manner to optimize compliance; and,
4. data transmission is accomplished in a timely manner conforming to the schedule designated by the Clinical Coordinating Center.

In order to successfully meet these responsibilities, it is assumed that the principal investigator will chair at his/her center, on a regular basis, joint conferences that include all co-investigators, the nurse coordinator, the clinical research nurse, the data manager, and social service representative.

Although quality control and assurance will be monitored by each section/scope of a clinical center, the principal investigator will regularly audit these issues with individual co-investigators and supporting staff members. It is the responsibility of the principal investigator to communicate to the Clinical Coordinating Center, issues related to quality control and assurance. The latter will include analysis of subject enrollment rate and compliance with follow-up.

The principal investigator will be responsible for monitoring the cost center of each clinical center in order to comply with the negotiated budget of the contract. The principal investigator will be vested with the authority to restructure the center's budget in that manner which will assure compliance with the contract protocol. It is assumed that the latter will be conducted following consultation with the co-investigators of the individual clinical center.

3.3 Clinical Center Data Management Staff

The Clinical Center Data Management staff consists of a nurse coordinator, clinical research nurse, social worker, data manager, data entry technician, and a patient interviewer/medical record abstractor. This staff works closely to coordinate patient visits, maintain patient compliance, compile accurate data, and transmit these data in a timely fashion.

3.3.1 Nurse Coordinator

3.3.1.1 Job Description

The nurse coordinator is a member of the multidisciplinary team of the P^2C^2 HIV study. Each clinical center is encouraged to hire a full-time nurse coordinator preferably with a Masters degree in Nursing and training in research

methodology. At least two years experience in the care of HIV patients and experience with the use of microcomputers is recommended.

3.3.1.2 Responsibilities

Working with the principal investigator, the nurse coordinator guarantees that the protocol is followed at the clinical center. The nurse coordinator is under the supervision of, and works closely with, the principal investigator and other members of the team to achieve the goals of the study.

The major responsibilities of the nurse coordinator are:

1. Administration - this includes setting up and managing the clinic, coordinating patient visits and serving as a liaison between the study team and other clinical personnel involved in the study;
2. Quality Control and Assurance - this includes reviewing the patients case report forms for completeness and accuracy and monitoring the adherence of the patients to the protocol;
3. Patient Care - this includes monitoring the patient's health and progress throughout the study, assisting in managing patient problems and assisting in maintaining compliance; and,
4. Research - this includes attending appropriate meetings to address questions and concerns regarding the study and developing abstracts for presentation at scientific meetings.

3.3.2 Clinical Research Nurse

3.3.2.1 Job Description

The clinical research nurse has a vital role in the clinical follow-up of

the study patients. Each clinical center is encouraged to hire a full-time clinical research nurse with a BSN and preferably work experience on research projects. The clinical research nurse should also have at least 1 year experience in the care of HIV patients.

3.3.2.2 Responsibilities

The clinical research nurse works under the supervision of the nurse coordinator. The major responsibilities of the clinical research nurse are:

1. To monitor and document study participant's health during the study including results of laboratory and diagnostic tests and changes in health status;
2. To participate in data collection by completing the data collection forms needed at each study visit; and,
3. To perform direct patient care as required by the study protocol.

3.3.3 Data Manager

3.3.3.1 Job Description

The data manager is a vital position within the P^2C^2 HIV study team and works under the supervision of the principal investigator. Each clinical center is encouraged to hire a full-time data manager. The individual should be proficient in the use of microcomputers and their peripherals, and have a very good working knowledge of database management, and word processing software packages. At least two years of work experience as a data manager is recommended. Experience with AIDS studies and a background in anatomy and physiology and medical terminology is helpful.

3.3.3.2 Responsibilities

The data manager will work closely with the nurse coordinator to guarantee that quality data are transmitted to the CCC.

The major responsibilities of the data manager are:

1. Communications - this involves maintaining contact with, responding to requests and understanding the procedures followed at the CCC;
2. Data Management - this involves maintaining study data files and complying with the CCC established procedures.
3. Quality Control - this involves setting up procedures to monitor the quality of the completion of data forms and of data entry, and maintaining study specific logs to track data management activities; and,
4. Supervision of data entry and storage - this involves making sure the data are entered in a timely fashion according to procedures, and maintaining an adequate backup system for study data files.

3.3.4 Data Entry Technician

3.3.4.1 Job Description

The data entry technician is under the supervision of the data manager and performs extremely confidential entry of research study information. This part-time position requires an individual with at least a high school diploma or equivalent and previous data entry experience. This position is optional as the data entry duties can be managed by the data manager and/or the nurse coordinator.

3.3.4.2 Responsibilities

The major responsibilities of the data entry technician are:

1. To become proficient with the data entry software and procedures;
2. To enter data from the study's data collection forms;
3. File data collection forms and records appropriately; and,
4. Maintain logs of data entry activities.

3.3.5 Patient Interviewer/Medical Record Abstractor

3.3.5.1 Job Description

The patient interviewer/abstractor is a member of the P^2C^2 HIV study team. Each center is encouraged to hire a full-time patient interviewer/abstractor. The individual should have a BSN with experience working on clinical research studies.

3.3.5.2 Responsibilities

Working closely with the nurse coordinator, the patient interviewer/abstractor completes all data collection forms which require an interview or review of past medical records.

The major responsibilities of the patient interviewer/abstractor are:

1. To conduct quality interviews as needed; and,
2. To abstract medical records for patient histories.

3.3.6 Social Worker

3.3.6.1 Job Description

The social worker plays an integral part in a hospital's support program.

The P^2C^2 HIV study will also require this support in dealing with the AIDS patient's family (parent[s], guardian[s]). Each clinical center, depending upon the existing experience and pool of social workers, will utilize a social worker with a Masters degree in Social Work who is already established within the institution. The social worker position will need to be a full-time position but may also participate in projects outside the P^2C^2 HIV study.

3.3.6.2 Responsibilities

The social worker's primary responsibility will be to assist in maintaining compliance. The social worker will attempt to maintain close contact with the patients' families. He or she will also assist in making reminder calls to families about the patient's scheduled appointments. When necessary, the social worker will also assist the family in complying with return appointments by making transportation arrangements. An additional responsibility of the social worker will be to aid the family in obtaining welfare benefits.

3.4 Training and Certification of Data Management Personnel

Data Management personnel (e.g., data manager, nurse coordinator) will attend a three day training session at the CCC for certification to guarantee uniformity of procedures. Only certified personnel will be allowed by the CCC to complete study forms, enter or transmit data and respond to queries.

For complete information regarding training and certification refer to Section 2.5.

3.5 Setting-Up the Clinical Center

It is recognized that each clinical center's set-up will vary, as will the amount of space available. The more organized and efficient the use of space, the more smoothly this study will operate. One of the nurse coordinator's initial tasks is to work with the principal investigator to survey the available space and assess how it can be used to meet the study's needs.

3.5.1 Patient Care

There must be adequate space to see the patient (i.e., child/mother.) At regular visits there should be space for the clinician, the patient and at least one other person at any given time. Many clinical centers may have use of an outpatient area, clinic room, hospital room, or clinical research center space. The nurse coordinator should make sure that the space the patient is being seen in is consistent with that clinical center's policies for patient care areas.

3.5.2 Office Space

There should be adequate office space for all members of the study team. The team may share space or have individual office areas. It is the responsibility of the principal investigator and the nurse coordinator to discuss this with the other study team members. The layout of office space will differ among clinical centers. There should be enough total room available to house one computer, as well as desks and supplies for the data entry technician, the data manager, patient interviewer/medical record abstractor, clinical research nurse, and nurse coordinator. In addition, room must be available for records for patient and mother data.

3.5.3 Supplies and Equipment

The nurse coordinator is responsible for making sure that all necessary supplies are available prior to entering patients on the protocol. Requirements and availability will vary at each center. Below is a suggested list of necessary supplies.

a. Desks:

All members of the study team will need the use of desk space. The study coordinator should meet with all team members including the principal investigator to discuss the available number of desks. A desk should be large enough to support a microcomputer, modem and printer.

b. Computer:

The study team will need one microcomputer, modem and printer which will be used for key entry and responses to data queries. This will be provided, installed and set up by the CCC prior to initiation of recruitment.

c. Data Jack:

A dedicated phone line is required to connect the modem and initiate the distributed data entry system and electronic mail.

d. File Cabinets:

The clinical center data management staff will need file cabinets which can be locked for security. There will need to be enough cabinet space available to store the following:

e. Forms:

1. **Blank Forms**

Originals of all forms will be provided by the CCC to the clinical centers so that the forms can be copied as needed. Forms should be filed in a system that the nurse coordinator has organized, and should be easily available to the other team members. The system should be such that it is readily apparent when a center is running low on a given form. File space must be available to hold the supply of blank forms. Other items to be filed here will include informed consents, patient handbooks and other educational items.

2. **Completed Forms**

While copies of completed forms from each patient visit may be kept in the patient's chart, the nurse coordinator will set up a file system to keep copies of all completed P^2C^2 HIV study forms. Due to the large number of completed forms, it is advisable to have one four drawer file cabinet available to hold completed copies of forms. In addition, the nurse coordinator may

want to use other files for record keeping and informational purposes.

f. Miscellaneous Office Supplies:

The nurse coordinator and other team members may choose to keep other supplies to help the study run smoothly. These would be items such as locally ordered appointment cards, business cards, stationery, and other usual office supplies. The nurse coordinator is not solely responsible for other team member's supplies, but he/she should be aware of what supplies are needed, how and where they are stored. The nurse coordinator should assist them in obtaining and storing whatever supplies are needed.

g. Equipment/Supply List for Sample Handling/Processing:

The nurse coordinator and other members of the team that recruit pregnant women and their children into the protocol will prepare a list of needed supplies and give it to the administrative secretary. The secretary will order the supplies for the recruitment and specimen team. Examples for serum samples include: syringes, needles, sterile swabs, heparin, and blood collection tubes. Specimens, to be sent to a central testing facility, will need freezer storage space (at -70°C) available at each clinical center.

3.6 Record Handling

3.6.1 Introduction

The data manager, organizes and maintains the P^2C^2 HIV study files. This is especially important since the data manager assumes operational responsibility for the integrity, confidentiality and security of the data. Each data manager will organize records differently, but he or she should work with the other team members in accommodating their needs. The data manager must become thoroughly familiar with where and how files are kept, and must have easy access to all files when necessary.

3.6.2 Confidentiality and Security of Patient Information

Confidentiality of data collected by the clinical centers and the Clinical Coordinating Center is a major priority of this multicenter study. This issue is particularly sensitive since the study involves subjects being diagnosed with the HIV infection or who are at high risk for developing the infection.

Particular care must be taken to ensure the confidentiality of participants in this study, to the extent possible, under applicable laws. All persons engaged in the collection, handling or dissemination of patient data shall be specifically informed of their responsibility to protect patient data and of the penalty for violation of this trust. Confidentiality statements will be signed by all employees and data users (see Appendix 2). Unauthorized acquisition, release, and/or discussion of any information to persons not in association with the contract is strictly prohibited, unless otherwise authorized by the National Heart, Lung and Blood Institute.

The collection of patient data, whether by interview, observation or review of documents, shall be conducted in a setting which offers maximum privacy and protects information from unauthorized individuals. Data will be housed in a secure area and will not be left unattended in areas accessible to unauthorized individuals. All patient information will be kept in locked files and will be accessible only to authorized personnel. Offices will be locked in off duty hours. Only authorized personnel will have access to such areas. All computerized files will be protected by means of a password system.

All correspondence (e.g., letters, forms, tapes, reports, etc.) containing patient information, mailed from the clinical centers to the CCC, will be marked "CONFIDENTIAL". Only authorized personnel will be allowed to handle such correspondence. Requests for data on patients via telephone will require proper identification and verification to assure that the requesting party is entitled to receive such information. A record of the request will be maintained at each clinical center by the data manager. Any destruction of correspondence containing patient data shall be according to an approved facility and will be accomplished by controlled incineration, shredding or other acceptable means of document disintegration. Data stored on electronic media will be bulk erased.

All data will be identified by code only. All data collection forms will utilize the code rather than a subjects name. Some data on each subject will be maintained for the clinical center to follow the patient but this will not be reported to the Clinical Coordinating Center.

3.6.3 Patient Study Files:

Each patient should have his or her own study file which is easily

identifiable. The nurse coordinator updates and maintains these files with the assistance of the data manager.

Each clinical center should develop a record system that suits its purposes. Two separate files will be created for each patient. One file will contain patient identifier information and will be kept in a separate locked file. A second file will be created for filing data forms.

Some suggestions for pertinent information for the patient files are:

a. Study Forms/Flow Sheets

b. Progress Notes

c. Laboratory Reports (local and central labs)

d. Informed Consent

e. Contact Persons

f. Death Certificates and/or Autopsy Reports

3.6.4 Miscellaneous Records

The nurse coordinator and other team members may choose to keep other types of study records. These will serve to monitor and account for P^2C^2 HIV study activities for areas important to that clinical center. Again, it is stressed that if team members keep records of certain activities, the nurse coordinator should keep track of these records.

Some activities for which it is helpful to keep logs include:

a. Quality Control Procedures

b. Data Transmission Reports

c. Queries and Responses

d. Patient Data Requests

e. Mailing of Forms

f. Computer Software Update Receipts

g. Electronic Mail

h. Correspondence

i. Supplies

j. Meeting Agendas and Minutes

3.7 Liaison Personnel

It is important for all clinical center study personnel to familiarize themselves with laboratory staff and other technologists (e.g., pulmonary, cardiology) who will be working with patients involved in the study. A good working relationship with these individuals will contribute to the success of the study.

3.8 Clinic Management

Since the nurse coordinator oversees the overall operation of the protocol, he or she will need to coordinate and manage the clinic in an organized fashion.

3.8.1 Scheduling of Activities

The nurse coordinator is responsible for scheduling all P^2C^2 HIV study activities. Since there will be several patients at each clinical center, it is important to organize the workload so that it is evenly distributed over the course of the week, and the data are accurate and timely.

When scheduling the visits in a given week, the nurse coordinator must take into consideration:

a. Coordination of activities among each team member, and

b. Obtaining the data in a timely fashion.

Since data must be transmitted within two working days of the visit, there must be time following each patient visit to complete forms and compile data. The nurse coordinator should work with other team members to set up a weekly visit schedule which accommodates this.

3.8.2 Coordination of Functions Between the Study Team Members

It is the nurse coordinator's responsibility to notify the other team members of the P^2C^2 HIV study activities. He or she serves as a liaison in communication, problem solving and scheduling. The nurse coordinator notifies the other team members of the patient visits and pertinent meetings. The coordinator should work with them to design a system which allows each team member to see the patient at different times, without conflict. Team meetings should be held weekly, and the nurse coordinator should work with the P^2C^2 HIV study team in scheduling these.

3.9 Data Management

The nurse coordinator should have the responsibility of assuring that the data collection forms are completed properly with special consideration of out of range clinical or laboratory values, or illogical entries. After the forms are completed the data manager assumes the responsibility of the data.

Data Management will generate patient scheduling lists, with the system provided by the CCC, so both the clinical and data management staffs are aware of when data forms need to be completed and when to expect completed data forms for keying. The clinical staff are responsible to complete the data forms as soon after the patient visit as possible. It will not be possible to complete all of the forms within one working day of a patient visit. Many of the forms are dependent upon laboratory results which may not be available immediately.

Completed forms should be given directly to the data manager. The data manager will log the received forms, and check for missing data items and illegible or illogical entries. Questions generated during data manager's review of the forms should be directed to the clinical research nurse for resolution. The forms should then be entered by the data entry technician. After the forms are entered, the data file should be printed and checked against the data collection forms for inconsistencies. After the data inconsistencies are resolved the file is ready for transmission to the CCC.

Data Management should develop a backup system for the data files that they generate. The CCC will provide general guidelines for such a system. This can be accomplished with a series of floppy disks and a log. For example, the backup files can be maintained on the hard drive of the computer and two disks until the backup disks are full. The hard disk is used for original data entry. Edits and

changes will be frequently backed up on a floppy disk #1. On Friday afternoons, a final copy of the hard disk files will be backed up on floppy disk #1. The floppy disk will also be taken off-site for storage. On Monday, floppy disk #1 will be left in storage while backup floppy disk #2 is used for the week. Floppy disks #1 and #2 are interchanged each week.

The Data Management staff should devise a quality control program for their center. Quality control systems are needed for:

1. Data entry;
2. Completion of data forms;
3. Abstraction of medical records; and,
4. Data backup and storage.

Data Management is also responsible for appropriately responding to CCC requests for rekeying of data forms, verifying pieces of data and other data entry/management procedures.

The Data Management staff is also responsible for maintaining communications with the CCC. A system for logging and filing E-mail messages should be developed.

3.10 Patient Management

3.10.1 Recruitment

The nurse coordinator is responsible for coordinating all patient recruitment activities in cooperation with the principal investigator and other P^2C^2 HIV study team members. The cooperation of all such individuals in recruitment efforts will directly reflect the success of the study.

3.10.1.1 Recruitment Resources

Recruitment of infants and children for this study should utilize the following resources:

1. NIH-funded ongoing research projects which have access to established patient's and referral patterns for enrolling new patients. Such projects include:
   a. Pediatric AIDS Clinical Trial Group (ACTG, NIAID);
   b. Randomized Clinical Trail of Intravenous Immunoglobulin vs. Placebo in HIV Infected Symptomatic Children (IVIG, NICHD); and
   c. Women and Infants Transmission Study (WITS), (NIAID, NICHD).
2. Obstetrical units within each clinical center which see high-risk pregnant women.
3. Patient registries consisting of HIV-infected patients.

3.10.1.2 Recruitment Enhancements

3.10.1.2.1 Compliance Through Patient Management

The nurse coordinator can help to enhance compliance primarily by building a good rapport with the patient's family (parent[s] or guardian[s]). The nurse coordinator, as part of the study team, is involved with the health management and education of the patient. He or she assists in assessing the family's level of knowledge about the child's disease, or risk of disease and, and the study procedures. Also, in conjunction with the study team, educates the family appropriately. The nurse coordinator also troubleshoots problems and provides support for the patient and family as necessary. Consideration of all the family

members is important to the study. Each member affects and is affected by the patient's illness or potential illness and their involvement with the study.

3.10.1.2.2 Compliance Through Clinic Management

Compliance is also enhanced if the clinic runs smoothly. Time management is essential. Patients should not be kept waiting for excessively long periods of time. In between patient visits, reminder calls or cards are essential. Not only do they reinforce study procedures, they are a good way to maintain contact with the patient and deal with problems.

Specific enticements should be used to enhance recruitment and follow-up of patients; these include taxi-cab vouchers, day care services and meal vouchers.

3.10.2 Informed Consent

3.10.2.1 Regulations

3.10.2.1.1 Department of Health and Human Services

It is the policy of the Department of Health and Human Services that studies which involve human subjects must be preceded by assurance that the individual's safety, health, and welfare (including the rights of privacy) must not be infringed. Participation must be voluntary and the potential benefits of the research must outweigh the inherent risks to the individual. Under the Department of Health and Human Services policy, each clinical center has the responsibility for the protection of human rights.

3.10.2.1.2 Clinical Center Investigational Review Board

The consent form, to be used by each clinical center, must satisfy each center's Investigational Review Board (IRB). It is impossible to provide a single statement that can be used by all physicians with all patients (i.e., mother/child) and all situations in this study. However, the consent form to be used by each clinical center must include, as minimum, the information contained in the consent form developed by the Steering Committee (see Appendix 7 of Protocol).

3.10.2.1.3 Project Office

A copy of the assurance of each clinical center's compliance with this policy is required by the Project Office prior to the initiation of the study. This policy specifies that an informed consent must be obtained from all patients prior to entry into the trial.

3.10.2.1.4 Clinical Coordinating Center

The Clinical Coordinating Center must receive a copy of each clinical center's local IRB approval of the consent forms before any patient can be entered into the study. In addition, annual renewals must be received by the CCC or entry of subjects will be revoked when the previous assurance expires.

3.10.2.2 Obtaining Informed Consent

The primary physician will obtain the informed consent. Considerable responsibility rests with this physician since the consent must be as informative and educational as possible. The patient's parent/guardian, as well as mothers

undergoing prenatal tests, must be made aware of all the ramifications of entry into the P^2C^2 HIV study and the inconveniences and risks involved, as best known to the investigators. The physician must also try to ensure that all the pros and cons of the study design and possible side effects are not so detailed that it confuses the parent/guardian to the extent that the physician, in essence, ends up making the decision for the parent/guardian.

Informed consents will be available in both English and Spanish. The consent will be reviewed with the parent/guardian prior to initiation of the study. In the instance the parent/guardian is unable to read, the consent will be read to them. An interpreter must be present in the event the person the informed consent is being obtained from does not speak English. Adequate time should be allowed for the parent/guardian to review the consent and ask questions.

If the patient were to move from the area that the clinical center is located in and relocates near another clinical center, and wishes to continue the study, a new informed consent must be obtained at the new center.

3.10.2.3 Authority to Give Informed Consent

Laws governing who may give informed consent differ from state to state. With the assistance of the NIH, each clinical center will clearly define who may give informed consent (e.g., guardians, foster parents). Each clinical center must also define whether or not an informed consent remains valid once the mother has expired. It will be the responsibility of each clinical center to follow these guidelines.

3.10.2.4 Types of Informed Consent

3.10.2.4.1 General Informed Consent

Initially, a general informed consent will be obtained depending upon whether or not the patient is in Group I or Group II. If the patient is being entered prenatally, the mother will also be required to give informed consent.

3.10.2.4.2 Informed Consent for Invasive Procedures

When invasive procedures are acquired, an informed consent specific to each test must also be obtained (e.g., endomyocardial biopsy, open lung biopsy, cardiac catheterization, bronchoalveolar lavage). The parent/guardian must be made aware of the potential for having these invasive procedures performed at the time the initial (general) consent is obtained. Participants will need to agree, prior to study entry, to all procedures or they will be excluded from the study. For all clinically indicated procedures, for which a specific informed consent is required, consent forms already in use at each institution will be used.

3.10.2.5 Filing Informed Consents

Informed consents must be handled as any other confidential document (see Section 3.6.2). A copy of the informed consent must be given to the parent/guardian after all signatures have been obtained. A copy of the informed consent must also be retained in the study folder at the clinical center.

3.10.3 Preparation for Patient Visits

The nurse coordinator, with assistance from the social worker, is responsible for ensuring that the patient is reminded of their appointment(s) prior to every scheduled monthly visit. The nurse coordinator or social worker may call the patient or ask the assistance of the clinical research nurse or have

the data manager send a reminder card. Reminder calls should occur two to four days prior to the visit. Any tests done at outside laboratories must be obtained for the clinical center's files.

Study visits should occur within designated time intervals, and it is essential no visit be missed because the patient forgot the appointment. Reminder cards will probably be sufficient once a patient has become familiar with study visits.

Prior to the patient visit, the research nurse should assemble the necessary forms for that particular visit. A schedule for required forms may be found in the forms manual. It is important to review the forms before the visit to assure all the required information can be obtained at the time of the visit and that all appropriate team members will be present for the visit.

3.10.4 Follow-up

Follow-up, of any duration, is difficult in an indigent population with chronic illness, poverty and drug abuse. Despite these factors, through the combined efforts of the study team, each patient will be aggressively followed. Prospective studies rely on the success of such follow-up.

Continued follow-up will be encouraged by the social worker who will maintain contact with families and assist them in keeping clinic appointments, obtaining social welfare benefits and obtaining transportation to hospitals. The CCC will generate a customized follow-up appointment schedule for each patient (see Section 2.9.1). The nurse coordinator must keep close track of patient visits during follow-up, as different tests and activities occur at different visits. Follow-up visits must be scheduled within ± 15 days of the scheduled

target date. The data manager will generate records of scheduled and missed appointments utilizing software provided by the CCC. These listings will be used to make reminder calls, assure accurate scheduling and provide follow-up for missed appointments.

Each clinical center will review patient adherence data quarterly in order to identify and resolve problems on an ongoing basis.

3.10.4.1 Patient Relocation

If a study patient moves to another U.S. geographic area served by a different clinical center, the patient should be reassigned to the care of the new center. A new consent must be obtained. The nurse coordinator should contact the Clinical Coordinating Center regarding the transfer. The former clinical center should supply the new clinical center with the patient's information.

If the patient should move to an area not served by another clinical center, every effort will be made to maintain the follow-up regimen specified in the protocol, and to document the subsequent clinical course. This will include trying to get patients back for visits and tests when feasible.

3.10.4.2 Lost to Follow-up

To help prevent loss to follow-up, the parent/guardian is asked to provide one or two names, addresses and telephone numbers of friends or relatives who do not live with the patient but who is likely to know of his or her whereabouts.

3.10.4.3 Patient Deaths

In the event of a patient death, the nurse coordinator should notify the Clinical Coordinating Center immediately with a telephone call or by electronic mail and subsequently document this by submission of the Death Notification Form (see Forms Manual).

3.11 Site Visits

Clinical centers can expect annual site visits to monitor quality control. The site visit team will consist of personnel from the NHLBI, from each of the other five clinical centers and from the CCC. NHLBI personnel, if in attendance, will be observers. Members of this team will have a diverse background (e.g., pulmonary, cardiology, data management, biostatistics, etc).

The site visit team will schedule the visit with the PI and suggest an agenda. The PI and the nurse coordinator at the clinical center may make revisions in the agenda according to local restraints. The nurse coordinator will coordinate, with the other study personnel, a schedule of the visit so that all are available, if needed. The nurse coordinator will also make arrangements to have the site visit team escorted to sites in the institution as well as those off campus.

The CCC will give advanced notice of any outstanding issues to the clinical centers so that those issues can be resolved prior to the visit.

Members of the site visit team will review each clinical center according to established criteria. The site visit will measure compliance with these established criteria. Quality control checks will be made on all facets of each clinical center's program, such as recruitment techniques, confidentiality

standards, chart content, data entry, and schedule of required tests/procedures (see Section 2.10).

The nurse coordinator assists the clinical center staff in preparing for the site visit. The nurse coordinator works with other personnel in making sure that all materials are well organized and accessible should the study team need to see any item. The nurse coordinator will assist the PI in preparing a short summary of each patient enrolled thus far, to include information on any problems encountered. The nurse coordinator will also work with other local investigators and technicians in preparing for any discussion related to laboratory procedures, pulmonary and/or cardiac testing.

The site visit will include time for the clinical center staff to raise study issues and problems for consideration by the site visit team. The nurse coordinator will see that issues are resolved, such as: frequency of missed visits, missing forms, missing data, missing informed consents, and missing responses to queries by the appropriate clinical center personnel. Any discrepancies should be resolved at the site visit.

At the end of the site visit there will be an executive session of the site visit team followed by feedback to the clinical center PI. The clinical center will receive a written report within two months of the site visit.

If a clinical center is found to have indicators of inadequate performance, such as high frequency of missed visits, large amounts of missing data, and/or a high rate of invalid data submitted, additional visits may be indicated.

3.12 Completion of Study

When the P^2C^2 HIV study patient follow-up ends, the patients will continue to be seen by the physicians who were part of the research team. The designation of specialty care of these physicians will vary depending upon the leading clinical problem of the patients and upon the particular center (e.g., infectious diseases/immunology, pulmonary, cardiology). Although the costs of the continuing medical care of these patients will revert to their primary support mechanisms, it is likely that additional NIH-sponsored clinical trial protocols may be available for enrollment.

4. Distributed Data Entry

4.1 Introduction

The P^2C^2 HIV study microcomputer software includes a distributed data entry system to collect data from clinical centers and transfer that data to the Clinical Coordinating Center (CCC) via telephone lines and an electronic mail (EM) system.

The microcomputer program menu driven system allows clinical participants to check the eligibility of subjects, and enter and verify data from study forms. It also provides an electronic mail facility which allows the transfer of messages between each clinical center and the Coordinating Center, as well as between the clinical centers.

The following sections provide detailed instructions regarding the use of the program from the various menus. This manual should be kept near the computer system at all times.

4.2 Using the Program

4.2.1 Menu Structure

As mentioned earlier, the computer system is menu driven. Menus appear throughout the program displaying all the possible choices available. A choice is made by keying the number corresponding to the desired action. Pressing the ENTER key will activate the choice made. At several menus the prompt has the additional message "blank to exit" which means the option to return to the previous menu may be accomplished by pressing the ENTER key (without typing in a number, or other character, first).

The program is started by typing "start" at the DOS prompt, and pressing the ENTER key. When the program first starts, the Main Menu appears on the screen. From the Main Menu the appropriate sub-menu can be chosen depending on the action needed to be performed at the time.

If the Main Menu is displayed on the screen, and selections are not made, the program will exit after 10 minutes and must be restarted from the DOS prompt. Throughout this manual the following screen exiting key sequence terms are used.

CTRL-END means to press and hold the CTRL KEY and then press the END key.

CTRL-HOME means to press and hold the CTRL KEY and then press the HOME key.

CTRL-ENTER means to press and hold the CTRL KEY and then press the ENTER key.

ESC refers to the key marked "ESC" in the upper left hand corner of the keyboard.

F5 thru F9 these are function keys, located on the upper left side of the keyboard; F5 = Unknown, F6 = Not Applicable, F7 = Not Done, F8 = Missing Date, F9 = Pending.

Please Note: It is important to remember that the computer should never be left at the end of the day with anything but the Main Menu on the screen. Doing so will prohibit automatic uploading of data to the CCC.

4.3 Data Entry

4.3.1 Enrolling a Patient

This section of the system is used to verify that the patient to be enrolled meets the criteria necessary for inclusion into the system as defined in Table 2 of the Protocol. Each question must be answered as it appears. If at any time an answer to a question does not fulfill the Protocol criteria, a message will appear on the screen indicating the problem. After noting the message, press the ENTER key and the system will return to the preceding menu. After successfully answering all the questions, the system will provide an I.D. number for the patient and the mother.

4.3.2 Forms Entry

The Data Entry option from the Main Menu is used for initial entry of a data form for a new patient. It is also the option used to make changes to data after a patient has been added to the permanent data base. Choose "Data Entry"

from the main menu, and then choose the appropriate form number from the Forms menu. When the screen appears, choose the "New Record" option. The cursor will move to the patient I.D. field, enter the patient's I.D. number, or press the TAB key and enter the last name.

When the form is completed, the computer will check the data and display a message if an improper value was entered. The cursor cannot be moved from the field until an appropriate value is entered.

If necessary to abort the data entry operation, press ALT-F10, and the form will be aborted. When completed, press CTRL-W to save the form or CTRL-Q to exit without saving. Choose "finished" when data entered is complete, and return to the Forms menu.

4.3.2.1 Verifying Data

Data must be verified before it will be uploaded to the Coordinating Center. Choose "Data Entry" from the main menu, and then choose the number of the form to be verified. When the screen appears, choose the "Verify" option and call up the form for the desired patient and date.

Proceed to enter the data as you did originally. If the value entered does not agree with the original data, then a message will appear and revisions can be made.

4.3.2.2 Editing Data

To make changes to data that has been entered, verified, and uploaded to the coordinating center, bring up the desired form and choose the "Edit" option. Use the arrow keys to move to the field to be changed. After making the changes, use the arrow key to pass through the remainder of the form. Then use CTRL-W to exit and save the changes.

4.3.2.3 Browsing Data

To view data without making any changes, use the "Browse" option. Use the Page Up and Page Down keys to view the desired fields. Changes cannot not be made using this option.

4.3.2.4 Missing Data (Procedure Not Done)

To identify a procedure/test as not done, choose the number of the form to be documented as not done. When the screen appears, select the "Missing" option from the menu bar. The identification number of the patient, date form completed, visit month (when applicable; see Forms Manual section 4.4.2 for details) and certification number of individual entering the form, are the only fields required when using this option. Press CTRL-W to save the entries. After the record has been saved, entry is complete. It will not be necessary to re-enter the data using "Verify" option.

4.3.2.5 Code Not in Selection List

4.3.2.5.1 General Codes Not in Selection List

When a valid code is entered into the computer, and a selection list is returned which does not include the code, the CCC must be notified immediately by E-mail. The error will be corrected by the CCC through a simple modification to the general reference file.

4.3.2.5.2 SNOMED Codes Not in Selection List

When a valid SNOMED code is entered into the computer, and is not included in the computer's selection list, the code of "XXXXXX" (TXXXXX for a site code and EXXXXX for organism code) should be selected. This code is displayed at the bottom of the selection window. By choosing this code, the data entry person is

indicating that a specific code is not included in the computer's general reference file.

When the above situation occurs the data entry person should perform the following steps:

1. Choose "XXXXXX" (TXXXXX or EXXXXX) to indicate the code is not in the selection list.

2. Backspace to the narrative field and enter the valid code immediately following the diagnosis.

Example: The code of M02910 is entered into the computer, but not found in the general reference file.

Enter:

Narrative: Increased density of lung M-02910
Site Code: T28000
Diagnosis Code: XXXXXX

The CCC will:

4. Update the general reference file by adding the code entered in the narrative field.

5. Download the file to the Centers.

6. Notify the Centers of the new code and the form which needs to be edited.

4.3.2.6 Memo Fields

Several forms have special fields which will allow entry of memos. Although memos should be kept to a minimum number of characters, memo fields are not restricted in size as are other fields.

4.3.2.6.1 Entering a Memo Field

To enter a memo field press CTRL HOME. This will access a pop-up window, at which time the memo can be entered. Once data are entered, the field will be exited by one of two tracks; either

saving the entry or exiting without saving. CTRL W or CTRL ENTER will save the data entered; CTRL Q will quit without saving.

4.3.2.6.2 Editing a Memo Field

To edit a memo field, the user will first access the memo field as indicated in the previous section. Once the changes are entered, press CTRL W or CTRL ENTER to save the changes. This process will save the changes to the memo field, only. To send the changes to the CCC, the record must also be flagged as a changed record. Following the last edit to the record, press CTRL W to exit and flag the record as changed. (NOTE: If the MEMO is exited and saved, but the RECORD is exited using CTRL Q [quit without saving] the changes to the memo field will be saved, however the changes will not be sent to the CCC.)

If a MEMO field is entered and edited, but the individual decides he/she does not want to save the changes, CTRL Q (quit) should be used to exit the memo without saving.

4.4 Electronic Mail

An Electronic Mail Facility is available to send mail to the Coordinating Center and other clinical centers. Choose Electronic mail from the Main Menu. Then choose the desired function. Throughout these instructions, references to keystrokes will appear, (example; "CTRL-HOME", "ALT-P", etc). For example, "CTRL-END" means press and hold the CTRL key, then press the END key. The "Delete message" and "Revise message" refers to messages that have been written during the day. These options allow the ability to make changes before they are sent out at night. Mail is distributed every weeknight along with data uploading.

4.4.1 Transmitting Messages

When sending a message, a screen will appear displaying the institutions to which the message can be sent. Choose the desired destination by entering the corresponding number at the prompt. Press the ENTER key without choosing a number to return to the previous menu. The cursor will proceed to the message window allowing the message to be typed. When completed, press CTRL-ENTER. Messages are then transmitted by telephone every evening, Monday thru Friday.

4.4.1.1 Editing Text in the Message Window

At various times when using the Electronic Mail Facility the message window will appear enabling the user to create or revise text. This section will provide information regarding the use of the Text Editor. The "Text Editor Key Functions" list below describes the keys and their functions.

When creating a message the "word wrap" feature will cause the text to automatically go to the next line.

To erase the entire message, press CTRL-HOME and position the cursor at the

beginning of the message. Press SHIFT-PAGEDOWN until the entire message is highlighted. Then press the DELETE key. Press CTRL-ENTER to return to the memo field and press ENTER to exit.

TEXT EDITOR KEY FUNCTIONS

Key	Function
Right Arrow	Moves cursor one character to the right
Left Arrow	Moves cursor one character to the left
Up Arrow	Moves cursor up one line
Down Arrow	Moves cursor down one line
Page Up	Moves cursor up one window of text
Page Dn	Moves cursor down one window of text
Home	Moves cursor to the beginning of current line
End	Moves cursor to the end of current line
Ctrl Rt Arrow	Moves cursor one word to the right
Ctrl Lt Arrow	Moves cursor one word to the left
Ctrl Home	Moves cursor to the beginning of text
Ctrl End	Moves cursor to the end of text
Insert	Toggles between Insert/Overstrike mode
Delete	Deletes character at cursor location
Ctrl-Backspace	Deletes word at cursor location
Backspace	Deletes character to left of cursor

4.4.1.2 Revising Messages

Once a message is created using the send message option, revisions can be made before transmitting that evening. When revising more than one message choose Revise message. Then proceed to edit the message as described in Section 4.4.1.1.

4.4.1.3 Cancelling Messages

When choosing "Cancel message" from the Mail menu, a screen is displayed listing all the memos pending to be sent. Choose the appropriate message to be deleted. A warning message is displayed, this is a special precautionary measure to prevent inadvertent deletion of a message. The message is then deleted, and the screen is recreated with new numbers for each message.

4.5 Read/Print Mail

When receiving mail, use this function to view. A screen appears displaying the incoming mail indicating the date, time and sender. When choosing the desired number, the message is displayed for viewing. If the message takes up more than one screen use scroll forward by first pressing CTRL-HOME, then PAGE-UP or PAGE-DOWN keys. Press ALT-P to print the message. Be sure the printer is online with sufficient paper.

After viewing the message, a question appears asking whether to mark it for deletion or save it to an archive file. When saving a message for later viewing use "Read/Delete Old Mail" feature from the Mail menu. The message will then remain available for viewing throughout the day. The message will be erased the following day.

4.5.1 Read/Delete Old Mail

To read or delete old mail use this option. The screens are similar to the previous ones mentioned. A warning message will appear when choosing to delete old mail.

4.5.1.2 Edit/Send Frequently Used Messages

Frequently used messages can be created, revised, deleted or transmitted by entering the following:

- When creating a message, first enter a subject. This should be a meaningful phrase that describes the message since it will be used later to identify the message being sent, revised or deleted.
- When revising, sending or deleting a message, a list of all the messages created are displayed by number and arranged alphabetically by subject. If the list takes up more than one screen, scroll forward and backward using the PAGE-UP and PAGE-DOWN keys.
- When sending a message to one or more destinations, enter a "1" in

the field next to the name of the institution. To delete the message replace the "1" with a "0".

4.6 Utilities

There is a section of the program, called "Utilities" that allows a variety of miscellaneous activities to be performed on the system.

4.7 Backup Procedures

A back-up of the data must be done weekly. This involves copying the data files from the hard disk to 3 1/2" diskettes using the Backup feature of the Utility menu.

Before backups can be performed, new diskettes must be formatted. This can be done using the Format diskette feature from the Utility menu. Format 10 diskettes. There will be 2 diskettes for each of 5 weeks. Each diskette will be labelled with its week number and disk number. For example, for week 1, one diskette will be labelled "WEEK 1, DISK 1 and the other one will be labelled "WEEK 1, DISK 2". For week 2, "WEEK 2, DISK 1" and "WEEK 2, DISK 2", and so on and so forth for all 5 weeks.

Backups should be done every Friday. When you choose the Backup feature from the Utility Menu, it will tell you which weekly set of diskettes you should use. When the computer prompts you to load the first diskette, insert the "DISK 1" diskette into the disk drive. Then press the ENTER key. As the study progresses, a weekly backup may require more than one diskette. If so, the computer will prompt you for DISK 2.

If for some reason, an error occurs during the backup procedure, an error message will be displayed. Try the backup again, and if the error recurs, make a note of the error number, and call the Coordinating Center.

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If for some reason, an error occurs during the backup procedure, an error message will be displayed. Try the backup again, and if the error recurs, make a note of the error number, and call the Coordinating Center.

5. Methods

Standardized techniques for methods described in the P^2C^2 HIV study are essential for accurate data analysis. This chapter is dedicated to describing the standardized techniques for completing the virology/immunology, cardiology, pulmonary, and postmortem methods as required by the Protocol. Additional comments may also be made regarding the equipment to be used for these methods, the methods to be used for interpreting such results, training required for employees to perform these tests/procedures, and quality control measures that will be taken to ensure that they have been properly executed.

5.1 Pulmonary

5.1.1 Chest Roentgenogram

5.1.1.1 Frequency of Study

For frequency of chest roentgenograms for this study, refer to Section 5.2.4.2. of the Protocol.

5.1.1.2 Method

Inspiratory chest roentgenograms will be obtained according to the age of the patient and clinical condition. The film screen system speed will be 200 to 400. The objective of the film quality is to obtain adequate penetration for visualization of lung parenchyma without use of a bright light for increased illumination.

5.1.1.3 Interpretation

The chest films will be read on the basis of criteria established by the pediatric radiologic consultant panel.

5.1.1.4 Training

The investigator at each center will be responsible for the technical training at their center.

5.1.1.5 Quality Control

A review of a representative selection of chest films will be performed by having a random sample of chest x-ray films from each Center re-read in a blended manner by two other study radiologists at other Clinical Centers. A third evaluation will be done by the radiologist at the Clinical Coordinating Center.

5.1.2 Oxygen Saturation by Pulse Oximetry and Arterial Blood Gas Analysis

5.1.2.1 Equipment and Supplies

1. Nellcor pulse oximeter (Nellcor Incorporated, Hayward, California) - model N-10 battery-operated, with display of pulse, oxygen saturation and heat sensitive paper recording of a ten-minute study of pulse and saturation.
2. Impedance cardiopulmonary monitor.

5.1.2.2 Method

Pulse oximetry is a non-invasive, continuous estimate for hemoglobin oxygen saturation method based on a modification of the Beer-Lambert law which states that the concentration of a solute dissolved in a clear solvent can be determined from the absorbance of a light of known wavelength transmitted through the solution. Laboratory oximeters use this principle to measure hemoglobin concentration by shining light of a single wavelength through a solution of lysed red blood cells. Once solutions of known concentrations of hemoglobin are used to construct a calibration curve, it is possible to determine the hemoglobin (Hb) concentrations of unknown solutions (Tremper, 1989). Oxyhemoglobin (HbO_2) measurement using this principle requires the measurement of transmission at two wavelengths. In clinical pulse oximetry, the arterial pulsatile component is measured through analysis of absorbance in the red and infrared wavelengths and, theoretically allows determination of arterial blood. With the eight wavelength band pulse oximeters, the correlation has been excellent (Strohl, 1986; Praud, 1989).

$$SpO_2 = \frac{HbO_2 \times 100\%}{HbO_2 + Hb + COHb + metHb}$$

The use of multiple wavelength analysis avoids the confounding variables of capillary and venous blood and eliminates the necessity of arterializing blood flow through a limb by warming it. However, this does not eliminate the problems

encountered by the inability to exclude the influence of carboxyhemoglobin and methemoglobin. The latter in concentrations, greater than 30%, will overwhelm the hemoxyglobin signal and cause the oximeter to read 85% regardless of the true saturation with oxygen.

The two major causes of artifacts in the pulse oximetry method are motion and light (Nellcor, 1986; Yelderman, 1983). The light detector must be in continuous contact with the skin of the patient and must be totally shielded from extraneous light (e.g., room or examination lighting) as the detector emits current whenever light excites its surface. There is a correction logic wired into the circuit as the oximeter first turns on a red light emitting diode (LED), then an infrared LED, and finally both a red and infrared LED to filter out the room light. This cycle is repeated continuously, hundreds of times a second. Light and especially motion can still cause troublesome artifacts. While in stable infants and children, pulse oximetry has been a reliable measurement of oxygen saturation in patients who are critically ill and/or experience peripheral circulatory problems, the absence of adequate peripheral blood flow can also lead to an inconclusive assessment of oxygen saturation (Swedlow et al, 1983; Ramanathan et al, 1987; Taylor et al, 1986; Fanconi, 1988). The pulse oximeter, to be used in this study, yields oxygen saturation measurements which when compared to co-oximeter measurements of oxygen saturation, overestimates saturation when the arterial oxygen saturation is less than 96% (Fanconi, 1988; Praud et al, 1989). This is thought to be due to the fact that it uses two wavelengths rather than eight wavelength analysis to determine oxygen saturation.

5.1.2.2.1 Frequency of Study

For frequency of pulse oximetry for this study, refer to Section 5.2.4.3 of the Protocol.

5.1.2.2.2 Procedure

The oxygen sensor is placed on the small finger of an upper extremity or on a toe and taped in place. Three impedance electrodes are placed on the chest, with two at the mid-axillary line in the level of the twelfth rib on opposite sides of the chest. The cardiopulmonary and oxygen saturation monitoring are begun. Heart and respiratory rates and oxygen saturation are recorded each minute for a ten minute period of study. When agreement between the impedance and pulse oximeter heart rates are within 3 beats per minute, the measurement of oxygen saturation is considered valid.

5.1.2.3 Interpretation

The values of SpO_2 fall into three ranges:

1.	Normal	-	SpO_2 >97%
2.	Indeterminate	-	SpO_2 96 - 97%
3.	Abnormal	-	SpO_2 < or = 95%

For the SpO_2 study to be considered valid, the pulse corresponding to a SpO_2 value must be accurate. Therefore, for each minute, during the 10 minute observation period, the pulse and the corresponding SpO_2 must be recorded. When review of the 10 minute record reveals the pulse in any minute to vary by more than 10% of the average pulse for the observation period, the SpO_2 for that minute is likely to be inaccurate. Another method to confirm the accuracy of the pulse determined by the oximeter is to perform simultaneous impedance monitoring of the heart rate. This method is the most accurate method for measuring heart rate. The pulse value from the oximeter must be within 2% of the impedance value or the oxygen saturation value is invalid.

When the oximetry recording is considered accurate and free from pulse artifact described above, and a SpO_2 value is indeterminate for 3 of the 10 minute monitoring period, another 10 minute observation period is indicated. When there is an abnormal tracing or a second 10 minute observation period yields

indeterminate values for 3 of the 10 minutes, the patient should receive a radial or brachial arterial puncture for blood gas determination. An exception to this guideline may be indicated in some patients closely followed by the physician, when the patient has a normal physical examination, a normal sleeping respiratory rate and a normal chest roentgenogram.

Local anesthesia with 1% lidocaine is advised when feasible for the arterial puncture. Blood gases will be drawn with the patients breathing air unless they require continuous oxygen administration. The oxygen flow at which the blood gas is drawn will be noted and reported. Both A-aDO_2 and a/A O_2 ratio will be calculated.

Normal blood gas values include:

pHa 7.37 - 7.42
$paCO_2$ 37 - 42
paO_2 >80 torr or an associated (A-a)O_2 gradient of less than 15 (after one month of age; normal PaO_2 at birth and during the first month after birth = or > 70 torr.

5.1.2.4 Training

The investigator at each center will be responsible for the technical training at their center.

5.1.2.5 Quality Control

In the measurement of arterial oxygen saturation, the pulse rate as determined by the saturation sensor attached to the patient's finger or toe must be within 2-4 beats per minute of the heart rate measurement from impedance monitoring. Saturation must meet this criteria for at least 6 of the 10 minute observation period. Respiratory rate will be determined in parallel with this monitoring as a check on the adequacy of patient ventilation during the study. The patient must have been breathing quietly at rest without breathholding during the portion of the study from which the data is accepted. With assisted ventilation, there must be no interruption of ventilation during the test period.

Arterial oxygen tension measurement is dependent on acceptable blood drawing technique. The blood must flow passively into the syringe and there must be evidence of pulsatility. The blood gas analyzer will have a two point calibration within 15 minutes of the analysis. Hemoglobin will be used to correct the bicarbonate and base deficit values.

5.1.3 Pulmonary Function Studies

The schedule and type of pulmonary function studies to be performed in Groups I, IIa and IIb are provided in this section. The studies selected for each age range are based on the child's ability to cooperate and to perform the studies. Not all children in an age group will be able to perform all of the studies for that age range, but an attempt should be made. All PFT studies will be performed at six month intervals according to the schedules provided in the Protocol (See Tables 9, 10 and 11)

Pulmonary Function Studies

Age	Equipment	Sedation	Method of Data Collection	Description
* 0 - 3 Years (0 to 36 months)	SensorMedics 2600	Chloral hydrate 80-100 mg/kg	Diskette	1) Partial expiratory flow-volume measurements 2) Compliance 3) Squeeze 4) FRC (nitrogen washout)
3 - 5 Years (37 to 60 months)	1) SensorMedics 2600 (linear pneumotachograph 0-100 l/min) 2) Mini-Wright Peak Flow Meter CURRENTLY UNDER REVIEW	None	Diskette (Peak flow data entered under comments section)	1) Peak expiratory flow: a) Calibration of meter b) Five maneuvers (three minimum) c) Best effort recorded 2) Partial expiratory flow volume measurements a) Seated position b) Five maneuvers (three minimum) c) Best effort recorded 3) FRC (nitrogen washout)
> 5 Years (≥ 61 months)	1) Mini-Wright Peak Flow Meter 2) Wedge spirometer, pneumotachograph or rolling seal spirometer	None	Data Collection Form	1) Spirometry measurements: a) PEF b) FVC c) FEV_1 d) $FEF_{25-75\%}$ 2) Five maneuvers (three minimum) 3) Two best efforts recorded 4) Seated position

* Group IIb patients are not tested at month 24, 30 and 36. Testing resumes without sedation at month 42.

5.1.3.1

SensorMedics 2600 Studies

The pulmonary function studies to be done in children from 0 - 3 years of age consist of measurements of total respiratory system resistance and compliance, measurements of forced expiratory flow from partial expiratory flow volume (PEFV) curves and measurements of functional residual capacity (FRC) by the nitrogen washout method.

SensorMedics Studies in children from 3 - 5 years of age will consist of tidal flow-volume loops with forced expirations. The child will perform five spirometry maneuvers (minimum of three efforts) in a seated position with a face mask. Measurements will be recorded using the Sensormedics equipment and the best of the five efforts will be saved.

Complete instructions on the performance of the pulmonary function studies, including expiratory flow-volume curves, resistance and total pulmonary compliance measurements are described in the Sensormedics Operators Manual. A brief description of the tests is included in Section 5.1.3.1.2.

Proceedings from the July 1993 PFT Workshop are found in Appendix 16 of this Manual. Included are frequently encountered problems, discussion and resolution achieved at the Workshop. Review this Appendix before proceeding with performance of PFT studies.

5.1.3.1.1 Equipment

To ensure consistent, reproducible results from pulmonary function testing in this study, all centers will use the same pulmonary function equipment. (#2600 Infant Pulmonary Function System and #2605 Infant Hugger System). See Appendix 3 for maintenance of equipment.

5.1.3.1.2 Methods

PEFV Curves:
(Schaeffer and Cerny, 1985; Adler and Wohl, 1978; Godfrey et al, 1983; LeSouef, 1986; Taussig et al, 1982; Tepper et al, 1987; Ratjen, 1989)

Following sedation with 80-100mg/kg of chloral hydrate (if the patient does not sleep within 20 minutes following medication, an additional dose, to bring the total to 120mg/kg, should be given) administered in a small amount of formula

(premedication can be repeated at 50% of the initial dose if there is no response within one hour), the infant is studied supine in an appropriately fitting inflatable jacket (Hammersmith Hospital). The jacket is connected with wide bore tubing to a pressure reservoir (see Sensormedics Manual). A 3-way valve is placed between the tubing to the jacket and pressure reservoir to allow rapid inflation of the jacket (Silverman). Pressure is measured in the jacket from a pressure tap connected to a pressure transducer (± 100 cm H_2O, Validyne, Northridge, CA). The rise time of the system, excluding the jacket for a sudden static pressure increment, is 50 milliseconds or less.

Flow is measured with a pneumotachograph (Fleisch 0, 00, 1) connected to a pressure transducer (± 2 cm H_2O, Validyne) and integrated to record tidal volume. Tidal flow-volume curves are collected to determine proper head, neck and jaw position for subsequent measurement. There should be less than or equal to 5% difference between V_I and V_E . If the loops are moving back and forth, this difference would be considered normal variation of the end-expiratory lung volume. If the loops are working in one direction only, a leak or pneumotach alinearity should be suspected, even if the error falls within the +/- 5% variance. The pneumotachograph is attached to an anesthesia face mask (Ohio Medical Products) placed over the infant's mouth and nose. Air leaks are prevented by using a rim of therapeutic putty (Allmed, Arlington, MA) around the edge of the mask.

To obtain PEFV curves, 2 or 3 tidal breaths are obtained and the jacket is pressurized for 3 seconds beginning with jacket pressures of 40cm H_2O. Jacket pressure is increased in 10-15 cm H_2O increments until further increases in flow are no longer obtained. A minimum of three curves, one of which was obtained

using a different hug pressure so as to demonstrate true flow limitation, should be obtained. The ideal goal is to obtain eight acceptable curves, one of which was obtained using a different hug pressure. At least 60 seconds is allowed between successive PEFV maneuvers to limit the effect of volume history on flow (Morgan et al, 1989).

The following signals are recorded:

1. Jacket pressure
2. Flow
3. Volume

Technique for determining whether flow limitation is achieved:

The maximal curves are superimposed and aligned by shifting volume. If there is overlap, the segment of the curve overlapping with another curve can be used.

The following values are reported:

Tidal volume, V-FRC, peak flow, mid-tidal volume flow, and slope of segment representing 1/2 of a tidal volume will be collected on at least 3 maneuvers.

Compliance and Resistance

Always use the 2600 software to insure the presence of the Hering-Breuer reflex before making compliance/resistance measurements. If % prolongation is less than 0, do not proceed with compliance/resistance measurements. Doing this will prevent wasting time attempting to make compliance measurements on children without a H-B reflex present.

Measurement of Compliance and Resistance of the Respiratory System: (Mortola et al, 1982; LeSouef, 1984A&B)

The sedated sleeping infant will be placed supine on a bed without a respiratory jacket in place. The infant may be loosely swaddled. An anesthesia mask with an attached pneumotachograph is place on the infant's face and sealed with therapeutic putty. Measurements will be made during quiet respiration. At end-inspiration, the airway is occluded for about 200 milliseconds. The occlusion is released and the infant expires passively. The assumption is that occlusion produces relaxation and that the system relaxes to a passively

determined volume (Zin et al, 1982). Thus, only occlusions during which the airway pressure reaches a plateau (P_{ao}) are selected for analysis. The difference between volume, at occlusion and the passively determined volume, is considered to be delta-V. The difference between the "plateau" pressure and atmosphere is delta-P. When the patient begins inspiration prior to the completion of a passive expiration, the straight line of the flow-volume relationship is extrapolated to zero flow in order to obtain the total passive expiratory volume (V_e). This correction is also employed in completed passive expirations to obtain the flow appropriate for the occlusion pressure (V_o). Ten to fifteen occlusions should be performed, of which five to seven should have good plateaus lasting 100 milliseconds of longer. C_{rs} is calculated by dividing the volume change (delta-V) of the end-expiratory level by the pressure change (delta-P) and then subtracting the compliance of the circuit, $C_{rs} = (dV/dP) - C$ circuit, and then converting to BTPS.

Therefore: $C_{rs} = V_e/P_{ao}$

$R_{rs} = P_{ao}/V_o$

$T_{rs} = C_{rs} \times R_{rs} = V_e \times P_{ao} \times V_o = V_e/V_o$

Each subject's reported result is average of at least three acceptable tracings.

Determination of Functional Residual Capacity (FRC) by the Nitrogen Washout Method (Gerhardt, 1985; Ronchetti, 1975)

The determination of FRC is by the open-circuit nitrogen washout method. The nitrogen needle valve should be peaked before each patient. After low and high volume calibration, the oxygen inflow is set at a flow equal to or slightly greater than the expected peak inspiratory flow rate of the patient. At the end of a tidal breath, the patient is turned into the oxygen inflow. Expired volume and alveolar nitrogen (N_2%) are displayed graphically and numerically and

recorded on a breath-by-breath basis. Since oxygen resident in the lung from a previous measurement lowers the amount of nitrogen in the lung at FRC, it will lower the measured FRC. Therefore, the minimum interval between repeat FRC tests (N2 wash-in time) will be three times the "wash-out" time from the previous test. A minimum of two FRC tests will be performed if the variability is within 5%; three if within 10%. When the nitrogen concentration falls below 1% the 3-way inspiratory valve is turned to permit the patient to breath room air. From the patient's initial alveolar nitrogen concentration and the final nitrogen concentration in the collecting system the patient's FRC and FRC/kg body weight are calculated (Hiatt, 1988).

$$FRC(N_2) = \frac{V_{N2}}{F_A \times N_{2,0} - F_A \times N_{2,1}}$$

Where:

V_{N2} - volume of nitrogen washed out

$F_AN_{2,0}$ - initial fraction of alveolar nitrogen

$F_AN_{2,1}$ - final fraction of alveolar nitrogen

Acceptable and unacceptable N2 washouts are defined as:

Acceptable	Unacceptable
1. Progressive decline of expired N2	1. Non-reproducible FRCs (implies minimum of 3 measurements)
2. Smooth washout curve	2. Spikes in N2 curve
3. No spikes in expired N2 tracing	
4. Multiple efforts show similar washout time and curve shape	

5.1.3.1.3 Interpretation

The pulmonary function data will be collected by a microcomputer. The same software for collection and calculation of the results will be employed at all centers participating in this study.

5.1.3.1.4 Training

Training in the performance of the pulmonary function studies, using the equipment purchased from a single manufacturer, will be accomplished with two training sessions for the investigators and their technical staff. One training site on the east coast and one on the west coast prior to the start of the clinical phase of the study.

5.1.3.1.5 Quality Control

The adequacy of the quality of pulmonary physiology measurements will be determined by first comparing results of pulmonary function tests from control patients (e.g., those with no other evidence of lung disease), in the five centers participating in this study, to published controls and then comparing the results between the centers. These results will be compared on a monthly basis by a group appointed by the pulmonary subcommittee and a complete, in-depth analysis will be performed by the Clinical Coordinating Center twice a year during the study period. Both intrasubject and intersubject analysis will be performed. At each pulmonary function study at least three repetitions of each pulmonary function parameter will be obtained before the best effort is selected. However, all three efforts will be reported to the CCC. A center reporting data which varies greater or less than 10% from the mean for all centers will be required to study their pulmonary function testing procedures to assess the nature of the discrepancy. They will also be asked to retrain their technical staff in the methodology for the test performance.

Quality control for the operation of the Sensormedics #2600 - Infant Pulmonary Function System, and the #2650 - Infant Hugger System are outlined in the Manufacturer's manual which is provided with the equipment. A summary

covering the calibration of the four major system parameters is found below.

The four measurement parameters which require recalibration and verification on the Sensormedics model #2600 - Pulmonary Function System and the model #2605 - Infant Hugger System.

1. **Pressure** is calibrated manually, once a month, using the analog-digital conversion board, calibration software supplied with the system and a water manometer (Dwyer Instruments, mode #1230-20 [0-20 cm H_2O]; Anaheim, CA). Monthly calibration is adequate due to the stability and minimal drift of the differential pressure transducer (Validyne Engineering Co., Northridge, CA).

2. **Volume** is calibrated before each test session using a 100 mL syringe (model #5510, Hans Rudolph, Kansas City, MO). The specific pneumotachograph utilized is selected on the basis of the weight of the patient:

Model #8311B	Flows 0 to 10 L/min.	Neonates
Model #4500B	Flows 0 to 30 L/min.	Infants (until 2 years of age or 13 kg)
Model #4700	Flows 0 to 100 L/min.	Infants (>13 kg)

Volume and flow calibrations are performed immediately prior to each study in order to check for drift from temperature, moisture in the system and condensation of moisture on the Monell screen of pneumotachograph. Volume calibration must be correct to within 3 ml of the instilled volume.

3. **Flow** is calculated directly by dividing the test volume instilled by the time and converting to ml./sec. Thus, the accuracy of the flow measurement is dependent on the accuracy of the volume calibration. The same holds true for the extrapolated values of compliance and resistance.

4. **Nitrogen FRC measurement** is performed with a T-piece breathing circuit through which pure oxygen (FiO_2 = 1.0) flows at a steady rate. The patient breathes oxygen from the circuit through a T-connector. Nitrogen calibration is performed using the Hans Rudolph 100 mL calibrating syringe and two standard gas mixtures. Zero point adjustment for N_2 is made with pure oxygen flooding the circuit and the high calibration mixture is achieved when the system is flooded with room air (FiO_2 = 79%). For this parameter calibration is performed prior to each study. The N_2 integral is measured at two points, 20 and 40 mL. Verification of calibration is determined when retesting at 40 and 50 mL is within 3% in the duplicate test. Calibration of the Model #2605 Infant Hugger is dependent on the proper calibration of the Validyne transducers and the 100 mL/min. pneumotachograph.

5.1.3.1.6 **Safety**

Upper airway obstruction in children may be more common in HIV infected children than in the normal population. It is important that prior to sedation of a child, the guardian be asked if the child snores, has noisy breathing or pauses in breathing during sleep. This will alert the investigators of infants who may experience airway difficulties during testing. In addition, the researchers should have bag mask ventilation equipment available whenever a child is being sedated for a study.

5.1.3.2 **Peak Flow Measurements**

Peak flow measurements will be made in children who are 3 years and older. The patient should be in a sitting position for these studies.

5.1.3.2.1 **Equipment**

A Mini-Wright peak flow meter will be used for peak flow studies. Either a disposable meter or a permanent meter with a disposable mouth piece should be used. The meter used must be calibrated.

5.1.3.2.2 **Quality Control**

Peak flow meters can be calibrated using a mechanically generated flow. Ideally, this flow should have a wave form that typifies a forced expiratory maneuver, but this may be expensive and impractical. The alternative is a "home-made" mechanical source which should deliver an "explosive" type flow. Example: Mt. Sinai uses a vacuum cleaner as the generator of the flow. This is connected to a rotameter with which they can achieve a desired flow rate. This is attached to a 3-way valve which is, in turn, connected to the peak flow meter. The valve is opened and closed quickly and the peak flow recorded.

Using this system, the generating flow rates should be 180 L/min and 300

L/min.

An alternative method is connecting a flow source to a pneumotach (0-1200 L/min) which is connected to a peak flow meter. The flow is achieved by using a pressurized (4.9 PSI) aluminum one gallon chamber filled with copper wool.

Whatever method is used, the peak flow meter should be calibrated in triplicate every three months.

5.1.3.3 Spirometry

5.1.3.3.1 Equipment

Any spirometer which meets the 1987 ATS specifications (Am Rev Respir Dis 136:1285-1298, 1987) may be used. A record of the type of equipment used by each Center will be kept on file at the Clinical Coordinating Center.

5.1.3.3.2 Method

Five efforts will be performed and the two best efforts will be recorded in the spirometry form. FVC, FEV_1, $FEF_{25-75\%}$ and PEF will be recorded for each effort. $FEF_{25-75\%}$ from the best (greatest FEV + FVC) of the five efforts will be used for analyses. All measurements will corrected for body temperature and pressure saturation (BTPS). The measurements will be performed pre and post-bronchodilator.

The participant will be studied in the seated position. Every effort should be made to perform spirometry at the same time of day throughout serial testing sessions.

The FVC is the total amount of air that can be expelled in one forced exhalation after a maximal inhalation. The accuracy of the FVC depends on a maximal inspiration and on a complete exhalation.

The patient's neck should be slightly extended and the spirometer hose

adjusted to the patient's height, so that the child sits erect and is not hunched over. The chin should be slightly elevated.

In order to prevent nasal leakage during expiration, a noseclip will be used during the maneuver. While wearing the noseclip, the child should be instructed to avoid swallowing as this may block the ears and cause discomfort. The noseclip should be removed between efforts.

Forced Vital Capacity:

The technician should explain to the patient that he/she is about to do a test to determine how much, and how forcefully, air can be exhaled from the lungs. Also, the technician should explain to the child that a noseclip will be placed on his/her nose and that he/she will need to do the following:

a) Take in a deep a breath, and when full,
b) Place the mouthpiece in his/her mouth *(assisted by technician)*
e) Close his/her lips tightly around the mouthpiece
d) Exhale through the mouthpiece into the spirometer, blasting the air out as hard, fast, smoothly, and completely as possible.

To insure that the child understands the maneuver, the technician should demonstrate the complete maneuver, stressing the importance of trying to obtain a complete inspiration and a forceful, complete and smooth exhalation.

When ready to proceed, place the noseclips on the child and coach the child to inhale as large a breath as possible.

When the child is ready to exhale, coach him/her to blast out the air at the onset of the maneuver until all the air is out. The technician should watch the patient, not the instrument, during the maneuver. Continue coaching until a volume plateau of at least 2 seconds with an exhalation time of a least 3 seconds is reached or an expiratory plateau zero volume change is demonstrated. If no plateau is demonstrated, the participant should exhale for as long as

possible. Terminate all expiratory efforts after 15 seconds. At the end of every maneuver, the results should be checked for acceptability and reproducibility.

The technician must check to be sure that the patient's lips form a tight seal around the mouthpiece. The lips should not be pursed in front of the mouthpiece, like a trumpet player's and neither the teeth nor the tongue should block the mouthpiece during the expiration. The mouthpiece should be placed on top of the tongue.

After each trial, encourage the child to try to get just a little more air in when inhaling, and to try to blast out harder and longer when exhaling on the next trial. Although the final results are checked for reproducibility, the emphasis should always be placed on maximal efforts, and not on simply reproducing what has already been recorded.

Always let the child rest as long as needed between trials. If the child has problems with the maneuver, determine the problem and re-instruct the child if necessary. For example did the tongue get in the way of the mouthpiece, or did air leak around the mouthpiece?

If coughing or flow transients are observed, the patient's throat may be dry (offer a drink of water), or he/she may not have tried to keep a smooth push while exhaling.

Recheck whether the participant's clothing is restricting his or her breathing. If the noseclip slides off, wash it with soap or alcohol pads and place it back on the nose.

A second demonstration by the technician of a smooth and complete exhalation versus a choppy and hesitating exhalation can help a child perform the maneuver better. Breathing along with the child may be helpful.

The participant is not to take in any additional breaths until the forced expiratory maneuver is finished. The participant should continue to actively push out air as long as possible. The participant should be able to exhale for a least three seconds and should continue to try to exhale until an expiratory plateau has been reached. The technician should continually urge the participant to "squeeze" his/her air out until the appearance of an obvious plateau in the volume-time curve resulting in no change in volume for at least 2 seconds with an exhalation time of at least 6 seconds, or when, for legitimate clinical reasons, the participant cannot or should not continue further exhalation, or the forced exhalation has continued for a reasonable duration (15 seconds in subject with severe airway obstruction).

Post-Bronchodilator Spirometry Testing:

Bronchodilator do not need to be administered if reproducible spirometry maneuvers are not achievable. If the test is normal and the maneuvers are reproducible, a bronchodilator is not necessary. However, if the FEV_1 or FEF_{25-75} values are below normal, but the shape of the flow volume curve demonstrates concavity indicating an increase in airway resistance, a bronchodilator should be used and the test repeated 15 to 20 minutes post-bronchodilator. Bronchodilator will be administered by using five inspiratory capacity breaths of undiluted Albuterol, with a three-second breath hold. The cheek should be held to prevent accumulation of aerosol in the mouth during the inspiration.

5.1.3.3.3 <u>Quality Control</u>

Technicians are a critical part of the pulmonary function testing system, since they must guide the child through a series of unfamiliar and uncomfortable procedures. The forced expiratory maneuver is highly dependent on patient's effort, and the technician must be able to judge the adequacy of the patient's effort. To make the spirometric testing results as accurate and consistent as possible, the testing should be done in a standardized fashion by all technicians, for all participants.

It is not appropriate to use a participant's previously collected data as

a target to shoot for at a subsequent visit. This will bias the results toward an upward slope over time. Each test session must be independent of all others. Spirometry testing will include three slow vital capacity measurements, followed by the forced expiratory maneuver.

Acceptability Criteria:

1. Less that 5% extrapolated volume (calculated from hard copy of volume-time trace)
2. Satisfactory peak effort (PEFR/FEF50>1.25)
3. No cough in first second expiration
4. Satisfactory end of test criteria (Expiratory time > 3 secs. and no volume change for a least two seconds.

Reproducibility Criteria:

1. Less than 5% or 100 ml difference between two largest FVC's
2. Less than 5% or 100 ml difference between two largest FEV_1's

Equipment Checks:

Daily calibration of the screening spirometer with a 3.00 liter syringe is required (emptied at three different rates if using a pneumotach). The measured volumes (FVC) from the three maneuvers should match each other within 3% (for pneumotachs and the mean of the three volumes (corrected to BTPS if appropriate) should be within 3% of 3.00 liters (gain check). Leak checks are required for volume sensing spirometer whenever they are cleaned, whenever tubing is changed or on the day of testing patients.

Leak Check:

Ideally, a leak check should be done on a volume displacement spirometer each time a clean breathing tube is attached. This may not always be possible if a clean hose is being used for each participant. At minimum, the first tube used each day must be checked for leaks after its last cleaning and within the past seven days. The procedure for leak-testing may vary slightly from one volume displacement spirometer to another, but most will be a variation of the following procedure:

> Attach a three liter syringe with plunger withdrawn (as in volume calibration) to the patient hose. Inject three liters of air into the spirometer. Place a weight (minimum 50 gm) on top of the spirometer wedge or bell. Record the spirometer volume reading. Wait one minute. Record spirometer volume. Volume loss of less than 30 ml/min. is acceptable. If an unacceptable leak rate is detected, cause for the leak must be sought in the hose, spirometer or connections. After correction of the suspected leak, an acceptable leak test must be performed.

5.1.4 Aerosolized Tc-99m DTPA Scintigraphy (discontinued February 10, 1993)

5.1.4.1 Equipment

A state of the art gamma camera - computer is in place in each of the participating institutions and will be used for these studies.

5.1.4.2 Method

The patient will be imaged while lying in the supine position over the face of the gamma camera. Using a 30mCi starting dose of Tc-99m DTPA in the nebulizer, have the children breathe through a tightly applied or strapped mask (such as a pediatric anesthesia mask). Crying results in deep breaths and is not a cause for concern. Attempt to obtain at least 50,000 counts per minute inhaled. If unsuccessful after 3 to 4 minutes, refill the nebulizer and try again. If still unsuccessful, strive for 30,000 counts per minute. The washout is then recorded dynamically with continuous 15 second computer frames for 30 minutes (120 frames at 15 sec. per frame); the more rapid the disappearance, the more permeable the epithelial layer. Regions of interest (ROI) for both whole lungs together, each whole lung separately and the peripheral third of each lung are drawn and time-activity curves are generated for each ROI. ROI should be drawn to exclude central deposition in large airways and to exclude single patchy deposition peripherally. However, if there are multiple patchy areas of deposition peripherally, then a representative ROI of the peripheral third of the lung should be drawn. Record the number of counts obtained, and the time in seconds to achieve these counts. To characterize the distribution of the radio aerosol on the initial preclearance image, record as uniform or nonuniform, whether central hyperdeposition is present or absent and whether peripheral penetration is good or poor. Please save the preclearance image and the clearance graph.

5.1.4.3 **Analysis**

Half-time clearances will be calculated for each lung using a single exponential fitted analysis for both whole lungs together, each whole lung separately and for the peripheral third of each lung for the first 8 minutes and the entire 30 minutes of the clearance study; percent change will be calculated from the above half-time clearances. [% change/minute = (0.693/T½) x 100] (Also see item 4, under Section 5.1.4.5).

5.1.4.4 **Training**

The technical skills already exist among the technical personnel to carry out the studies. The only exception may be the skill to apply a face mask to small children and getting them to use it prior to initiating the study, a skill that can be learned individually at each institution.

5.1.4.5 **Quality Control**

To assure uniformity of technique and results in the DTPA aerosol studies, quality control records will be kept in log books at each site and interchangeable PC discs. Records will be kept in four major quality control areas.

1. Tc99m-DTPA radiochemical quality testing: Using 100% acetone solvent and simple paper strip chromatography, the percent free TcO4 in each DTPA preparation will be determined prior to each study and afterward on any residual nebulizer fluid for each study. Using chromatography results as a quantitative basis for free Tc, clearance results will be corrected for any rapidly clearing Tc that might have been present. Corrected and uncorrected DTPA clearance rates will be recorded.

2. Gamma camera performance will be documented by including photos of a corrected Tc-99m flood field from a point source obtained from the camera the day of the study. If an uncorrected field is also available, this should be sent as well, along with the time to collect both flood images.

3. At least once, Camera-computer quantitative performance will be assessed by obtaining a 3 minute digital flood field using the point source method. This flood will be compared visually with the analog flood to look for computer-introduced areas of inhomogeneity. A second 3 minute digital flood also will be collected in twelve increments of 15 seconds each. These floods will be compared visually with the other floods and the total counts from the incremental collection will be added to determine if they match the total counts collected over the single 3 minute collection period.

4. Data analysis - clearance rates will be determined in percent/min and as half-time of clearance. If motion is detected, such that curve analysis is uninterpretable, the ROIs will be repeated by hand for each frame, or the study will be eliminated. A new time-activity curve and a new clearance rate will then be generated from the corrected ROI.

5.1.5 Bronchoscopy and Bronchoalveolar Lavage

5.1.5.1 Equipment

Standard equipment to be used include a pediatric bronchoscope, a light source, a cardiac monitor, and an oximeter.

5.1.5.2 Method

Following informed consent, the sedated patient will be examined under local anesthesia. Oxygen saturation will be maintained with supplemental oxygen as necessary. Bronchoscopy will be performed to evaluate systematically the upper airways, tracheal anatomy, and airway dynamics. Bronchoalveolar lavage (BAL) will be performed following wedging of the bronchoscope into a segmental bronchus of the most involved lobe documented by the radiograph or the right middle lobe bronchus or the lingular bronchus, if the radiograph is diffusely abnormal. Lavage will be performed using aliquots of non-bacteriostatic normal saline kept at room temperature (1-2 ml/kg or a maximum of 10 ml aliquots). The patient will be suctioned until ≥ 30cc of saline is obtained.

The intubated patient will be examined following the above guidelines. Ventilation will continue through a Bodai side arm and the bronchoscope will be inserted through the endotracheal tube to enter the distal airways directly.

Intubated patients whose endotracheal tube is too small to allow use of the flexible bronchoscope (< or - 0) will have an appropriately sized catheter inserted through the endotracheal tube and wedged. Aliquots of sterile nonbacteriostatic saline will be instilled and suctioned in a manner identical to that using the bronchoscope.

All specimens will be treated according to established institutional guidelines for the handling of infectious material.

5.1.5.3 Specimen Handling

The lavage fluid will be treated according to established institutional guidelines for the handling of infectious material. The lavage fluid received in the laboratory will be pooled and then aliquots taken for the required studies.

1. Cell count and differential
   Red blood cell count and white blood cell count and differential should be done by standard methods used in the laboratory.

2. Cytospin preparations
   Cytospins should be made and stained for evaluation of cellular elements, and with one or more of the following to identify bacteria, fungi, pneumocystis, and mycobacteria. The responsible pathologist should ensure that the specimen is handled appropriately, that the cytospins are reviewed for the required elements and that the reporting forms for the P^2C^2 HIV study are completed.
   Stains for cytospin evaluation:
   For cellular elements - Giemsa. Proportion of alveolar macrophages, respiratory epithelial cells and leukocytes.
   For bacteria - Gram.
   For mycobacteria - Fite, Ziehl-Nielsen.
   For yeast and fungi - Methenamine silver, PAS, Gridley, Calcofluor White.
   For pneumocystis - Methenamine silver, Toluidine Blue.

3. Cultures
   Aliquots will be removed for culture for aerobic, anaerobic, fungal, mycobacterial, and viral pathogens, and for Legionella culture.

4. Preservation and Storage of Unused Fluid and Cells
   The remaining lavage fluid should be handled according to one of the following two protocols for preservation and storage of cells and fluid.

A. Simplified Protocol for Storage of BAL Cells and Fluid

Equipment required: -70^0 freezer
liquid nitrogen tank
sterile screw capped freezer vials

Cell medium: RPMI 1640 with 10% fetal calf serum and 10% DMSO. The medium must be made up first, then filtered through a 0.22u filter. (Note: DMSO cannot be filtered undiluted because it destroys the membrane. Failure to filter may result in fungal growth originating from DMSO)

Preparation:

1. The medium must be cold and kept on ice.

2. Centrifuge the lavage fluid to pellet the cells and decant the supernate into sterile screw capped freezer vials. These should be labeled appropriately and stored at -70°.

3. Resuspend the cells in RPMI as prepared above at a concentration of approximately 10-20 x 10^6 cells/ml, place 1.0 ml in each freezer vial and label vials appropriately.

4. Place the vials in the -70° freezer and leave them overnight.

5. The next day place the vials in liquid nitrogen.

B. Alternate Protocol for Freezing Cells

Equipment: Cryomed automated freezer model 1010 with chart drive, microcomputer and freezing chamber
Cryomed tank, model LL450
Cryomed
51529 Birch Street
New Baltimore, MI 48447
(313) 725-4614
Waterbath at 37°C
Freezer vials, screw cap, plastic

Cell medium: RPMI 1640 with 10% fetal calf serum and antibiotics.

Procedure:

1. Centrifuge lavage fluid as above to pellet the cells, decant the supernate into screw cap freezer vials, appropriately label and store in freezer at -70°C.

2. Resuspend the cells in a small amount of the cell medium and count the cells. Reconstitute the cells to a concentration of 20-40 X 10^6 cells in medium and place 0.5 ml in each freezer vial. Appropriately label vials.

3. Add 0.5 ml complete medium as above with 20% DMSO added. The medium must be made up first, then filtered through a 0.22u filter. (Note: DMSO cannot be filtered undiluted because it destroys the membrane. Failure to filter may result in fungal growth originating from DMSO.) The total volume in each vial should be 1.0 ml.

4. Place the vials in the programmed cell freezing chamber.

5. Cycle down to -90°C.

6. Store frozen cells in liquid nitrogen.

C. To Reconstitute Frozen Cells

1. Thaw cells rapidly in a 37°C waterbath.

2. Add 2-3 X volume of complete medium (without DMSO).

3. Centrifuge.

4. Resuspend in 3-4 ml complete medium (without DMSO).

5. Place in 37°C waterbath for 20-30 minutes to allow DMSO to leach out of the cells.

6. Centrifuge cells and wash.

5.1.5.4 Quality Control

The pediatric bronchoscope will be maintained according to the manufacturers specifications. Cold or gas sterilization will follow vigorous cleaning following each procedure. For cold sterilization the fiberoptic bronchoscope should be soaked in glutaraldehyde solution for at least 20 minutes. The light source will be maintained per manufacturer's specifications. Cardiac monitors and oximeters will be maintained and calibrated per manufacturer's specifications.

Each procedure will occur in the appropriate location determined by the individual clinical centers with adequate personnel to monitor the patient following informed consent. Informed consent will be obtained. Patients old

enough to comprehend will be addressed directly to familiarize them with the procedure to improve compliance and minimize fear.

Review of BAL fluid for consistency of diagnosis will be by a panel of three pathologists from the participating institutions. When there is disagreement a consensus diagnosis will be made and data accordingly revised. Standardized methods with appropriate controls will be employed by all centers.

5.1.6 Open Lung Biopsy

All patients who undergo open lung biopsy for any reason will have a BAL the same day so that the direct comparison of the value of these methods may be made and recommendations for their use in the future may be clarified.

5.1.6.1 Method

The pathologist will be notified in advance of a scheduled lung biopsy on a P^2C^2 HIV study patient so that the appropriate reagents for fixation and processing can be made up in advance of the biopsy procedure.

Open lung biopsy will yield a specimen approximately 1 x 1 x 1 cm. The biopsy will be from an area of active disease as judged radiographically and in general should not be obtained from the tip of the lingula or the tip of the right middle lobe.

The biopsy will be divided approximately as follows:

- 50% for culture (viral, mycobacterial, fungal, bacterial).
- 10% frozen (in OCT in liquid nitrogen/2-methyl butane) for marker studies not currently available on fixed tissue.
- 10% fixed in glutaraldehyde for electron microscopy.
- 30% inflation fixed with 4% paraformaldehyde made with RNase free reagents.

Preparation:

1. The medium must be cold and kept on ice.

2. Centrifuge the lavage fluid to pellet the cells and decant the supernate into sterile screw capped freezer vials. These should be labeled appropriately and stored at -70^0.

3. Resuspend the cells in RPMI as prepared above at a concentration of approximately 10-20 x 10^6 cells/ml, place 1.0 ml in each freezer vial and label vials appropriately.

4. Place the vials in the -70^0 freezer and leave them overnight.

5. The next day place the vials in liquid nitrogen.

B. Alternate Protocol for Freezing Cells

Equipment: Cryomed automated freezer model 1010 with chart drive, microcomputer and freezing chamber
Cryomed tank, model LL450
Cryomed
51529 Birch Street
New Baltimore, MI 48447
(313) 725-4614
Waterbath at 37^0C
Freezer vials, screw cap, plastic

Cell medium: RPMI 1640 with 10% fetal calf serum and antibiotics.

Procedure:

1. Centrifuge lavage fluid as above to pellet the cells, decant the supernate into screw cap freezer vials, appropriately label and store in freezer at -70^0C.

2. Resuspend the cells in a small amount of the cell medium and count the cells. Reconstitute the cells to a concentration of 20-40 X 10^6 cells in medium and place 0.5 ml in each freezer vial. Appropriately label vials.

3. Add 0.5 ml complete medium as above with 20% DMSO added. The medium must be made up first, then filtered through a 0.22u filter. (Note: DMSO cannot be filtered undiluted because it destroys the membrane. Failure to filter may result in fungal growth originating from DMSO.) The total volume in each vial should be 1.0 ml.

4. Place the vials in the programmed cell freezing chamber.

5. Cycle down to -90°C.

6. Store frozen cells in liquid nitrogen.

C. To Reconstitute Frozen Cells

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6. Centrifuge cells and wash.

5.1.5.4 Quality Control

The pediatric bronchoscope will be maintained according to the manufacturers specifications. Cold or gas sterilization will follow vigorous cleaning following each procedure. For cold sterilization the fiberoptic bronchoscope should be soaked in glutaraldehyde solution for at least 20 minutes. The light source will be maintained per manufacturer's specifications. Cardiac monitors and oximeters will be maintained and calibrated per manufacturer's specifications.

Each procedure will occur in the appropriate location determined by the individual clinical centers with adequate personnel to monitor the patient following informed consent. Informed consent will be obtained. Patients old

4. Pour off 60% ETOH and pour 80% RNase-free ETOH into container and place on ice for 10 minutes. Repeat.

5. Store specimens in 80% RNase-free ETOH at 4°C prior to processing.

Tissue Processing - Done in a Tissue-Tek VIP:

1. 70% ETOH - 20 minutes at 36°C
2. 95% ETOH - 20 minutes at 36°C.
3. 95% ETOH - 30 minutes at 36°C.
4. 100% ETOH - 20 minutes at 36°C.
5. 100% ETOH - 30 minutes at 36°C.
6. 1/2 100% ETOH and 1/2 chloroform - 30 minutes at 36°C.
7. Chloroform - 30 minutes at 36°C.
8. Chloroform - 30 minutes at 36°C.
9. Paraffin - 20 minutes at 60°C.
10. Paraffin - 20 minutes at 60°C.
11. Paraffin - 20 minutes at 60°C.
12. Embed.

Preparation of Slides

1. Clean slides overnight in 10% Extran.

2. Wash slides for 2 hours in running warm tap water and dry the slides in an oven at 160°C (not very long). Cool to room temperature.

3. Immerse slides in freshly prepared solution of 2% 3-aminopropyl triethoxysilane made up in absolute acetone for one hour.

4. Wash slides two times in acetone and then RNase-free H^2O.

5. Dry slides in an oven at 42°C overnight. Store at room temperature in a slide box.

Notes

1. Only the RNase free parformaldehyde need be made up fresh at regular intervals. All the other reagents using RNase free water in their preparation can be made up in quantity and stored for prolonged periods.

2. Gloves should be worn throughout the preparation of reagents and the handling of specimen bottles and specimens, as RNase is ubiquitous and may be transferred from bare hands to the glassware.

5.1.6.2 Interpretation

The responsible pathologist at each clinical center will ensure that the lung biopsy is reviewed and the appropriate forms completed for the P^2C^2 HIV study.

5.1.6.3 Quality Control

Standard methodology and appropriate controls will be employed for all special stains, in situ hybridization and immunohistochemical procedures.

Review of diagnostic lung biopsies for consistency of diagnosis will be by a panel of three pathologists from the participating institutions. When there is a disagreement, a consensus diagnosis will be made and data accordingly revised.

5.2 Cardiac

5.2.1 Fetal Echocardiography (discontinued December 19. 1992)

5.2.1.1 Equipment

Ultrasound Equipment Requirements:

High resolution ultrasound scanners employing 3.5 and 5.0 mHz frequency transducers with capabilities for pulsed Doppler ultrasound are required. Pulsed Doppler requirements include ability for baseline shift and full spectral output.

Additional carotid/brachial pulse amplifiers with linear responses over the usual pressure range are required to interpolate end-systolic pressure from blood pressure measurement. This measurement is necessary to calculate end-systolic wall stress.

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The responsible pathologist at each clinical center will ensure that the lung biopsy is reviewed and the appropriate forms completed for the P^2C^2 HIV study.

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Standard methodology and appropriate controls will be employed for all special stains, in situ hybridization and immunohistochemical procedures.

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Additional carotid/brachial pulse amplifiers with linear responses over the usual pressure range are required to interpolate end-systolic pressure from blood pressure measurement. This measurement is necessary to calculate end-systolic wall stress.

All studies are recorded on 1/2" standard (or super) VHS recording transcribed and to standard 1/2" VHS videotape for record analysis and quality control. Tape studies are numbered with the examining center's initials, the preassigned fetal code number, and clear reference to starting and ending times recorded.

5.2.1.2 Methods

Fetal ultrasound will be performed with the highest frequency transducer consistent with sound penetration. After preliminary scanning for fetal body parts, biparental diameter, and femur length, the fetal position will be assessed. The transducer will then be placed in a position to obtain major cardiac long-axis, and four-chamber views. From these approaches, the measurement described below can be obtained.

5.2.1.2.1 Fetal Age and Growth Measurement

Fetal age and growth - Menstrual age is reported for comparison with the following measurements to determine fetal age and growth:

1. Femur length (Ott, 1984); and
2. Skull biparietal diameter (Jentry et al, 1984)
   (The conversion tables found in Appendix 14 will be used. The figures from Table 5-5 and 5-6 will be averaged to obtain the estimated gestational age.)

5.2.1.2.2 Fetal Dysmaturity Measurements

An index of umbilical arterial pulsatility, such as peak systolic to diastolic ratio (the A/B ratio), is determined from the umbilical vessels by

placing the sample volume as close to the placental insertion as possible (Abramowicz et al, 1989; Campbell et al, 1987; Schulman et al, 1984; Stuart et al, 1980; Trudinger et al, 1985; Trudinger, Giles and Cook, 1985; Trudinger, 1987). This measurement has been used as an indication of fetal dysmaturity.

5.2.1.2.3 Fetal Cardiac Measurements

1. Ventricular wall thickness and cavity dimensions:

From a 4 chamber view, free walls of the right and left ventricular and septal thicknesses are measured from an end diastolic frame (maximum dimension) at the level just below the annulus of the atrioventricular valves (Shime et al, 1986; Schmidt et al, 1989; Allan et al, 1985; De Vore et al, 1984). Left and right ventricular cavity dimensions are also made at this time. The end systolic cavity dimension (minimum dimension) is measured from the same points as end diastolic dimensions. From these cavity dimensions fractional shortening index (FS) of both the left and right ventricle is calculated from the formula % FS = (EDD-ESD)/EDD where % FS is the percentage of shortening of the ventricle and EDD and ESD are the end diastolic and end systolic dimensions of the respective ventricles (Schmidt et al, 1989). Cavity dimensions and wall thicknesses are compared to established normal standards (Allan et al, 1985; De Vore et al, 1984; Shime et al, 1986). If it is not possible to obtain a 4 chamber view of sufficient quality, dimensions are obtained from a short axis view at the level of the mitral valve leaflets near their annular attachments. Ventricular and septal end diastolic and end systolic wall thickness are measured from this view. Again, using maximum dimensions obtained to

represent the end diastolic frame, slight cranial angulation is required to measure the tricuspid valve annulus diameter. Right ventricular wall dimension is measured just distal to the annulus in this plane. End diastolic and end systolic cavity dimensions of the right ventricular cavity are also measured at this position.

2. Aortic and pulmonary artery dimensions:

Aortic and pulmonary diameters will be measured in systole from several planes including 4 chamber planes with cranial tilt, short axis views and sagittal views of the great vessels. The systolic portion of the cardiac cycle will be used to make these measurements.

3. Cardiac Doppler measurements:

Doppler spectral/velocity data will be recorded with the Doppler reference cursor parallel to axial flow or with on-line angle correction of discrepancy between Doppler reference cursor orientation and axial flow across the 4 cardiac valves. (De Smedt et al, 1987; Meijboom et al, 1985). For the atrioventricular valves, peak early diastolic velocity (E) and peak late atrial velocity (A) will be measured, the E:A ratio, and diastolic velocity time integral determined (Reed et al, 1986; Reed, Sahn et al, 1986; De Smedt et al, 1987; Meijboom et al, 1985). For all valves, the velocity time integral (VTI) and peak velocity will be measured averaging 3 cycles. Heart rate will be calculated from the aortic signal. Combined left and right ventricular outputs may then be calculated from the formula $Q=(\pi/4)(D)^2 \times VTI \times HR$ (Goldberg et al, 1985).

In addition, the flow signal velocity area proximal to the atrioventricular valves is interrogated to determine the presence of mitral and tricuspid regurgitation. Severity is assessed by qualitative grading of the regurgitant jet. The presence or absence of pericardial effusion is noted. Assessment for hydrops fetalis is made (Chinn, 1988).

5.2.1.3 Interpretation

1. Two-Dimensional Echo Methods:

   Measurements in axial resolution will be measured by the leading edge method; the middle echo from the endocardium will be the boundary for non-axial measurements.

2. Doppler Methods:

   Peak velocity measurements will be used to measure E and A velocities. Velocity time integrals will be measured using modal velocity.

5.2.1.4 Training

All centers approved for this proposal have ultrasonographers qualified to perform, examine, and measure fetal echocardiograms. Training specific for this study is not necessary.

5.2.1.5 Quality Control

Studies recorded on videotape will be randomly requested by the Clinical Coordinating Center and measurements reviewed by the cardiologists involved in this project, who will critique the technique and/or measurement.

5.2.2 Postnatal Echocardiography

5.2.2.1 Equipment

A high resolution ultrasound scanner using a 3.5, 5.0 or 7.5 mHz transducer and equipped with pulsed Doppler, 2-D directed M-mode, ECG, pulse and phono channels will be used. Hard copy capability for simultaneous 2-D directed M-mode, phono, pulse, and ECG will be available either in the form of a high quality strip chart recorder or a large format page printer. A high fidelity external pulse transducer (SPR-428, Millar Instruments, Inc.) will be used with an automated blood pressure recorder (Dinamap Vital Signs Monitor 1846 SX, Critikon, Inc.) to obtain the calibrated carotid pulse tracing.

5.2.2.2 Methods

5.2.2.2.1 General 2-D and Doppler Exam

This portion of the exam is designed to detect masses associated with the heart and pericardial effusion, to evaluate valve function and structural defects, and to evaluate qualitatively regional left ventricular function. The 2-D echo and Doppler exams are recorded on 1/2" VHS video cassette tape for subsequent review. The heart is scanned in parasternal long and short axis views and apical 2 and 4 chamber views. Subxyphoid long and short axis views are used where possible. Valve morphology and function is assessed by imaging and pulsed

and/or color Doppler. Doppler interrogation of the atrioventricular valves is performed in apical and parasternal long axis views with attention directed to both the atrial and ventricular aspects of the valve. Semilunar valves are examined in parasternal long and short axis views and apical views, angling anteriorly to include the pulmonary valve where possible. The subxyphoid coronal and short axis views provide an alternative window for the pulmonary valve. Again, attention is directed to both aspects of the valves. Left ventricular regional wall motion is evaluated by scanning in parasternal long and short axis and apical 2 and 4 chamber views. Subxyphoid long and short axis views provide an alternative window. Particular attention will be directed toward obtaining the long axis dimension of the left ventricle with clear visualization of the endocardium.

5.2.2.2.2 Left Ventricular Function Exam

This part of the exam is designed to provide the physiologic data needed to calculate indices of ventricular function and to assess left ventricular loading conditions. The quality of the recording is highly dependent on the level of cooperation of the patient. It is uncommon for children below about 3 years of age to be able to cooperate sufficiently. It is usually necessary to sedate such patients.

High speed (100 mm/sec) stripchart hard copy or large format page printer of 2-D echo directed M-mode recordings of the left ventricular minor axis are obtained simultaneously with recordings of the electrocardiogram, phonocardiogram, indirect carotid or axillary pulse tracing, and peripheral blood pressure. Blood pressure is obtained using a Dinamap 845 Vital Signs Monitor. Indirect pulse tracing is obtained using a Millar pulse transducer (SPR-428, Millar Instruments).

The following should be obtained:

1. Long and short axis views of the left ventricle from apex, left parasternal, and subxyphoid positions (in young patients) for documentation of regional wall motion abnormalities, calculation of LV Mass and volume, and determination of circumferential stress. Technical

note: Display the apical and subxyphoid images as they would be shown in an angiogram, that is, with the base of the heart on top.

2. 2-D measurement of mitral diastolic (AP dimension from long-axis parasternal and lateral dimension from four-chamber apical) and aortic systolic (from long-axis parasternal) annulus sizes. Technical note: Make 2 or 3 measurements during the study rather than on an off-line review station to avoid errors in scale entry and to enable use of the highest quality pictures. Cine loop mode is useful for those machines so equipped.

3. Pulsed doppler above and below the mitral and aortic valve (all from apex) to document any valvar abnormalities as well as permitting calculation of Doppler outputs and Doppler indices of diastolic function. Technical note: Doppler should be recorded at 100 mm/sec paper speed, recording simultaneously on paper and tape. If the echo machine permits recording the simultaneous 2D image in large sector during the paper recording (as the H-P machine does) then this is preferable. The 2-D update trigger should be reduced to at least every 4-5 beats to allow at least 2 sequential beats to be recorded without blank space in the doppler. Mitral sampling for diastolic indices is at the level of the annulus during diastole.

4. Continuous wave doppler just above the aortic valve (from high-right parasternal or suprasternal notch) with simultaneous recording of phonocardiogram, carotid pulse tracing, and automated blood pressure for calculation of left ventricular stroke volume, stroke work, and power. Technical note: As in #3, record on both media at high sweep speed. At least three blood pressure recordings are obtained, with the machine set to obtain recordings one per minute, preferable beginning before the data recording and continuing after.

5. 2-D directed m-mode is obtained from parasternal short axis and recorded

at the minor diameter of the ellipse, obtained simultaneously with electrocardiogram, phonocardiogram, pulse tracing, and automated blood pressure for calculation of left ventricular size, thickness, afterload, preload, contractility, and diastolic function indices. Technical note: As in #3, record on both media at high sweep speed and large 2-D sector size. Phono transducer filter and position is adjusted to optimize recording of aortic valve closure. At least three blood pressure recordings are obtained, preferably beginning before the data recording and continuing after.

5.2.2.2.3 Blood Pressure Measurement

1. Blood pressure (BP) will be obtained simultaneously with wall stress or immediately after wall stress in sedated and non-sedated patients. If the patient moves, wakes up, or changes position, the hemodynamic conditions may change and affect the results of the final analysis.
2. The BP cuff should be applied to the patient at the beginning of the study. Monitor accuracy is dependent on use of proper cuff size. Measure the limb circumference and select the proper cuff.

Cuff Type	Range
Infant	8 - 11 cm
Child	12 - 16 cm
Small Adult	17 - 22 cm
Adult	23 - 33 cm

3. Position the artery mark on the cuff on the patient's artery before inflation.
4. Four BP measurements will be taken simultaneously or immediately following wall stress. The first measurement will be excluded. The average of the remaining three BP measurements will be recorded.

5.2.2.3 Interpretation

Videotape and stripchart or page print recordings and blood pressure data will be sent to a central analysis site (The Children's Hospital, Boston) for analysis of LV size, function, loading conditions and contractility.

5.2.2.4 Training

Personnel from The Children's Hospital, Boston will be available to make up to two trips to each of the study centers to assist in training personnel in the LV function methodology.

5.2.2.5 Quality Control

An experienced physician echocardiographer and pediatric echocardiography technologist will be designated at each Clinical Center and, whenever possible, will be present for studies on protocol patients to ensure that appropriate and medically acceptable patient care procedures and laboratory safety principles are followed.

Each center will record and document postnatal echocardiograms in accordance with its own protocols for such studies. Blood pressure measurement will be made according to Methods Section 5.2.2.2.3. Current individuals designated to monitor quality assurance will continue at their own institutions, to accomplish the following:

1. Ensure examinations are performed in a complete and uniform manner;
2. Facilitate the acquisition and recording of data needed for this particular contract protocol and obtained from enrolled patients; and,
3. Provide ongoing "on-site" training for all local personnel involved in the performance of these studies to assure interobserver reproducibility (especially in view of the relatively long course of this protocol).

5.2.2.5.1 Maintenance and Calibration of Dinamapp BP Monitors

The Dinamapp Vital Sign Monitors will be maintained according to instructions provided in the service manual. A calibration check will be performed at six month intervals (see Appendix 17).

5.2.3 Electrocardiogram

5.2.3.1 Equipment

A standard electrocardiogram (at least 12 leads) will be performed using an electrocardiograph that employs analog to digital conversion (e.g., Marquette, Hewlett-Packard).

5.2.3.2 Method

Temporal and voltage indices of rate, chamber size, QRS vector, T wave configuration, and rhythm will be obtained by conventional techniques.

5.2.3.3 Interpretation

The maximum duration of any pause will be recorded. ST depression is measured at 40 milliseconds after J Point. The following guidelines will be used when documenting sinus tachycardia, sinus bradycardia, atrial enlargement and hypertrophy:

Sinus tachycardia – Rate which exceeds the 98th percentile of the Davignon Tables (See Appendix 10 and 15)

Sinus bradycardia – Any rate below the 2nd percentile of the Davignon Tables (See Appendix 10 and 15)

Atrial enlargement – Right: P wave in any lead > 2.5 mm
Left: P terminal forces in lead V_1 > 1 mm

Hypertrophy –

Right:
a) qR in V_{4R}, V_{3R} or V_1
b) Positive T wave in V_1 over the age of 1 week
c) R wave in V_1 > 98th percentile for age in absence of a biphasic QRS complex (RS ratio > mean for age)
d) S wave in V_6 (SV_6) > 98th percentile for age in absence of a biphasic QRS complex

Left:
a) R V_6 > 98th percentile for age in absence of a biphasic QRS complex
b) S V_1 > 98th percentile for age in absence of a biphasic QRS complex

Biventricular hypertrophy:
a) Independent criteria for right and left ventricular hypertrophy
b) Any biphasic QRS complex > 60 mm (i.e. R+S)

5.2.3.4 Training

The investigator at each center will be responsible for the technical training at their center.

5.2.3.5 Quality Control

During the yearly audit of each center, 10% of the ECG's will be examined to be sure that the tracing is free of motion artifact for at least 10 seconds and that the measurements of the amplitude agree within 100 microvolts.

5.2.4 Holter

5.2.4.1 Equipment

A standard Holter recorder capable of recording two simultaneous leads will be used. These leads should include a lead II equivalent or MCL5, and an anterior chest lead such as MCL1.

5.2.4.2 Method

At least 18 hours of interpretable data should be recorded. Holter studies less than 18 hours are unacceptable, and the study should be repeated.

5.2.4.3 Interpretation

The rhythm will be interpreted by standard criteria (Garson, 1983). The frequency of premature beats is defined as follows: infrequent (less than 10 per day), moderate (10 per day/one per hour), and frequent (greater than 1 per hour). For any episodes of tachycardia the number of episodes, and the rate, and the duration of the longest episode will be recorded. The following criteria will be used to define sinus tachycardia: 0 - 1 year of age > 230/minute; over 1 year of age > 190/minute *(rate averaged over 6 seconds)*

5.2.4.4 Training

The investigator at each center will be responsible for the technical training at their center.

5.2.4.5 Quality Control

Holter reports will be reviewed in each center. Any abnormalities in the numeric report (e.g., number of premature beats) must be reviewed and the number of beats verified by a physician. The high heart rate, low heart rate and any abnormalities of conduction of rhythm must be documented with electrocardiographic tracings. Any Holter report with a disturbance of conduction or rhythm (other than sinus tachycardia or sinus bradycardia) will be sent to the Clinical Coordinating Center for review.

5.2.5 Cardiac Catheterization and Endomyocardial Biopsy

5.2.5.1 Method

The pathologist will be notified in advance of any scheduled endomyocardial biopsy on a P^2C^2 HIV study patient so that the appropriate reagents for fixation and processing can be made up in advance of the biopsy procedure.

Analysis of endomyocardial biopsy samples will be performed in each clinical center. It is anticipated that the number of fragments obtained at biopsy will be no fewer than four and will in general be six or more. Biopsy samples of less than three fragments will be considered inadequate for evaluation. For three fragments, all will be fixed in Rnase free paraformaldehyde and processed for light microscopy according to the protocol for preservation of RNA in human tissue (see 5.1.6.1.1 Procedure for Preservation of RNA in Human Tissues). A fourth fragment will be frozen in OCT in 2-methylbutane in liquid nitrogen and stored at -70°C or lower. A fifth fragment will be placed in glutaraldehyde for processing into resin blocks. A sixth will be frozen. A seventh will be added to the fragments fixed for light microscopy. An eighth will be added to the frozen fragments.

The portions fixed for light microscopy will be processed according to the protocol for RNA preservation. Histologic sections will be cut and stained with hematoxylin and eosin (at

least 8 sections on at least 2 slides); where appropriate special stains for structural elements (trichrome, Movat, VVG, etc) and organisms will be done. Electron microscopy and frozen material will be utilized for appropriate diagnostic studies. If they are not used, the glutaraldehyde fixed tissue will be processed into resin blocks and stored, and the frozen tissue will be retained at -70°C.

The responsible pathologist will ensure that the biopsy specimen is evaluated for histologic abnormalities and the appropriate forms for the P^2C^2 HIV study are completed.

5.2.5.2 Interpretation

Cardiac pressure data saturation measurement, oximetry and cineangiograms will be interpreted by all the cardiologists involved in this proposal. All are capable of and qualified to record and interpret data from the cardiac catheterization.

5.2.5.3 Quality Control

Review of biopsies for consistency of diagnosis will be by a panel of three pathologists from the participating institutions. When there is a disagreement, a consensus diagnosis will be made and data accordingly revised. Standardized methods with appropria controls will be employed by all centers.

5.3 Virologic and Immunologic

5.3.1 ELISA

The enzyme-linked immunoabsorbant assay (ELISA) for serum antibodies is a standardized screening test for detecting present or past infection with HIV. It is a test that is overly sensitive in that it tends to overpredict the number of positive subjects and must be confirmed by the more specific Western Blot test described in Section 5.3.2. A limited number of commercial testing kits have been approved by the Food and Drug Administration (FDA) and Center for Disease Control (CDC) for the screening of individuals.

5.3.1.1 Method

The method employs the standard equipment for any ELISA determinations. Essentially, serum is added to microwells in a plastic dish or plate which has been coated with HIV antigens. After reaction and washing reagents containing a colored dye are added to the wells, the resulting solutions are read at a certain wave length in a special spectrophotometer which is computerized.

5.3.1.2 Interpretation

The test is read as positive when a certain optical density is recorded (see Appendix 4). When a serum is determined to be positive, the test must be repeated before the test result is returned as positive to the physician of record.

5.3.1.3 Quality Control

Each ELISA testing laboratory must be certified by the College of American Pathologists, the American Association of Blood Banks, or the Joint Council of Accreditation of Hospitals and are inspected periodically by the FDA. Appropriate positives and negatives must be included in each test determination. Appendix 4 contains supplementary information on one testing system used by The Methodist Hospital in Houston where mandatory HIV screening is performed on all patients upon admission.

5.3.2 Western Blot

Interpretation and use of the Western Blot Assay for Serodiagnosis of Human Immunodeficiency Virus Type 1 Infections (Center for Disease Control, 1989)

5.3.2.1 Method

The P^2C^2 HIV study has identified the Western Blot test (DuPont) as the method for serodiagnosis of Human Immunodeficiency Virus Type 1 (HIV-1) infections. This test is the only commercial test to be licensed by the Food and Drug Administration.

The Western Blot assay is a method in which individual proteins of an HIV-1 lysate are separated according to size by polyacrylamide gel electrophoresis. The viral proteins are then transferred onto nitrocellulose paper and reacted with the patient's serum. Any antibody from the patient's serum is detected by an antihuman immunoglobulin G (IgG) antibody conjugated with an enzyme that in the presence of substrate will produce a colored band. Positive and negative control specimens are run simultaneously to allow identification of viral proteins.

5.3.2.2 Interpretation

Clinical center laboratories should report test results as positive, intermediate or negative. These should be in accordance with the currently licensed Dupont Western Blot test as follows:

Positive: when detected bands include p24 and p31, and gp 41 or gp 120/160.

Negative: *absence of any and all bands, not just viral bands.

Indeterminate: presence of any other band(s) that fail to meet the positive criteria.

*This interpretation is essential because some observed bands may reflect the presence of antibodies to HIV regulatory proteins or may indicate partially

processed or degraded viral structural proteins.

5.3.2.3 Quality Control

All tests for HIV testing (ELISA and Western Blot) will be performed using FDA-certified testing kits and in testing laboratories certified by the American Board of Pathology (ABP) and College of American Pathologists (CAP). Quality control will be exercised by regular testing of unknown sera in accordance with standard ABP and CAP practice and in the routine use of both positive and negative controls in every testing panel (see Appendix 5 for example of Quality Control.)

5.3.3 Serum Ig's

Serum Ig's will be measured in clinical pathology laboratories affiliated with the five clinical centers or in designated reference laboratories approved by the centers.

5.3.3.1 Method

The methods of measuring serum Ig's (IgG, IgA, IgM) will depend upon the testing laboratory. In general, there are two methods: kinetic nephelometry (e.g., Beckman Immunochemical System at Texas Children's Hospital) or standard single radial immunodiffusion.

5.3.3.2 Interpretation

In terms of kinetic nephelometry, the principle of measurement is the light scattering produced by protein molecules. After suitable calibration the

machine readout is in terms of mg/dl. Age-matched control samples of Ig concentrations will be used to assess whether patients are hypogammaglobulinemic or hypergammaglobulinemic. Similarly with single radial immunodiffusion techniques, which measure precipitin lines at given distances from the origin of the sample, age-matched control values will be used.

5.3.3.3 Quality Control

Each testing system has a standard number of controls of known Ig concentration to ensure accurate readings. In this manner the testing laboratory will be able to determine whether their procedures are producing acceptable data. In addition, each laboratory already has in place an organized system of quality control checks, which includes the periodic testing of unknown samples. Part of the continuing re-accreditation of the testing laboratories by the College of American Pathologists is the regular review of laboratory data by external reviewers. This mechanism will provide the final measure of quality control of the laboratory which measures serum Ig levels.

5.3.4 HIV Culture

HIV culture is the definitive test for the presence of HIV. Patient peripheral blood, cerebral spinal fluid, lymph nodes, or other body secretions or biopsies can be examined for presence of the virus. Although new technologies may supplant HIV culture, at the moment, it is the gold standard.

5.3.4.1 Method

The methods of culturing HIV and performing the readout of HIV cultures (p24 Ag) will be those of the ACTG Virology Committee. Each clinical center involved in this study has an ACTG unit and utilizes the accredited laboratories for HIV cultures. The precise methods are contained in the ACTG Virology Manual, Version 6, Nov. 6, 1989 (see Appendix 6).

5.3.4.2 Interpretation

HIV cultures will be assessed for positive or negative status according to the HIV p24 Ag level of HIV culture supernatants. The criteria are contained in the ACTG Virology Manual, Version 6, Nov. 6, 1989 (see Appendix 6).

5.3.4.3 Quality Control

F. Blaine Hollinger, M.D., Baylor College of Medicine is in charge of certification of laboratories performing HIV cultures and p24 Ag determinations for the ACTG. The criteria for accreditation and quality control are contained in the ACTG Virology Manual, Version 6, Nov. 6, 1989 (see Appendix 6).

5.3.5 CD3, CD4, CD8

The principal T cell subsets are comprised of total T cells (CD3), helper T cells (CD4), and suppressor T cells (CD8). The CD4 lymphocyte count is being used as a surrogate marker for advancing HIV infection and will be measured for the pediatric patients enrolled in the NHLBI contract. In addition CD19 (B4) and CD20 (B1) subsets of B lymphocytes will be measured.

5.3.5.1 Method

The methods of analyzing patients peripheral blood white cells for CD3, CD4, and CD8 will be those prescribed for the immunology testing laboratories of the ACTG. The precise methods are contained in the Flow Cytometry Report, ACTG immunology Committee, Oct. 6, 1988 (see Appendix 7).

5.3.5.2 Interpretation

The interpretation of the data obtained by flow cytometry is contained in the Flow Cytometry Report, ACTG Immunology Committee, Oct. 6, 1988 (see Appendix 7).

5.3.5.3 Quality Control

Extensive quality control testing system is already in place for the immunology laboratories affiliated with the ACTG. FAST Systems, Inc., 211 Perry Parkway, Gaithersburg, MD, 20877, (301) 977-0536 has been contracted to send unknown specimens to immunology laboratories and grade their performance. A sample monthly report is enclosed (see Appendix 8).

5.3.6 Delayed Hypersensitivity Skin Tests (Including Purified Protein Derivative Skin Test)

The delayed hypersensitivity-type skin reaction is important for the evaluation of cellular immune responsiveness in vivo. Delayed-type hypersensitivity is manifested in the skin as an immunologically specific inflammatory reaction that requires 24 to 48 hours to reach maximal intensity.

5.3.6.1 Method

The classic means of acquiring delayed-type hypersensitivity is by infection, and the classic infection is with Mycobacterium or other obligate or facultative intracellular parasitic microorganisms, such as viruses or fungi. This assay will be performed by injecting 0.1 ml of antigen solution (e.g., purified protein derivative of the tubercle bacillus) intradermally. Then erythema and induration appear and increase progressively; after reaching maximum intensity and size, the response gradually subsides over several days. The results are recorded at either 48 or 72 hours in terms of millimeters of induration and erythema. A positive reaction is one in which more than 5 mm of induration is observed at the test site 24 or 48 hours after injection of antigen (except for PPD where the induration must be 10 mm to be considered positive). DHST is performed on study patients at 1 year of age and older, only.

Table 1

Recall Antigens for Delayed Skin Testing

Antigen	Trade Name	Intermediate	Secondary	Source
Candida	Dermatophytin 0	1:100	1:10	Hollister-Stier Laboratories, Spokane, WA
PPD	PPD (stabilized solution)	1 μg/ml	50 μg/ml	Connaught Medical Research Lab Toronto, CN
Tetanus toxoid	Tetanus toxoid	1:100	...	Wyeth Labs, Philadelphia, PA

*PPD indicates purified protein derivative.

5.3.6.2 Interpretation

Most investigators use a panel of five or six "recall" antigens to evaluate the cellular immunocompetence of a subject. The most common antigens used are Candida, coccidioidin, mumps, Trichophyton, and purified protein derivative of tuberculin. More than 90% of the normal population will show a positive response to two or more of these "recall" antigens. (Infants and young children less than 2 years of age are an exception to this rule). Each clinical center will have its own set of antigens but the minimum number to be used by every center will include Candida, PPD, and tetanus. The reading of the skin tests will be performed by medical personnel at either 48 or 72 hours.

5.3.6.3 Quality Control

Each clinical center must enforce its own quality control procedures. The Quality Control committee of this project will meet to compare and standardize testing procedures and quality control checks of each center.

5.3.7 Cytomegalovirus Urine Culture/Serology

5.3.7.1 Method

Urine specimen: can be stored at 4° C for up to 1 week; do not freeze!

5.3.7.1.1 CMV Urine Culture Procedure for isolation of CMV from fresh Urine:

1. Collect 3 ml of voided urine. Keep cold on wet ice during transport, or keep refrigerated at 4° C until transport; do not freeze.

2. Add 3 ml of urine to 0.5 ml of antibiotic solution.
   Antibiotic Solution: 1 ml Gentamicin Sulfate; 2 ml Amphotericin B (0.2 mg/ml); 47 ml 1X HBSS
   Centrigue at 2800 rpm x 10 min.

3. Label 2 tubes of human foreskin fibroblast cells and 1 tube of HEp2 cells, remove all but 0.5 ml of media, then inoculate 0.2 ml of treated sample into each tube.

4. Absorb x 2 hr., then refeed tubes with 1.5 ml maintenance media.

5. Place tubes on a rollor drum and examine daily for cytopathic effect; maintenance media is changed once or twice weekly; passage is performed if toxicity develops; cultures are held 30 days.

6. CMV is identified by its characteristic cytopathic effect on fibroblast cell line and failure to grow HEp2 cell line.

5.3.7.1.2 CMV Urine Culture Storage Procedure for isolation of CMV from frozen Urine:

1. Add 3 ml of urine to 0.5 ml of antibiotic solution (as described in section 5.3.7.1.1) to decrease chance of bacterial or fungal contamination in specimen.

2. Add equal volume of glycerine 50% to pretreated urine specimen. Do not fill vial all the way to the top. Allow space in the vial for frozen liquid to expand.
   Glycerine 50%: 100 ml glycerine; 100 ml sd H_2O; Mix, Sterilize in autoclave; Allow to cool; Store 4°

3. Freeze specimen at -70°C to -90°C. Specimens also may be preserved at -190° in liquid nitrogen.

4. To reclaim virus from original specimen, thaw quickly in 37° water bath.

5. Once specimen is thawed, inoculate 0.2 ml into each of 2 fibroblast cell culture tubes. A HEp2 cell culture tube also may be inoculated at the same time. Follow instructions in section 5.3.7.1.1. Glycerine can be toxic to cell culture

monolayers, so observe closely and wash, refeed, or passage when necessary.

5.3.7.1.3 CMV Serology

Method for Serology for CMV: The serologic tests for CMV will be performed with an ELISA assay and optical density measurement will be established for negative, borderline, low, medium, and high antibody values. It is also acceptable to do antibody titers in lieu of the ELISA.

5.3.7.2 Reagents and Supplies for CMV Cultures

1. Cell Culture: Human Fetal Lung Fibroblasts Change in tubes (Whittaker M.A. Bioproducts, cat. #72-218D)
2. Medium:
   M.E.M. (Flow MEM Eagle modified w/Earle's salts, without glutamine, cat. #12-162-49) - 100 ml
   Glutamine 100x stock - 1 ml (add proportional amount q3 days)
   FCS, HI - 2 ml
   5% $NaHCO_3$ - 1 ml
   Pen/Strep. stock - 1 ml
   Amphotericin B stock - 0.5 ml
   Store at 4 C°
3. Stock Solutions (check sterility of all stock solutions):
   Glutamine 100x:
   L-Glutamine (Schwartz/Mann, cat. #817023) - 30 grams
   Sterile H_2O - 1 liter
   Filter (0.2 μm), store at -20 C° in 1 ml aliquots
   FCS, HI (heat activated): Fetal Calf Serum (Flow "Collect Silver Fetal Bovine Serum", cat. #29-161-49) - 100 ml
   Heat inactivate at 56 C° for 30 minutes
   Label as HI, store at 4 C°
   Check new lots for tissue culture toxicity
   Pen/Strep Stock:
   Penicillin G Na, cell culture tested (Sigma P 3032) - 10 million units
   Streptomycin sulfate, cell culture tested (Sigma S 9137) - 5 grams
   Sterile H_2O - 250 ml
   Store at -20 C° in 1 ml aliquots
   Amphotericin B Stock:
   Amphotericin B, tissue culture grade (Squibb 43760) - 50 mg
   Sterile H_2O - 20 ml
   Dissolve thoroughly, store at -20 C° in 0.5 ml aliquots
   5%$NaHCO_3$:
   $NaHCO_3$ - 50 grams
   Sterile H_2O - 1 liter
   Autoclave, store at room temperature.
   Sterile H_2O (for stock solutions) aliquot in 500 ml bottles, autoclave and store at room temperature.
4. Sterile pipets (1 ml)

5.3.7.3 Interpretation

5.3.7.3.1 CMV Culture

Culture is positive with typical CPE.

5.3.7.3.2 CMV Serology

The optical density of the ELISA plate will be measured and recorded by a digitalized and automated spectrophotometer – scanner (microplate reader). The following is an example of the readout of the test used at Texas Children's Hospital:

CMV-IgG

Negative: ≤ 0.99 – No evidence of past or present infection.

Positive: ≥ 1.00 – Indicates past or present CMV infection. CMV-IgM antibody identification may be helpful in determining a recent or current infection.

CMV-IgM

Negative: ≤ 0.29 – Negative for CMV-specific IgM.

Equivocal: 0.30 -0.59 May indicate a recent past infection or cross-reaction of antibody. Requires correlation within the patient's clinical context.

Positive: ≥ 0.60 – Positive for CMV-specific IgM. Indicates a recent infection 6 weeks to 6 months ago.

Antibody Results	Interpretation
CMV-IgG Positive and CMV-IgM Positive	3 = positive, active
CMV-IgG Positive and CMV-IgM Negative	2 = positive, past
CMV-IgG Positive and CMV-IgM Equivocal	1 = positive, NOS
CMV-IgG Negative and CMV-IgM Negative	0 = negative
CMV-IgG Negative and CMV-IgM Positive	4 = unsatisfactory
CMV-IgG Negative and CMV-IgM Equivocal	4 = unsatisfactory

5.3.7.4 Quality Control

An inter-laboratory survey will be conducted bi-annually to assure the comparability and uniformity of CMV antibody testing and CMV culture. The survey will be conducted by Gail Demmler, M.D., Director, Diagnostic Virology Laboratory, Texas Children's Hospital. Survey results will be reported to the Immunology/Infectious Disease Subcommittee.

5.3.7.4.1 CMV Culture

Coded samples will be distributed to each laboratory performing CMV urine culture. The urine samples will contain known quantities of live virus that have been added to a fresh urine specimen. The laboratories will process the samples as they would any other urine samples received in the laboratory. A results form and methods sheet will be completed and returned to Dr. Demmler within 28 days.

5.3.7.4.2 CMV Serology

Coded samples will be distributed to each laboratory performing CMV antibody testing. The serum samples will be actual patient samples that may or may not contain CMV-specific IgG and IgM antibodies. The laboratories will process the samples as they would any other serum samples received in the laboratory. A results form and methods sheet will be completed and returned to Dr. Demmler within 28 days.

5.3.8 Epstein-Barr Virus Culture/Serology

5.3.8.1 Method

Each institution should send the stored sera and the oropharyngeal secretion specimens by overnight carrier on dry ice, once a month, to reach the University of Texas Health Science Center, Department of Pediatrics laboratory Tuesday through Thursday. Specimens are to be sent to:

Ciro V. Sumaya, M.D.
University of Texas Health Science Center
Department of Pediatrics
7703 Floyd Curl Drive
San Antonio, Texas 78284-7811

The sender will inform Ms. Yasmin Ench (Laboratory Technician) or Cindy Buecker (Administrative Assistant), at 512-567-5250, when the specimens are mailed.

5.3.8.1.1 EBV Culture

The subject should have the following specimens obtained for EBV studies: oropharyngeal secretions obtained via a throat washing (preferably) or a throat swabbing (if unable to gargle) plus a blood specimen.

For throat swabs, 4 ml of transport medium* (* RPMI 1640 with 20% fetal bovine serum, 50 mcg/ml gentamicin and 5 mcg/ml fungizone) should be pipetted into a sterile plastic vial. The patient's oropharynx should be swabbed with 2 cotton swabs and the swabs should be broken off and left in the transport medium.

For throat washing, a kit will be provided from Dr. Jenson's laboratory containing the following items: 10X RPMI 1640 in 0.8 ml aliquot in a 15 ml polypropylene tube (store in refrigerator); fetal bovine serum (FBS) with antibiotics in 2 ml aliquot (store at -20° C). Thaw out FBS prior to use. One vial of each is required for each washing.

The patient should be asked to gargle with 7.2 ml of sterile water from a sterile specimen cup. The patient should be instructed to tip his/her head back and gargle with the mouth open for a few (3-4) seconds. The throat washing is then to be expectorated back into the specimen cup.

The gargle from the specimen cup is poured into the tube containing 10X RPMI medium. The contents of 1 vial thawed FBS is poured into the tube containing the RPMI and gargle. (The outside of the FBS vial is to be wiped clean before pouring into the RPMI tube.) The contents of the tube are mixed. Label the tube with the patient's ID number and the collection date.

The throat swabbing or throat washing specimen must immediately be placed in a -70°C freezer.

5.3.8.1.2 EBV Serology

Blood should be collected in a red top tube and allowed to clot at room temperature. Serum should be separated and stored in sterile vials at -20°C, preferably in a non frost-free freezer. The minimum volume of serum required for the EBV serological profile is 0.4 ml.

Routinely, 3 ml of blood should be drawn from older children, and at least 1.5 ml from infants. Serum should be transported on dry ice.

5.3.8.2 Interpretation

5.3.8.2.1 EBV Culture

Cultures are positive if there are signs of transformation, i.e., cell proliferation and resultant clumping. Cultures are usually positive at four weeks. Concomitant negative and positive controls should be run.

5.3.8.2.2 EBV Serology

Concomitant negative and positive controls should be run. Each commercial kit has established dilutions for positive and negative reactions for the immunofluorescence measurements (see Appendix 9), or established optical density readings in the case of the ELISA method.

5.3.8.3 Equipment for EBV Culture

EBV cultures require a centrifuge, laminar flow hood, water jacketed CO2 incubator, and inverted microscope. EBV cultures requires the same equipment as HIV tests.

5.3.8.4 Quality Control

5.3.8.4.1 EBV Culture

Each institution must enforce their own quality control program for EBV virus culture or have this culture done in a reference laboratory that meets institutional quality control criteria. Appropriate positive and negative control cultures are performed periodically to document accuracy of these laboratories. The Quality Control Committee of this project will meet to compare and standardize testing procedures and quality control checks of each clinical center.

5.3.8.4.2 EBV Serology

Each institution must enforce their own quality control program for EBV serology or have this serology done in a laboratory that meets institutional quality control. The Quality Control Committee will review these procedures.

5.3.9 Complete Blood Count

The Complete Blood Count (CBC) will be comprised of a total count of white blood cells (WBC), the differential count of the WBC, the hemoglobin, the hematocrit, the red blood cell (RBC) indices, and the platelet count.

5.3.9.1 Method

Standard techniques will be used to perform the CBC. Each clinical center has a CAP-approved laboratory which performs the CBC test.

5.3.9.2 Interpretation

Each CBC will be performed using automated technology, e.g. Coulter Counter. This will provide printed readouts of pertinent values. In special cases where machine readouts are not possible or suspected of being in error, a clinical pathologist/hematologist will be consulted for interpretation.

5.3.9.3 Quality Control

Each clinical center must enforce its own quality control procedures. The Quality Control committee of this project will meet to compare and standardize testing procedures and quality control checks of each center.

5.3.10 Erythrocyte Sedimentation Rate

Measurement of the increased rate of settling of erythrocytes is an important laboratory test in evaluation of disease activity in patients with connective-tissue diseases, certain infections, and neoplastic diseases.

5.3.10.1 Method

The most dependable and accurate method for measuring the erythrocyte sedimentation rate (ESR) is the Westergren technique. This simple, nonspecific test measures the rate of settling of RBCs in anticoagulated whole blood.

5.3.10.2 Interpretation

The ESR is increased in many illnesses, primarily because of increases in plasma protein such as fibrinogen or macroglobulins. The normal values are 0 to 9 mm/h for men, 0 to 20 mm/h for women, 0 to 6 mm/h for newborns, 0 to 20 mm/h for children. Elevated ESR is seen in infection, inflammatory conditions, cancer, lymphoma, multiple myeloma, and pregnancy (third month to one month postpartum). It is a useful measure to follow the course of previously diagnosed specific illnesses. It may be "falsely low" in conditions in which RBCs do not undergo rouleau formation, i.e., sickle cell anemia and hereditary spherocytosis.

5.3.10.3 Quality Control

Each clinical center must enforce its own quality control procedures.

5.3.11 Lactic Dehydrogenase

Elevated serum lactic dehydrogenase (LDH) levels have been shown to correlate with pulmonary infection due to Pneumocystis Carinii in children as well as adults with HIV infection.

5.3.11.1 Method

Total serum LDH activity (expressed in international units [IU] per liter) will be measured by standard techniques.

5.3.11.2 Interpretation

The following values, (Texas Children's Hospital) or a similar set of values at other centers, will be used to define the normal range.

Age	IU/L
Newborn	210-1240
1-30 days	115-490
1 mo-2 yr	85-365
2-12 yr	53-240
>12 yr	24-120

5.3.11.3 Quality Control

Each clinical center must enforce its own quality control procedures, etc.

5.3.12 Serum

Serum will be obtained at times of intercurrent illness, stored, and saved for later use, such as antibody titers to an infectious organism. It may be advantageous for centers to store serum specimens at alternate times for ancillary studies in the future. Any surplus serum should be stored according to the method listed below with a maximum of 2 ml (4 vials) stored.

5.3.12.1 Method

Universal blood precautions should be used in collecting, clotting, centrifuging and storing serum specimens. Three ml of blood will yield 1 ml of serum from infants.

1. Spin down sample to be saved at 2500 rpm.
2. Aliquot 0.5 ml serum samples into NUNC vials (smallest size).

3. Label vial directly and enter in log sheet.

4. The serum specimens will be stored at -70° C.

Notes: NUNC vials are the best choice; these are screw-cap vials with a rubber gasket so that there is no problem with spillage or desiccation of the specimen. They also have a white area for direct labeling so that the problem of loss of the label in the freezer is not an issue. The vials come in both a flat bottom and a round bottom version. They are relatively expensive; there may be less expensive versions available. The less good choice are the snap top plastic vials. These have all the disadvantages the NUNC vial overcomes. The tops may come open in the freezer permitting spillage or desiccation of the specimen, and these vials are difficult to directly label, so the label may be lost in the freezer as well.

Stored quantities smaller than 0.25 ml are not likely to be useful. There are problems with artificial concentration when small quantities are frozen. There is also likely to be loss of volume when small quantities are stored in larger volume containers due to wetting of the container making retrieval of the stored volume difficult. Any later testing done on stored samples is likely to require at least 0.2 ml, as duplicate determinations should be made even for tests requiring only small volumes.

5.3.12.2 Quality Control

To preserve the usefulness of the serum specimens all freezers will be hooked up to emergency power in case of a power failure.

5.3.13 Polymerase Chain Reaction (PCR) Blood Storage (discontinued 3/15/93)

To determine the timing of HIV infection in Group II children, blood specimens will be obtained within 48 hours of birth (represents in utero infection if positive) and at 3 months (represents perinatal infection if positive while <48 hour sample was negative). Only infant blood can be tested; cord blood may not be used for fear of maternal blood contamination. The filter paper dots of blood will be collected and saved at -20° C for future PCR testing.

5.3.13.1 Method

Serum will be stored for PCR testing with two filter paper dots. This is done by placing 50ul of EDTA whole blood (purple-top tube) on two separate squares of filter paper. (Heparinized whole blood may be used for the samples. This is not, however, the preferred anticoagulant since heparin may inhibit PCR detection. Blood with no anticoagulant should not be used). After air drying, each paper is placed in a separate "zip-lock" bag with desiccant (packet of silica gel) and stored at -20° C. Each bag must be labelled with the ID number, date of specimen and type of anticoagulant (EDTA or heparin). Note that grocery store zip-lock bags are not acceptable; the bags must be gas-impermeable. Do not seal labels or forms in the bag without sufficient desiccant, since this is deleterious to the samples.

Specimens will remain stored at -20° C for future testing. The methodology for PCR testing is yet to be determined.

Notes: Suppliers of materials for PCR testing

3x4 inch filter paper, S&S #903 Catalog #15440
Schleicher and Schuell, Keane, NH 1-800-245-4024

Bitran Saranex Series S bags, 6x6 inch Catalog #B1210-12
Baxter Scientific

Silica gel packets Catalog #S 8394
Sigma Chemical

5.3.13.2 Quality Control

To be completed on determination of methodology for PCR testing.

5.4 Postmortem Examination

Whenever permission for postmortem examination is obtained, the examination will be done in a timely fashion and in as complete a manner as the obtained consent permits. In addition to the standard autopsy examination and whatever special studies are appropriate to the individual case, study patients will have a number of cultures taken and tissue samples retained according to the following standard protocol.

A. Cultures
1. Bacterial - lung, myocardium, and blood.
2. Mycobacterial - lung, myocardium, and blood.
3. Fungal - lung, myocardium, and blood.
4. Viral - lung, myocardium, blood, spleen, and nasopharyngeal swab.
5. Pericardial and pleural effusions, if present, will also be obtained for bacterial, fungal and viral culture.

Tissue rather than swabs will be submitted for culture unless swabs are specified. If blood cannot be obtained for the above studies, a portion of spleen will be submitted instead.

B. Frozen tissue
Sections of the following tissues will be obtained, frozen in OCT in 2-methylbutane in liquid nitrogen and stored frozen at -70°C for possible ancillary studies:
1. Myocardium - 2 samples, to parallel sections RVI and LVI obtained for light microscopy (see Cardiovascular Examination, Light Microscopy below for details). These will include the right and left circumflex coronary arteries.
2. Coronary artery - one sample, midportion of the LAD (see Cardiovascular Examination, Light Microscopy below for details).
3. Mesentery (for small systemic vessels)
4. Lung
5. Thymus
6. Spleen
7. Renal cortex and medulla
8. Cerebellum with meninges
9. Frontal cortex with meninges
10. Skeletal muscle, quadriceps preferred
11. Mid-portion middle cerebral artery (see Cardiovascular Examination, Light Microscopy - Systemic Vessels for details)
12. Cells and fluid from pericardial and pleural effusions, when present, will be stored in a manner similar to those obtained at BAL (see 5.1.5.3 Protocol for Storage of BAL Cells and Fluid)

C. Tissue for fixation in RNase-free 4% paraformaldehyde
Sections of the following tissues will be obtained and fixed in RNase-free paraformaldehyde according to the protocol for the preservation of RNA in human tissues (see 5.1.6.1 Procedure for the Preservation of RNA in Human Tissues):

1. Myocardium (2 samples, RVI and LVI, as described in Cardiovascular Examination, Light Microscopy below)
2. Coronary Artery (1 sample, LAD, as described in Cardiovascular Examination, Light Microscopy below).
3. Lung (2 samples, one from each lung, inflation fix via tuberculin syringe, see 5.1.6.1 for details)
4. Systemic Arteries (4 samples, right and left renal arteries, mesentery, and carotid artery, as described in Cardiovascular Examination below).
5. Kidney - one sample of cortex and medulla with the corner of a pyramid to include arcuate artery.
6. Spleen
7. Skeletal muscle - quadriceps preferred, cross section.
8. Thymus
9. Left carotid artery at circle of Willis
10. Middle cerebral artery
11. Frontal cortex with meninges
12. Cerebellum with meninges

D. Tissue for fixation in glutaraldehyde
Small portions of the following tissues will be obtained, fixed in glutaraldehyde and processed into resin blocks for possible electron microscopic examination:

1. Myocardium - small, 0.1 cm, random samples of left and right ventricular myocardium, two from each ventricle, at least one to include the endocardial surface.
2. Coronary artery - two small, 0.1 cm, samples, one from the mid right coronary artery and one from the mid left anterior descending coronary artery.
3. Lung - small, 0.1 cm random sample, from abnormal area if present.
4. Kidney - two, 0.1 cm samples, one from the corticomedullary junction at the corner of a renal pyramid to evaluate arcuate arteries and one from the cortex for smaller vessels.
5. Skeletal muscle - two small, 0.1 cm samples, quadriceps muscle preferred.

5.4.2 Transbronchial Inflation Fixation of the Lung

The lungs will be removed intact with the mainstem bronchi and distal trachea. Tissue samples will be obtained for culture, freezing and fixation with RNase-free paraformaldehyde and glutaraldehyde (see above), these samples will be taken from one region of each lung. The bronchi will be divided at the carina

and the lungs weighed. The mainstem bronchi will be cannulated and the lungs will be placed on an apparatus for transbronchial inflation fixation (see Appendix 11; Basic Procedure for Lung Cannulation and Figure 1). The lungs will remain on the apparatus for at least 24 hours. When they are removed, they will be volumed immediately by weighing suspended in water. They will be sectioned sagittally (see Appendix 11; Figure 2), and the midsagittal section of each lung will be sampled for histologic examination (see Appendix 11; Figure 3 and 4). At least three sections will be taken from the midsagittal section of each lung (right upper lobe, right middle lobe, right lower lobe, left upper lobe, lingula, left lower lobe). The sections will be photographed underwater and then processed and stained with hematoxylin and eosin.

5.4.3 Cardiovascular Examination

In order to provide a systematic and standardized method for examination of the cardiovascular system, the following protocol will be followed:

I. Gross Examination

As part of the external examination of the heart, the origin of the arch vessels, the pulmonary venous connections, and the systemic venous connections will be examined with the heart in situ. After removal of the heart and lungs en bloc, external measurements will include the maximal breadth, midanterior base-apex length, and antero-posterior cardiac dimensions. Hemodynamic flow patterns will be assessed by measuring the diameters of the main pulmonary artery (MPA), ascending aorta (AA), ductus arteriosus (PDA-E), and aortic arch between the left common carotid and subclavian arteries (TA) (See Appendix 12; Figure 1). The internal diameter of the ductus, if patent, will also be measured (PDA-I). Branch pulmonary artery external diameters will be measured only if clinical

and/or gross observations suggest them to be abnormal. Additional measurements will be recorded for any other structures deemed to be dilated, narrowed, or too small.

All hearts will be opened in the fresh state and in the direction of blood flow, i.e. right atrium to right ventricle and pulmonary artery; left atrium to left ventricle and ascending aorta, modified as necessary for malformed hearts. The first incision will extend from the inferior vena cava to the tip of the right atrial appendage, thereby sparing the superior vena cava and sinus node. The left atrial incision will begin at the base of the left atrial appendage and extend across the free wall into the right pulmonary veins then back into the left veins. The incisions into the right and left ventricular inflow tracts will be made at the acute and obtuse margins respectively, extending between the anterior and posterior papillary muscles in each ventricle. The outflow tract incision in the right ventricle will be made through the moderator band so as to place the anterior papillary muscle on the flap of anterior free wall. Similarly the left ventricular outflow tract incision will extend between the anterolateral papillary muscle and ventricular septum, placing the papillary muscle on the free wall flap. The origin and course of the epicardial coronary arteries will be traced and the vessels sectioned at 0.2 to 0.5 cm intervals to look for luminal narrowing and/or calcification. The entire aorta, main pulmonary artery and both right and left pulmonary artery branches will be opened longitudinally and examined for lesions.

The heart will be removed from the lungs and dissected free of the parietal pericardium. If there are no lesions of arteries or veins in either the systemic or pulmonary circulations, a 1.0 cm portion of the great arteries, superior and

inferior venae cavae, and 0.5 cm rim of pulmonary veins will be left attached to the heart. Heart weight will be recorded (grams). If vascular malformations or lesions are identified, a longer segment of the involved vessel(s) will be left attached to the heart so as to leave the entire lesion intact. All malformations will be described in appropriate detail.

Internal cardiac dimensions to be measured (Lev, et al, Arch Pathol 72:17, 1961) include (in centimeters):

A. Ventricular wall thicknesses:
   1. Inflow and outflow tracts measured 1.0 cm below the respective cardiac valves; and the point of maximal thickness, all exclusive of trabeculations.

B. Atrioventricular valve circumferences:
   1. A string measurement of linear length of the annulus will ensure accurate and consistent measurements. (TV and MV)

C. Semilunar valve circumferences:
   1. A string measurement of linear length of the annulus along the superior margin of each cusp, i.e., along the sinotubular junction. (AV and PV)

D. Chamber dimensions (in order to provide more objective criteria for chamber dilation, etc.) (See Appendix 12; Figures 2 and 3)
   1. RVIT: measured from the tricuspid annulus at the midpoint of the posterior free wall to the apex of the cavity (exclusive of wall thickness).
   2. RVOT: measured from chamber apex to the base of the septal cusp of the pulmonary valve.
   3. LVIT: measured from the mitral annulus at the midpoint of the posterior (inferior) free wall to the chamber apex.
   d. LVOT: measured from the chamber apex to the base of the right aortic cusp.

II. Light Microscopy

In order to ensure adequate and consistent microscopic evaluation, the following sections will be taken and fixed as indicated with RNase-free paraformaldehyde according to 5.1.6.1.1 Procedure for Preservation of RNA in Human Tissues, with 10% buffered formalin, or frozen. These sections are mentioned above in the general autopsy protocol and appear on the appended (See Appendix 13).

A. Myocardial Sections:

1. RNase-free paraformaldehyde:
   a. RVI taken from the acute margin of the heart extending across the atrioventricular groove to include the right coronary artery, a small rim of right atrium, and the tricuspid valve.
   b. LVI - taken from the obtuse margin inflow incision into the left ventricle to include the left circumflex coronary artery, a small rim of left atrium, and mitral valve.
   c. A representative section of any gross lesion.
2. Formalin:
   a. RVO - taken from the outflow incision to include the pulmonary valve and proximal wall of the main pulmonary artery.
   b. RVA - taken near the apex to include the anterior papillary muscle.
   c. LVO - taken from the outflow incision to include the aortic valve and proximal ascending aorta. Note that this section may also include the proximal left circumflex coronary artery. If space allows, include a portion of the anterolateral papillary muscle.
   d. LVA - taken near the apex to include the posteromedial papillary muscle either en bloc or as a separate piece.
   e. LVP - if needed (see LVO) to section the anterior and/or posterior papillary muscles.
   f. VS - taken perpendicular to the long axis of the ventricular septum and at the point of maximal thickness.
   g. Additional sections of any gross lesions.
3. Frozen tissue:
   a. RVI-2 - taken parallel to section RVI (see above)
   b. LVI-2 - taken parallel to section LVI (see above)

B. Coronary Arteries:

1. RNase-free paraformaldehyde:
   a. LAD - The left anterior descending coronary artery will be sectioned as described in the gross examination. At least three segments including proximal, mid and distal will be submitted.
2. Formalin:
   a. LCA - The short segment of the left main coronary artery will be sectioned as described in the gross examination and at least one segment submitted.
   b. RCA - In addition to the section obtained above in RVI, the right coronary artery will be sectioned as described in the gross examination, at least three segments including proximal, mid and distal will be submitted.
   c. LCX - In addition to the section obtained above in LVI, the left circumflex coronary artery will be sectioned as described in the gross examination, at least three segments including proximal, mid and distal will be submitted.

d. PD - The posterior descending coronary artery will be sectioned as described in the gross examination and at least three segments (proximal, mid and distal) submitted.

3. Glutaraldehyde:
   a. RCA - one segment.
   b. LAD - one segment.
4. Frozen tissue:
   a. LAD - One section from the midportion of the vessel.

C. Systemic vessels:
1. RNase-free paraformaldehyde:
   a. Renal arteries - The main renal arteries will be sectioned at 0.2 to 0.5 cm intervals and one to three segments of each submitted.
   b. Carotid arteries - One segment of the left carotid artery at the aortic arch and one at the circle of Willis will be submitted.
   c. Middle cerebral artery - The middle cerebral artery will be dissected from its origin at the carotid to the trifurcation and sectioned at 0.2 to 0.5 cm intervals; at least three segments (proximal, mid and distal) will be submitted.
   d. Mesenteric arteries - A section of the midportion of the mesentery will be submitted.
2. Frozen tissue:
   a. Mesenteric arteries - A section of the midportion of the mesentery.
   b. Middle cerebral artery - A section from the midportion.

III. GROSS DISSECTION OF THE CONDUCTION SYSTEM

The region of the sinoatrial node and atrioventricular conduction axis through the proximal right and left bundle branches will be excised en bloc and stored in 10% phosphate buffered formalin for ancillary study.

A. Sinoatrial node (See Appendix 12; Figure 4):
The initial incision into the right atrium and the 1.0 cm length of superior vena cava that has been retained with the heart will leave the region of the SA node intact (the junction of the superior cava with the right atrium at the sulcus terminalis). A wide circumferential incision 1.0 cm below this caval-right atrial junction will excise this region en bloc. This tissue is to be stored in formalin for future dissection into appropriate sections for histologic examination (ancillary study).

B. Atrioventricular Conduction Axis (See Appendix 12; Figure 5 and 6):
All previously described atrial and ventricular incisions and histologic sections have spared this region of the heart. The general region includes the coronary sinus and atrial septum anterior to it, muscular atrioventricular septum with contiguous tricuspid septal leaflet and mitral anterior leaflet, membranous

septum with overlying aortic valve and underlying muscular subaortic ventricular septum from the left ventricular aspect, and tricuspid valve anteroseptal commissure with contiguous papillary muscle of the conus from the right ventricular aspect. A generous en bloc excision of this region requires four (4) major incisions:

1. The first incision is made within the opened right atrium and right ventricle. It should begin just above and posterior to the coronary sinus and extend inferiorly into the ventricle, parallel to the long axis of the ventricular septum (perpendicular to the tricuspid annulus) to include at least 1.0 cm to 1.5 cm of subjacent ventricular septum.
2. The second incision will be made roughly parallel to the first. It extends into the outflow portion of the ventricular septum; the septum in this area is curved so that it will be important to determine the appropriate position of the incision from both the right and left ventricular aspect.
   a. From the right ventricle the incision will be made into the anterior leaflet of the tricuspid valve, just anterior to the papillary muscle of the conus, and extending through the subjacent ventricular septum. The right bundle branch courses in relation to the papillary muscle of the conus so it is important to include this muscle with the bloc.
   b. From the left ventricle the incision should be clearly anterior to the membranous septum with at least a 0.1 to 0.2 cm muscular margin. The incision should be placed at least through the midportion of the right coronary cusp to ensure inclusion of the entire membranous septum and underlying branching bundle portion of the conduction system.
3. The third incision is made in the opened right atrium and extends across the atrial septum, above the coronary sinus and parallel to the tricuspid annulus to connect the atrial extent of the two previous incisions. From the left sided chambers it will be necessary to extend the incision into the aortic valve, above or within the right aortic sinus to encompass the anterior (outflow) extent of the membranous septum.
4. The fourth incision is made in the opened right ventricle and is also parallel to the tricuspid annulus. It should be at the level of the free margin of the septal tricuspid leaflet and inferior to the papillary muscle of the conus. From the left ventricular aspect it should extend at least 0.5 cm below the inferior margin of the membranous septum.
5. To completely free this bloc from all surrounding structures it may be necessary to cut attached chordae tendineae or atrial free wall connections.

DO NOT TRIM OR FURTHER SECTION THE EXCISED TISSUE. It will be further divided into appropriate blocks at the time of submission for histologic study. Store the tissue in formalin. 8

CHAPTER 6:

PUBLICATIONS

MANUAL OF OPERATIONS

(Chapter 6 divider on order)

6. Publications Policy

6.1 Review Process and Time table

All press releases, interviews, presentations and publications that require review by the PPS should be sent directly to the Chairman of the PPS, who will immediately communicate with the other members of the Subcommittee. Deadline for submission of material to the PPS will be at least four weeks prior to submission date for manuscripts, at least three weeks prior to submission for abstracts, and at least three weeks prior to presentation for presentations (Section 6.5). At least three reviewers will be selected from the study-wide list of investigators. When necessary, non-study members may be selected as reviewers at the discretion of the PPS. Reviewers will be chosen for their expertise in the subject matter of the particular document and for their understanding of the study as a whole. In general, a Cardiology, Pulmonary and Immunology/Infectious Disease Subcommittee member will be included as reviewers. However, members of other study subcommittees (Radiology, Nuclear Medicine, Pathology, etc) may be included as needed. All data presentations and analyses will be reviewed by a biostatistician at the Coordinating Center. Each reviewer will be asked to judge whether or not the publication as written will affect the study's process, its acceptance or the interpretation of its results. Reviewers will be asked to provide written comments, including recommended approval or disapproval and recommended changes, within five days for abstracts and presentations, and within two weeks for manuscripts. The PPS shall decide (1) whether the scientific content and interpretation of the data are acceptable; (2) whether the contributions of members, representatives, and consultants are properly acknowledged; and (3) whether the publication or presentation is in the best interest of the study.

The chairman or a designated member of the PPS shall then notify the principal author of the majority decision of the PPS which may include approval, approval contingent upon specific revisions, approval with suggestions for revision and resubmission, or disapproval. After revisions have been made and approval is given by the PPS, and the Chairman of the PDSMB and NHLBI Project Officer are sent written notification of the decision.

Investigators who challenge a Publications/Presentation Subcommittee decision will be able to appeal to the Chairman of the Steering Committee. The Policy, Data and Safety Monitoring Board of the NHLBI will be the final arbiter. In instances where the PPS does not reach a majority decision, the matter will be deferred for decision by the Steering Committee at its next meeting or conference telephone call, with the Policy Board as the final arbiter.

Membership in the Steering Committee implies agreement to abide by these procedures for all publications based on study data. These provisions apply to reports of writing subcommittees as well as other reports based on study data.

6.2 Reporting Methods and Editorial Review Requirements

6.2.1 Press Releases

Local press releases will not be reviewed by the PPS. Nevertheless, all press releases must limit their substantive content to information items that have been described in the most recent request for proposals. A copy of each prepared release must be sent to the NHLBI Project Officer prior to release, for approval and for updating the Program Office files on current knowledge to be used for responses to national queries. Also, copies of all press releases should be retained and sent to the Project Officer with Quarterly Reports as required by the contracts. A central file of all press releases will be maintained by the Project Officer.

Project-wide press releases will be initiated by the NHLBI Project Officer and reviewed by the PPS, and if there are any questions, by the Steering Committee.

6.2.2 Interviews

Interviews are subject to the same editorial rules as press releases. Local information concerning participation by local organizations can be provided to encourage cooperation and acceptance of the program. The ethics and legalities of medical confidentiality apply to the names and risk statuses of individual participants.

6.2.3 Presentations

Material for all presentations given outside closed academic communities must be reviewed by the PPS. These include presentations given to scientific, professional or public groups. Particular attention is drawn to presentation of material when proceedings of the meeting or workshop are likely to be published or publicized.

Presentations are subject to the same rules as press releases. If a presentation is limited to substantive information in the RFP and has no added interpretation or inferences, it can be given without prior review by the PPS. Any discussion of the study or ancillary projects that goes beyond those items of information must be submitted for review at least three weeks prior to the date of presentation. The PPS will identify scientific and professional forums where presentations about the study should be made on behalf of the Steering Committee. It will bring these proposed forums for presentation to the Steering Committee for approval. From a list of volunteer investigators the PPS will identify one or more persons to prepare and present the material. The written presentation must be reviewed by the PPS before it is presented.

6.2.4 Publications

All publications of primary and ancillary studies will be prepared under the direction of the PPS. Any work supported wholly or partially by the study

must be submitted to the PPS for review prior to submission. Categories of publications (Type A, B, C, or D) are described as follows:

Type A. Reports of the major outcomes of the study, prepared as study-wide documents, which draw on data collected by all centers.

Type B. Reports addressing in detail one aspect of the study, using data from all centers.

Type C. Reports of data derived from patients enrolled in the study, but where the focus of the paper is on data collected outside the P^2C^2 Study Protocol at a subset of the centers. (e.g., substudies or ancillary studies). These papers may include data collected as part of the study protocol as long as it is determined that this does not compromise any planned Type A or B publications.

Type D. Case studies involving protocol-mandated data collected on P^2C^2 patients at a single center.

6.3 Authorship and Acknowledgement Policy

The authors of study publications will be as listed below, for each type of publication:

Type A Abstracts: Authorship listed as "The Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted Human Immunodeficiency Virus (HIV) Infection Study Group." If the conference requires that an individual be listed, then the Chairman of the writing group will be listed, followed by "for the Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted Human Immunodeficiency Virus (HIV) Study Group."

Type A Publications: Authorship will be listed as "The Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted Human Immunodeficiency (HIV) Infection Study Group", with reference to a footnote stating that members of the group are listed in the appendix, and identifying the NHLBI Project Officer as the person to whom reprint requests should be sent.

The P^2C^2 HIV Study participant box, described in Section 6.4) must be included as an appendix to these papers.

Type B Abstracts and Presentations "[List of writing committee], for The Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted Human

Immunodeficiency Virus (HIV) Infection Study Group"

The participants box will be included as an appendix to these papers if this can be arranged with the publisher. Otherwise a reference will be made to one of the papers where the box is included.

Type C Abstracts: List of individual authors.

Type C Publications: List of individual authors. These papers should include an acknowledgement to the study.

Type D Abstracts: List of individual authors.

Type D Publications: Authorship will be a list of individual authors, without reference to the P^2C^2 HIV Study. These papers should include the following footnote:

"The authors acknowledge the support structure of the National Heart, Lung and Blood Institute-sponsored Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted Human Immunodeficiency Virus (HIV) Infection Study (P2C2 HIV Study). The data presented herein, although gathered during the course of the P2C2 HIV Study, do not address hypotheses of the study but, rather represent novel findings of an individual patient which are being reported at this time because they may have relevance for current therapeutic approaches to complications of HIV infection in children".

6.4 Listing of Professional Participants in the P^2C^2 HIV Participants Box

The P^2C^2 HIV Study participants box will list all professionals that have participated in the study for a minimum of one year. The participants for each participating center/cite will be listed together, with the center principal investigator listed first, and identified as "P.I." followed by the other center staff listed alphabetically. Each participant will be listed only by his/her professional and academic degrees, not by the specific position which he/she held in the study. The participants will be listed in the following order:

NIH
Steering Committee Chair
Clinical Coordinating Center
Clinical Centers (alphabetical order)
Central EBV Laboratory

6.5 Procedures for Initiation and Review of Abstracts or Publications

It is the policy of the P2C2 HIV study that preparation of all publications or presentations, other than materials prepared for local publicity purposes, must be assigned to specific individuals or appointed writing committees, and that all such materials must be reviewed and approved by the Publications Subcommittee before publication.

6.5.1 Initiation of Abstracts or Publications

All participants in the study are invited to suggest topics appropriate for preparation as abstracts, peer-reviewed papers, or chapters and reviews from the study. When the topic deals specifically with cardiology, pulmonary, or immunology/infectious disease data, the topic should be suggested to the chairman of the appropriate (i.e. Cardiology, Pulmonary, or Immunology/Infectious Diseases) Subcommittee. The chairman is responsible for reviewing the request with members of the subcommittee to make them aware of the proposal, to determine if the content conflicts with other currently established writing groups, and to solicit interest from members of the committee who would be interesting in being on the writing committee. The subcommittee chairman then is responsible for recommending the topic and a writing group to the Publications/Presentations Subcommittee. For more general topics, the suggestion for an abstract or writing group should be made directly to the Chairman of the Publications/Presentations Subcommittee. Final approval to proceed with preparation of an abstract, peer-reviewed paper or chapter, and selection of the appropriate writing committee, is made by the Publications/Presentations Subcommittee. This approval is required before analysis requests are made to the Clinical Coordinating Center.

6.5.2 Timetable for Abstract Submission

Abstracts should be suggested to the Publications/Presentations Subcommittee approximately three months prior to the intended submission deadline. Requests for data analysis should be made by the writing committee to the Clinical Coordinating Center approximately two months prior to the intended submission deadline. (See Figure 1)

6.5.3 Presentation/Publications Review Procedures

The review process will be as follows:

6.5.3.1 Abstracts

1. Abstracts are submitted to the CCC for review at least <u>three weeks before the submission deadline</u>. Approval of the relevant Subcommittee must be obtained prior to submission to the CCC.

2. The Study Coordinator at the CCC coordinates distribution of

abstracts/manuscripts.

3. Author faxes the abstract to the CCC, and calls the Study Coordinator to notify the Coordinating Center that the abstract has been sent.

4. Receipt of the abstract is logged at the CCC and tracking of its review is begun. The author(s) and NHLBI are notified by E-mail or fax that the abstract was received.

5. The Study Coordinator at the CCC contacts the PPS Chairman who identifies three primary reviewers, and calls them to confirm their availability. The CCC faxes abstracts to the reviewers and to all members of the Steering Committee.

6. Review is completed by the primary reviewers within five days.

7. Responses are submitted to the PPS Chairman, who collates them and sends the result to the author, along with one of the following categories of approval:

   Approve as submitted
   Approve with recommended modifications
   Approve with required modifications
   Disapprove

   A copy of this material is sent to the CCC.

8. Abstracts approved with required or recommended modifications will be returned to the CCC for distribution to P.I.'s, NHLBI staff, subcommittee members and the Chairman of the PDSMB, but will not be re-reviewed.

6.5.3.2 Manuscripts

1. Manuscripts are submitted to the CCC for review at <u>least four weeks before the desired submission date</u>. Approval of the relevant Subcommittee must be obtained prior to submission to the CCC. The journal to which the manuscript will be sent is identified in a cover letter.

2. A process similar to steps 2-5 for abstracts is used except that manuscripts are mailed to the CCC rather than being faxed.

3. Upon receipt of a manuscript at the CCC, the Study Coordinator informs the PPS Chairman, who selects three reviewers. At least one member of the Steering Committee should participate as a reviewer of a study-related manuscript.

4. The CCC sends a copy of the paper and a list of the reviewers to the

Chairman of the PDSMB.

5. Manuscripts are express mailed to the reviewers who complete their reviews, within two weeks.

6. Responses are submitted to the Chairman of the PPS who collates them and sends the result of the review to the author, the NHLBI Project Officer, the Steering Committee Chairman, and the CCC. Categories of approval will be the same as those described in #7 for abstracts.

7. Investigators are reminded that copies of correspondence with journals concerning study-related manuscripts should be sent to the PPS Chairman.

8. Final manuscripts should be sent to the Study Coordinator at the CCC for distribution to the P.I.'s, NHLBI staff and subcommitee members following their acceptance for publication.

6.5.3.3 Presentations

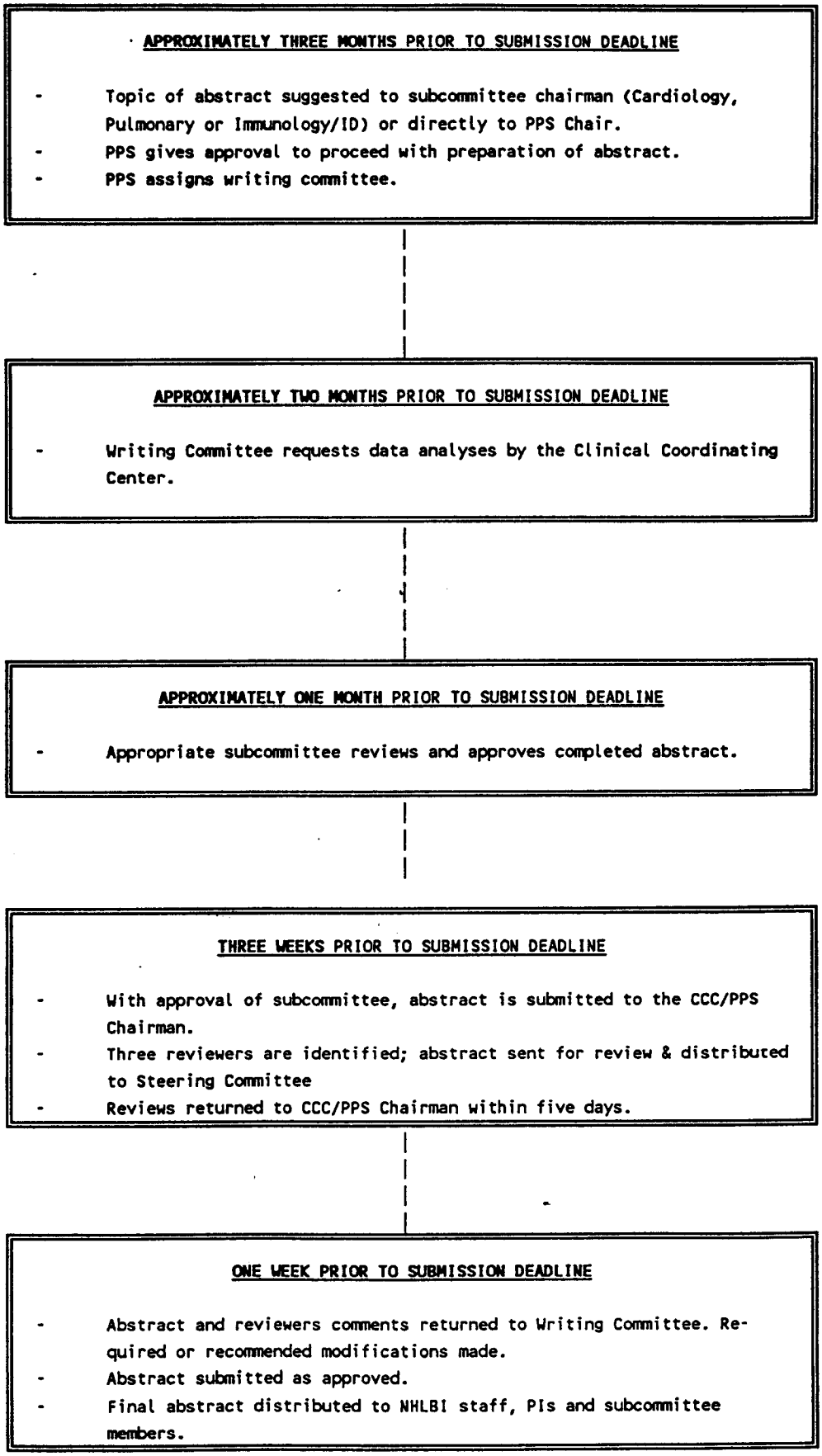
1. An outline of the talk and a hard copy of any text or figures to be used, along with a clear statement of any conclusions or recommendations and the "message" intended for the audience should be sent by fax to the CCC at least three weeks before the planned presentation.

2. A review process analogous to that used for abstracts will be followed.

3. Investigators are reminded that presentation should include acknowledgement of the NHLBI/P^2C^2 HIV Study Group.

FIGURE 1

This timetable for abstract preparation and submission includes deadlines which must be met in order to allow all investigators the opportunity to participate in the publication of P^2C^2HIV data, as well as to produce the best science.



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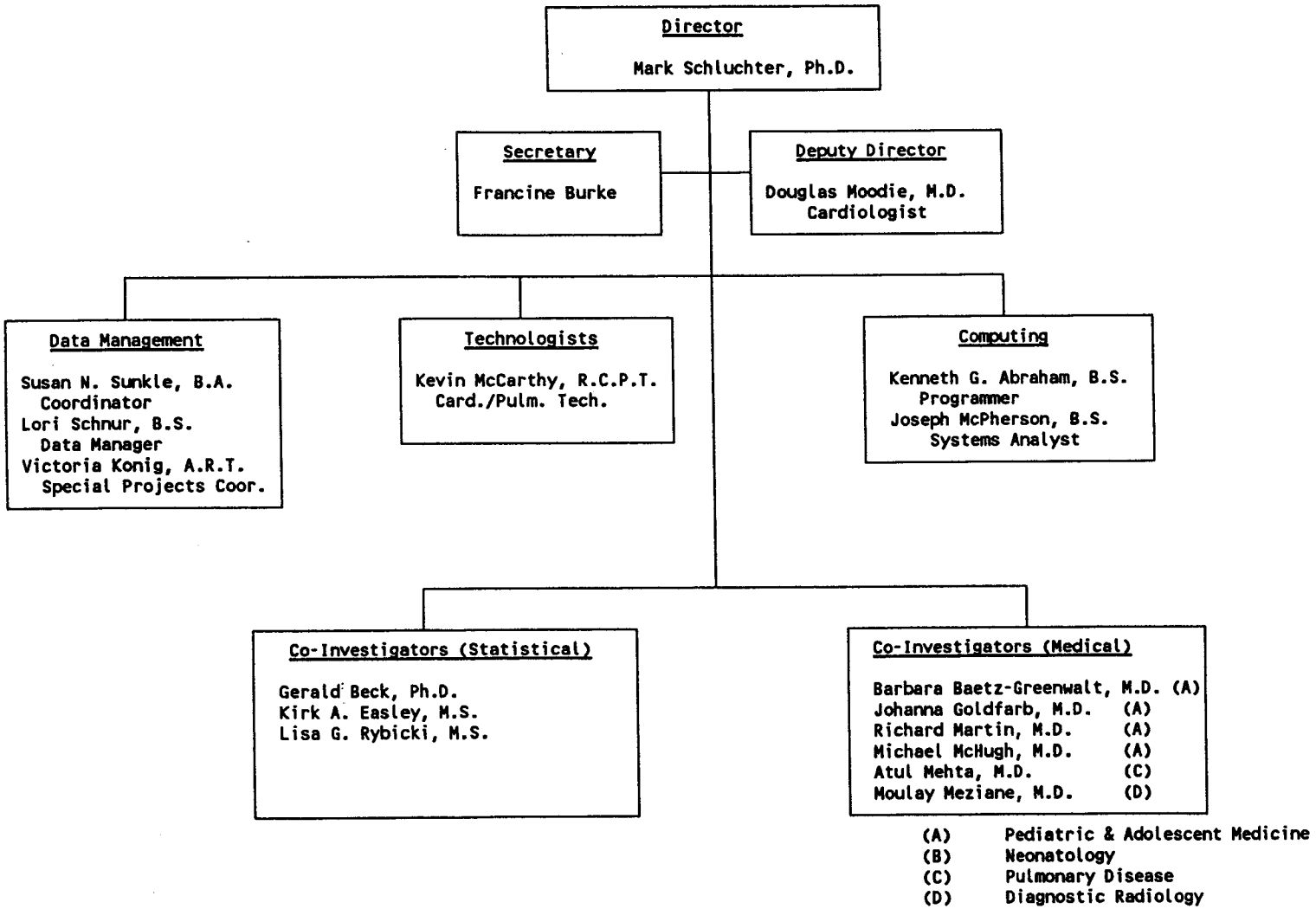
APPENDIX 1

P^2C^2HIV

Clinical Coordinating Center

Table of Organization



(A) Pediatric & Adolescent Medicine
(B) Neonatology
(C) Pulmonary Disease
(D) Diagnostic Radiology

APPENDIX 2

CONFIDENTIALITY STATEMENT

I _____ as an employee of _____ understand the nature of the project that I am involved with: Pediatric Pulmonary and Cardiovascular Complications of Vertically Transmitted Human Immunodeficiency Virus (HIV) infection.

I understand and agree that all information is to be held strictly confidential and that any unauthorized acquisition, release, and/or discussion of any information is strictly prohibited, unless authorized by the National Heart, Lung and Blood Institute.

I have reviewed and fully understand all rules and regulations regarding confidentiality as outlined in the Protocol, Chapter 6, section 6.

I also understand that any proven violation of this trust will result in the termination of my involvement with this project, and that I may be subject to other disciplinary action according to this institution's policy.

Employee Signature: _____

Date: _____

Position: _____

Witness Signature: _____

(Rev. 7/6/90)

APPENDIX 3

A. Maintenance

Occlusion Valve and FRC Valve

Both the Occlusion Valve and the FRC Valve should be cleaned and sterilized between patients. For best results, the valves should be sterilized in a cold liquid sterilization solution such as Control III®, according to the directions on the solution container (usually soaked for 10 minutes). The valves usually do not have to be disassembled for this procedure. If they are disassebled allow to dry thoroughly before reassembling. All O-rings should be lubricated with a silicone lubricant before reassembling also.

The following procedure may be used to sterilize the Double Sliding (Occlusion) Valve on the 2600 Pediatric Pulmonary Laboratory. The temperature of the disinfectant used must not be greater than 41° Centigrade.

1. Remove the pneumotach and tubing from the Double Sliding Valve.
2. Remove the Pneumotach Adapter and Ventilator Port from the Double Sliding Valve Assembly.
3. Slide the "Red Cap" onto the patient connection end of the Double Sliding Valve as shown in Figure 14.1.
4. Fill a 10cc syringe with disinfectant.
5. Attach the syringe to the Double Sliding Valve as shown in Figure 14.1.
6. Inject the disinfectant into the Double Sliding Valve until it flows from the Ventilator Port of the Double Sliding Valve.

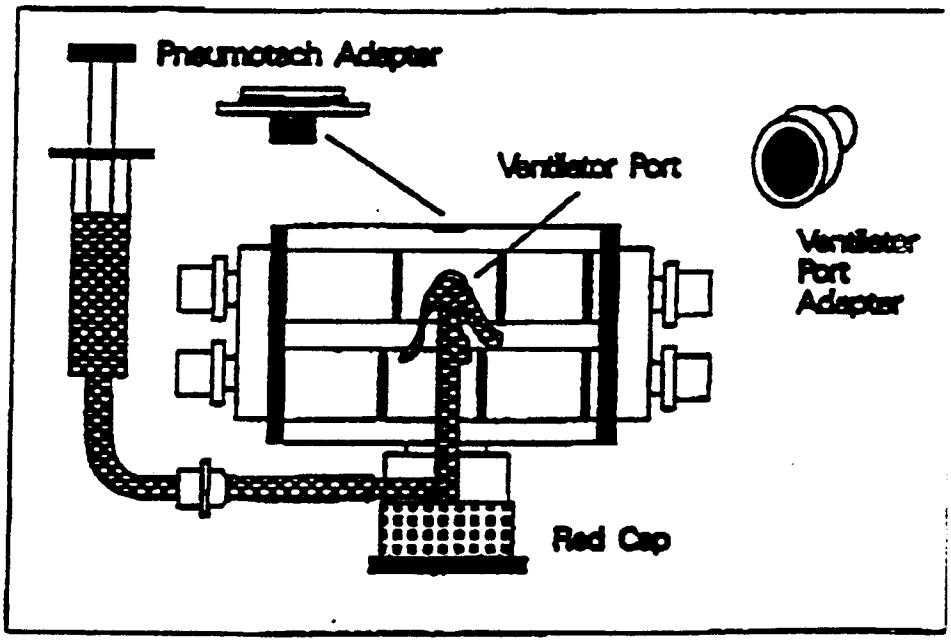


Figure 14.1. Occlusion Valve Cleaning

7. Position the "Pistons" inside the Double Sliding Valve as shown in Figure 14.2. This can be accomplished by attaching a syringe to the Pneumatic port.

8. Inject the disinfectant into the Double Sliding Valve until it flows from the Pneumotach Port of the Double Sliding Valve.

9. Allow the appropriate amount of time for the disinfectant to work and then flush the Double Sliding Valve with clean water.

10. Position the "Piston" inside the Double Sliding Valve as shown in Figure 14.1.

11. Flush the Double Sliding Valve with clean water.

12. Blow out all channels until dry. Do this with the "Piston" in both positions.

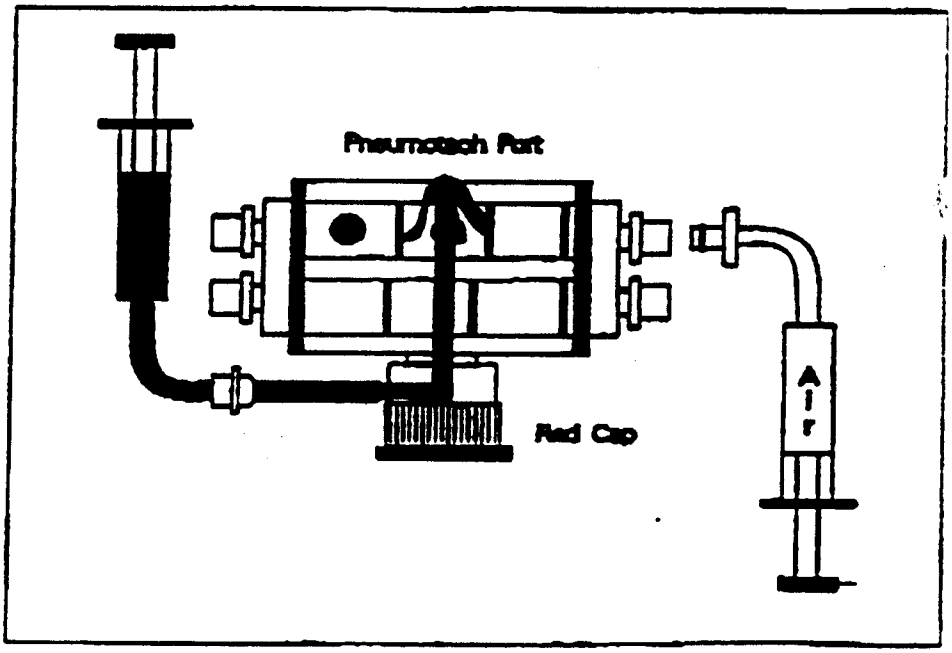


Figure 14.2. Occlusion Valve Cleaning

Pneumotachs

The Pneumotach(s) should be cleaned and sterilized between patients. For best results, the pneumotachs should be sterilized in a cold liquid sterilization solution such as Control III®, according to the directions on the solution container (usually soaked for 10 minutes). The pneumotachs should be disassembled for this procedure and allowed to dry thoroughly before reassembling. Special care should be given to removal of the resistive screens so that they are not accidentally torn and ruined.

Refer to figures 14.3 and 14.4 for disassembling the 10 LPM and 30 LPM Pneumotachs.

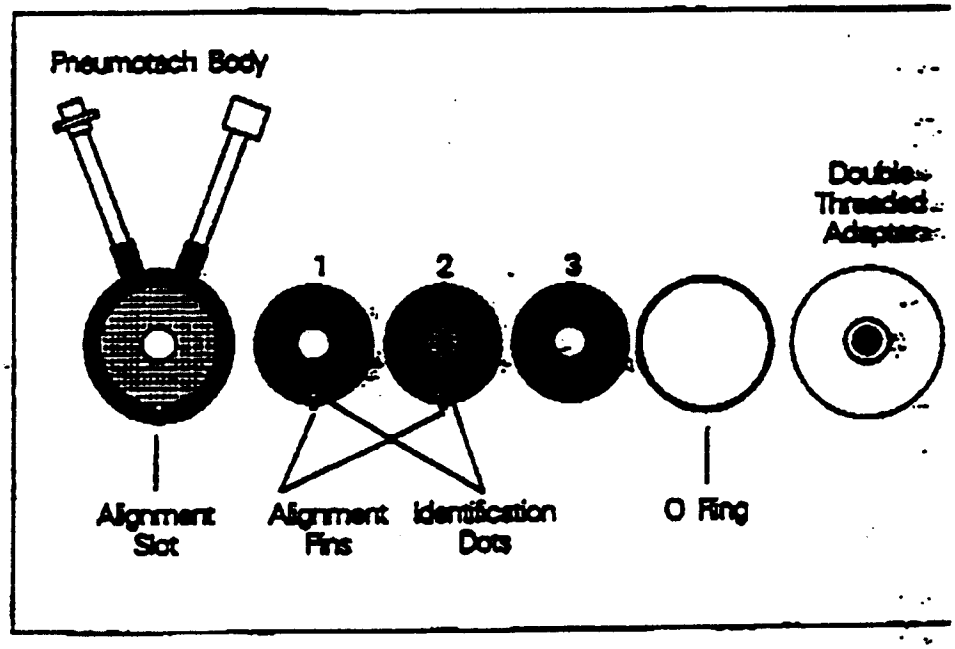


Figure 13.3. Exploded view of 10 LPM Pneumotach.

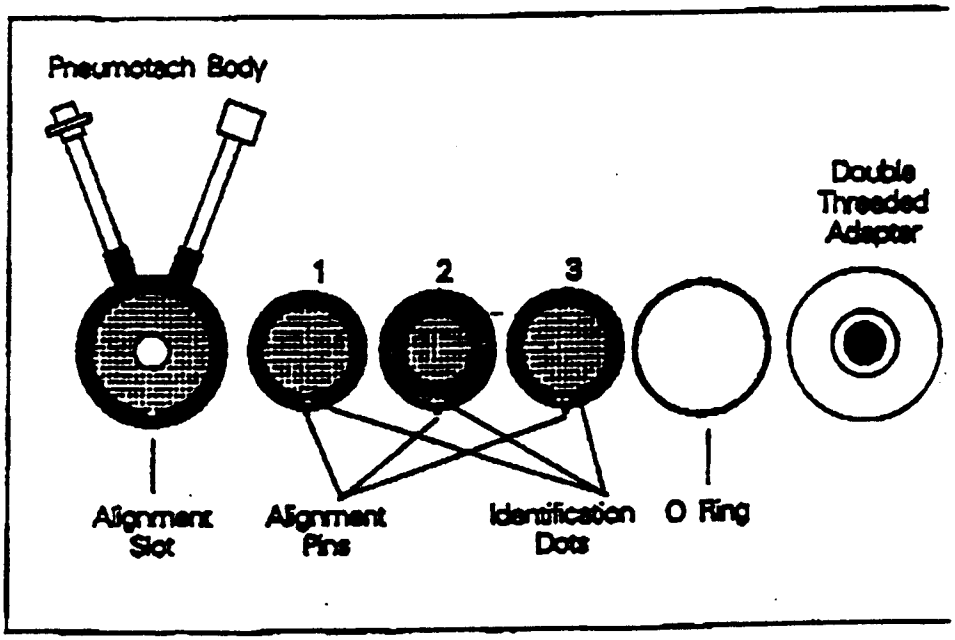


Figure 13.4. Exploded view of 30 LPM Pneumotach.

APPENDIX 4

ANTI-HIV ELISA BY ORGANON VIRONOSTIKA MICROELISA SYSTEM

PRINCIPLE

HIV antigen is derived from HIV virus propagated in T-lymphocyte culture. After the virus is purified by ultracentrifugation and inactivated by disruption, it is used to coat the microelisa wells contained in the Vironostika HIV ELISA test.

With the addition of a diluted test sample containing Anti-HIV, immune complexes form through the interaction of the Anti-HIV in the sample and the solid phase HIV. Following incubation, the sample is aspirated and the well washed with buffer. Subsequently, anti-human globulin (goat) conjugated with horseradish peroxidase (HRP) is added which binds to the antibody-antigen complex during the second incubation. Following a wash and incubation with 2,2,'-azino-di-[3-ethyl-benzthiazoline-sulfonate] (ABTS) substrate, a green color is produced. The enzyme reaction is stopped by the addition of a fluoride solution. The amount of Anti-HIV present in the sample is proportional to the amount of color developed.

SPECIMEN/TEST REQUEST

The test should be ordered in the laboratory computer system as ANTI HIV.

SPECIMEN

No special patient preparation is necessary. Fasting is not required. Serum or plasma derived from blood anticoagulated with heparin, CPD (citrate phosphate dextrose) or EDTA (ethylenediaminetetraacetate) may be used. Specimens may be stored for up to one week at 2-8 degrees C.

MATERIALS AND REAGENTS

Microelisa strips - 12 HIV antigen coated wells per strip.

Diluent concentrate - Normal goat serum in Phosphate buffered saline; contains 0.05% Thimerosal.

Tween-20, 5% - Contains 0.1% Thimerosal.

Diluent reagent water.

Positive Control serum (Human, inactivated) Lyophilized.

UILSIM - Lyophilized specimen inactivator medium.

Wash concentrate - contains 2.5% surfactant.

Peroxidase conjugated goat antihuman immunoglobulins (EnzAbody) in phospnate buffer - Lyophilized.

ABTS substrate - Lyophilized 2,2'-azino-di[3-ethyl-benztniazoline-sulfonate.

ABTS diluent - contains hydrogen peroxide.

Stop solution - contains 0.28% Sodium fluoride.

Clamp and rod - closure for foil microelisa tray packs.

Plate sealers - adhesive covers for microelisa plates.

Cavro automated diluter/dispenser system

Aspirator/wash system with waste trap and vacuum source.

12 Channel variable volume 50 to 200 uL pipet system.

Mircoelisa plate reader.

V shaped troughs

QUALITY CONTROL

Eacn strip holder with up to 8, twelve well strips will nave one Positive control(PC) and three Negative control(NC) samples run. The NC absorbance values must be greater than or equal to .100 (405nm) or .040 (690 nm) and be less than or equal to .400 (405 nm) or .200 (690 nm). The computer software is programmed to evaluate the three negative control values and to eliminate any single outlier using the following formula:

$$(NC1+NC2+NC3)/3=NCX$$
$$NCX \times 0.5 = NCLL$$
$$NCX \times 1.5 = NCUL$$

Where NCX is the Negative Control Mean
NCLL is the Negative Control Lower Limit
NCUL is the Negative Control Upper Limit

Any Negative Control value less than the NCLL or greater than the NCUL is eliminated from the calculation and the mean is recalculated using the two remaining acceptable values.

The Positive control (PC) value must be greater than or equal to .700 (405 nm) or .300 (690nm) for the run to be valid. The run must be repeated if the PC absorbance value does not meet this requirement.

A test run is valid if the PC and NC values are qualified and PC - NCX >= .500 (405 nm) or PC - NCX >= .200 (690nm)

If this equation is not true, technique may be suspect and the run must be repeated.

PROCEDURE

Reagent Preparation

Prepare all reagents before beginning the assay procedure. Reagents and samples should be at ambient temperature (20-25 deg. C.) before beginning test and can remain at that temperature during the testing. The reagents should be returned to the refrigerator after use.

Diluent

1. Check diluent concentrate to see if visible crystals have formed in the solution. If crystals have formed, resolubilize concentrate by warming at 37 degree C. until the crystals dissolve.

2. Add one vial of diluent concentrate and 1 bottle of Tween-20 to 1 bottle of diluent reagent water. Mix by inverting several times. Avoid excessive foaming.

3. Relabel the bottle DILUENT. Add 28 days to the date of preparation and record that as the expiration date on the bottle.

Positive and Negative Controls

1. Pipet 500 uL prepared DILUENT into each Negative and Positive Control serum vial to be reconstituted. Mix the contents thoroughly.

2. Add 28 days to the date of reconstitution and record that as the expiration date on each vial label.

DILSIM

1. Fill DILSIM bottle to the neck with prepared DILUENT (60 mL). Cap the bottle tightly and warm at 40-50 deg.C. in a water bath or under a stream of hot water for 20-30 minutes.

2. After warming, vortex the bottle vigorously until the cake is completely solubilized.

3. Add 28 days to the date of reconstitution and record that as the expiration date on the bottle label.

Wash Solution

1. Dilute the wash concentrate 1:50 with distilled water in a clean container by adding one bottle of wash concentrate to 2940 mL of distilled or deionized water.

2. Label the container WASH SOLUTION and add 28 days to the date of preparation and record that as the expiration date on the container label.

EnzAbody

1. Pipette 50 mL of prepared DILUENT to one vial of EnzAbody. Mix by inverting several times. Avoid excessive foaming.

2. Add 28 days to the date of preparation and record on the vial label.

ABTS Substrate

1. Allow ABTS diluent and ABTS substrate to warm to room temperature. Add ABTS diluent (1 bottle) to 1 bottle of ABTS substrate. Mix by inversion.

   Caution: To minimize auto-oxidation, reconstitute with ABTS diluent that has warmed to room temperature. DO NOT add cold ABTS substrate to test wells.

2. Add 14 days to the date of reconstitution and record date on bottle label.

   Note: Do not reconstitute the second bottle of ABTS substrate until the first has been depleted.

Stop Solution

No preparation is necessary. Contains Sodium fluoride. Avoid contact with skin. Should contact occur, wash area with water immediately.

Wash Procedure

1. Incomplete washing will adversely affect the test outcome.

2. The wash procedure consists of an initial aspiration of the well contents followed by a filling of the wells (0.3mL) with diluted wash solution. This process is repeated four times.

3. After the last aspiration of each wash step, invert the strip holder and tap firmly on absorbant paper taking care not to dislodge any of the strips. Any excess wash solution present can be removed by blotting with absorbant paper.

4. Vacuum aspirate the well contents and wash buffer into a waste flask containing Sodium hypochlorite solution, final concentration not less than 1%.

5. Routine maintenance of aspiration/wash system is strongly recommended to prevent carryover of antibody from specimens containing a high concentration of antibody to non-reactive specimens.

6. The aspiration/wash system should be flushed with copious amounts of water upon completion of the final wash process of the assay.

PROCEDURE

1. As specimens come to the department they are entered into the Laboratory Computer System, if necessary, and a label for a 1 dram vial is printed using the BLP program.

2. When all specimens for the run are in hand, a copy of all pending requests for Anti-HIV testing is printed using the Laboratory Computer System's BSB program. Arrange specimen vials in the same order as the BSB printout.

3. Enter into the Organon computer the specimen or unit number from each vial to be tested. It is IMPORTANT that this data be entered from the vials and not from a printed list.

4. When all specimens are entered into the Organon computer, print a worklist from that computer.

5. Fit strip holder with with the required number of microelisa strips based on the number of samples to be tested plus one well for a positive control and three wells for negative controls. Strips contain twelve wells and cannot be separated into groups of less than twelve wells.

6. Prepare a 1:75 dilution of sample or control to be tested in DILSIM. This is done using the Cavro diluter which aspirates 3.0 uL of sample and then delivers that and 225 uL of DILSIM to a test well.

   CAUTION: Do not allow the microelisa well to dry once the assay has begun.

   NOTE: Gloves and an eye shield (either prescription glasses or lab goggles) must be worn when performing dilutions.

7. When all samples and controls have been delivered to the proper test wells, mix contents of the wells by gently tapping the stripholder, then cover the strips with an adhesive plate sealer. Incubate at 37 deg.C. for 90-100 minutes.

8. When the first incubation is complete, wash each well four times with diluted wash solution using the Dynawash II washer. Be sure to slowly aspirate the reactants prior to addition of the wash solution. (Refer to wash procedure, above)

9. Pipet 150 uL of reconstituted EnzAbody solution into each well using the TiterTech 12 tip dispenser.

CAUTION: Do not allow EnzAbody to contaminate the substrate. If the same equipment is used to add both reagents, new disposable tips must be used.

10. Mix contents of the wells by gently tapping the stripholder, then cover with a new adhesive plate sealer. Incubate at 37 deg.C. for 30-35 minutes. Take ATBS substrate out of refrigerator so that it will be warmed to room temperature when needed.

11. When the second incubation is complete, wash each well with diluted wash solution as in step 4 above.

12. Pipet 150 uL of prepared ABTS substrate into each well using the TiterTech 12 tip dispenser. DO NOT flush remaining ATBS substrate from pipet tips back into TiterTech reagent boat. DO NOT mix or agitate. DO NOT cover with a plate sealer.

13. Incubate at 20-25 deg.C. for 10 to 12 minutes.

14. At the end of the third incubation, add 150 uL of Stop solution to each well using the TiterTech dispenser (Maintain the same sequence and timing used for the substrate addition in step 8 above) and gently tap stripholder to mix. Plate must be read within two hours.

15. Call up the plate reading program from the Main Menu of the Organon computer software. Follow the directions displayed on the computer screen which will lead to the placing of the stripholder of test wells into the reader. The program will blank the microelisa reader on air and read the absorbance of the solution in each well at 405 or 690 nm.

CALCULATION AND EVALUATION OF RESULTS

Calculation

Each plate of wells must have its own Positive and Negative Controls and be calculated individually.

Qualification of Negative and Positive Control values and testing of validity of the run are detailed in the Quality Control section above.

If the Negative and Positive control values are qualified and the run is valid the Minimum Reactive Value is calculated as follows:

NCX + 0.270 (at 405 nm) = Minimum Reactive Value
NCX + 0.110 (at 690 nm) = Minimum Reactive Value

A test sample is non-reactive if the sample absorbance is less than the Minimum Reactive Value. A test sample is considered reactive if the sample absorbance is equal to or greater than the Minimum Reactive Value.

Interpretation of Results

1. Specimens with absorbance values less than the Minimum Reactive Value are considered non-reactive by the criteria of the test and may be considered negative for the HIV antibody. No further testing is required.

2. Specimens with absorbance values equal to or greater than the Minimum Reactive Value are considered reactive (initially reactive) by the criteria of the test. However, before interpretation, the original sample source should be retested in duplicate. If either of the retests is reactive, the specimen is considered repeatably reactive.

3. Initially reactive specimens which do not react in either of the duplicate retests are considered negative for Anti-HIV.

4. If the specimen is repeatably reactive, the probability that Anti-HIV is present is high, especially in subjects at increased risk for HIV infection or in specimens with very high absorbance values. In most settings, it is appropriate to investigate repeatably reactive specimens by additional, more specific or supplemental tests. Those specimens found repeatable reactive by ELISA and positive by supplemental testing are considered positive for Anti-HIV. The interpretation of samples repeatably reactive by ELISA but negative with supplemental testing is unclear; further clarification may be obtained by testing another specimen taken three to six months later.

REPORTING OF RESULTS

The final output from the Organon computer printer will be a listing of all specimens tested on the run. Quality Control data regarding the Negative Control Mean, the Positive Control Mean, the Positive - Negative Control Value and the Minimum Reactive Value are displayed at the top of the form. The setup and readout technologist identification are also displayed. Next to be printed is a listing of the well position, specimen identification, absorbance and interpretation for all controls and specimens on the run.

To report the results of the run, the technologist will call the BSB program on the Laboratory Computer System. Results are reported based on the identification number assigned. Each absorbance value is checked against the Minimum Reactive Value to verify that the Organon Computer interpretation is accurate. If results agree, the results of all non-reactive tests may be reported. The default result is NEG and can be entered by pressing the ENTER key.

Samples giving a REACTIVE result on initial testing must be repeated in duplicate on the next available run. If both retested samples give a non-reactive result, the specimen may be reported in the Laboratory Computer System as NEGRPTX2 (NEG RPT). If one or both of the retested samples is REACTIVE, the specimen is reported as POSRPTX2 (POS RPT) with a footnote to indicate that the Western Blot has been ordered and is pending. A sample of the Repeatably REACTIVE specimen should be sent to Gulf Coast Regional Blood Center with a completed copy of one of their test request forms via the Blood Bank (Brown Second Floor Room 254) as soon as is practical. The Western Blot should be entered into the Laboratory Computer System by way of the LOE program using the same accession or unit number that the Anti-HIV specimen carried.

Any patient or donor found repeatably reactive for Anti-HIV must be written on the "Positive" clipboard and reported to the patient's physician (or to Dr. Yawn in the case of a blood donor). This report is made by telephone. The name of the person receiving the call in the physician's office (if other than the physician himself) must be noted on the "Positive" clipboard. In addition, Infection Control is telephoned and notified of the repeatably reactive result and the name of the Infection Control nurse noted on the "Positive" clipboard. Repeat reactive reports from the 11-7 shift should be left for the 7-3 technologist to call, but must be written on the "Positive" clipboard by the 11-7 technologist.

PROCEDURAL NOTES AND PRECAUTIONS

1. All test kit components used in an assay must be of the same master lot number. Materials should not be used after the expiration date shown on the package label. Components and test specimens should be at ambient temperature (20-25 deg. C.) before testing begins. Return reagents to refrigerator (2-8 deg. C.) after use.

2. Strips of the microelisa plate are removable. Remove strips of antigen coated wells not needed and replace with antigen free strips so that strip holder contains 96 wells . Store unused antigen coated strips in foil pouch with the clamp and rod closure. Before testing begins, the user should inspect the microelisa strip holder and ensure that all wells are secure. Strip holders should be handled with care to ensure that no strip is dislodged during the test process.

3. Microelisa strips may be used only once.

4. To avoid contamination, do not touch the top of strips or edge of wells with with fingers or pipet tips.

5. All reagents and specimens must be well mixed before use.

6. Use a separate disposable pipet tip for each sample.

7. All pipetting steps should be performed with the utmost care and accuracy. Cross-contamination between reagents will invalidate test results.

8. Avoid microbial or any other contamination of reagents by using aseptic technique to remove aliquots from original vials.

9. Refrain from opening door of incubator during the incubation time.

10. Dispose of all materials as biohazardous waste.

11. Check accuracy of the automated diluter system (Cavro) by weight each quarter and with eppendorf pipettors once per day.

LIMITATIONS OF THE PROCEDURE

The Vironostika HIV ELISA antibody procedure and the Interpretation of Results must be followed closely when testing for the presence of Anti-HIV in plasma or serum from individual subjects. Because the ELISA was designed to test individual units of blood or plasma, most data regarding its interpretation were derived from testing individual samples. Insufficient data are available to interpret tests performed on other body fluids, pooled blood or processed plasma, and products made from such pools; testing of these specimens is not recommended.

Vironostika HIV ELISA detects antibodies to HIV in blood and thus is useful in screening blood and plasma donated for transfusion and further manufacture, in evaluating patients with signs or symptoms of AIDS, and in establishing prior infection with HIV. Clinical studies continue to clarify and refine the interpretation and medical significance of the presence of HIV antibodies.

For most uses, it is recommended that repeatably reactive specimens be investigated by an additional more specific or supplemental test. A person who has antibodies to HIV is presumed to be infected with the virus and appropriate counseling and medical evaluation should be offered. Such an evaluation should be considered an important part of HIV antibody testing and should include test result confirmation on a freshly drawn sample.

AIDS and AIDS-related conditions are clinical syndromes and their diagnosis can only be established clinically. ELISA testing alone cannot be used to diagnose AIDS, even if the recommended investigation of reactive specimens suggests a high probability that the antibody to HIV is present. A negative test result at any point in the investigation of individual subjects does not preclude the possibility of exposure to, or infection with, HIV. The risk of an asymptomatic person with a repeatably reactive serum developing AIDS or AIDS-related disease is not known.

Data obtained from testing persons both at increased and low risk for HIV infection suggest that repeatably reactive specimens with high absorbance by ELISA are more likely to demonstrate the presence of the HIV antibodies by additional more specific or supplemental testing. Reactivity at or only slightly above the Minimum Reactive Value is more frequently nonspecific, especially in samples obtained from persons at low risk for HIV infection; however, the presence of antibodies in some of these specimens can be demonstrated by additional more specific or supplemental testing.

APPENDIX 5

The Blood Center

Bill T. Teague, BS MT (ASCP) SBB
President
Chief Executive Officer
Clarence P. Alfrey, M.D.
Medical Director

January 15, 1989

David Yawn, M.D.
Pathologist
The Methodist Hospital
6565 Fannin
Houston, Texas 77030

Dear Dr. Yawn:

Quality Control

Negative and weakly reactive control strips are run with every run of Western Blots. A strongly reactive control is run at least once with each new lot of Western Blots.

Proficiency Surveys

We participate in the following Western Blot proficiency programs:

- College of American Pathologists Blood Bank Proficiency Program
- Centers for Disease Control model Performance Evaluation program: HIV-I
- State of New York Department of Health Diagnostic Immunology Program

If you have any questions please call me at (713) 791-6285.

Sincerely,

H. David Fortenberry
Director
Laboratory Services

DF/rf

GULF COAST REGIONAL BLOOD CENTER
CLINICAL RESEARCH AND DEVELOPMENT PROCEDURES

5-100 HIV WESTERN BLOT PROCEDURE
For detection of antibodies to HIV

01 INTENDED USE
The HIV Western Blot test is utilized on human serum or plasma specimens found to be repeatably reactive using a screening procedure such as enzyme-linked immunosorbent assay (ELISA).

02 PRINCIPLE OF THE TEST
The Western Blot test will detect antibodies to HIV when present in human serum or plasma. Individual nitrocellulose strips coated with inactivated virus (H9/HTLV-III B T-Lymphocyte Cell Line) are incubated with serum or plasma specimens, and controls. During incubation, if HIV antibodies are present in the specimen, they will bind to the viral antigens bound to the nitrocellulose strips. The strips are washed to remove unbound material. Visualization of the Human Immunoglobulins specifically bound to HIV proteins is accomplished using a series of reactions with goat anti-human IgG conjugated with biotin, avidin conjugated with horseradish peroxidase (HRP), and the HRP substrate 4-chloro-1-naphthol. If antibodies to any of the major HIV antigens are present in the specimen in sufficient concentration, bands corresponding to the position of one or more of the following HIV proteins (P) or glycoproteins (GP) will be seen on the nitrocellulose strip: P17, P24, P31, GP41. P51, P55, P66, GP120, GP160.

03 REAGENTS
a. Nitrocellulose strips - coated with separated antigenic protein from partially purified inactivated HIV.

b. Non-reactive control.

c. Weakly reactive control - low titer.

d. Strongly reactive control - high titer.

e. Wash buffer - 20 x concentrate
Diluted contains 0.02 M Tris + 0.1 N NaCl + 0.1 N NaCl + 0.3% tween 20 + 0.005% thimerosal (Preservative) Ph 7.4.

f. Blotting buffer - 10x concentrate.
Diluted contains 0.02 M tris + 0.1 M NaCl + Heat inactivated normal goat serum + 0.01% thimerosal (Preservative), Ph 7.4.

g. Conjugate 1 - Biotinylated goat anti-human IgG antibodies

h. Conjugate 2 - Avidin conjugated horseradish peroxidase.

i. Substrate A - 7.8 mm solution 4-chloro-1-naphthol in alcohol.

j. Substrate B - Aqueous hydrogen peroxide (0.02%) in citrate buffer.

k. Blotting powder - nonfat dry milk (non-sterile).

04 PRECAUTIONS

a. Handle all specimens, strips, and controls as if capable of transmitting an infectious agent.

b. Do not pipet my mouth.

c. Wear gloves during the testing procedure and when disinfecting/decontaminating.

d. Wipe spills immediately with a 1:5 (=1%) dilution of liquid household bleach.

e. Dispose of all contaminated materials as biohazard waste and incinerate.

f. Liquid waste must be disinfected with an equal volume of 5% sodium hypochlorite solution (Liquid household bleach) for 60 minutes before discarding.

g. Substrate should not come in contact with skin.

05 MATERIALS REQUIRED

a. HIV Western Blot Test Kit.

b. Rocker or rotary platform

c. Pipets (10 ml)

d. Pipet tips and pipettor (0-50 microliters)

e. Aspirator with disinfectant trap.

f. Plastic forceps.

06 PROCEDURAL NOTES

a. The kit supplies only enough reagents for exact testing. Do not make extra amounts.

b. Working blotting buffer.
Do not shake the working blotting buffer (unless absolutely necessary) because the bubbles formed results in insufficient quantity for testing. Instead, make the working blotting buffer 15-30 minutes before use to allow for proper dissolution of powder.

c. Conjugate
Use separate tubes to make each of the conjugate solutions. Do not use the same container.

d. Substrate
1. Avoid contact with metal objects which cause the reduction of peroxide.

2. Shield the working substrate solution from sunlight during preparation and use within 30 minutes of mixing.

07 PROCEDURE

a. Allow reagents to reach room temperature before use (approximately 30 minutes).

b. Prepare the working blotting buffer needed for the 1st day. Let dissolve while proceeding to the next step.

# STRIPS	BLOTTING BUFFER	+	DI WATER	+	BLOTTING POWDER
1	0.2ml		1.8ml		0.1G
2	0.4		3.6		0.2
3	0.6		5.4		0.3
4	0.8		7.2		0.4
5	1.0		9.0		0.5
6	1.2		10.8		0.6
7	1.4		12.6		0.7
8	1.6		14.4		0.8
9	1.8		16.2		0.9

c. Add 2ml diluted wash buffer to each well.

Diluted wash buffer =
60 ml (1 bottle) wash buffer + 1140 ml D.I. water
or
1 volume wash buffer + 19 volumes D.I. water

Diluted wash buffer may be stored at room temperature for 3 months.

d. Using plastic forceps, carefully remove a nitrocellulose strip from the vial and place numbered side up into a well containing 2 ml diluted wash buffer.

e. Incubate the strips for 30 minutes at room temperature, then remove the buffer by aspiration.

f. Add 2.0 ml of working blotting buffer (prepared in step b) to each well. Place the tray on a rotator for 5-10 minutes at room temperature. Set the rotator for gentle agitation (approximately 50 RPM's). Orient the tray so that reagents mix lengthwise along the strips.

g. Fill out the HIV Western Blot worksheet (GC315) and assign samples a strip number.

h. Add 20 microliters of each undiluted specimen or control to a well containing its assigned strip in working blotting buffer. A negative control and weak reactive control must be included with each run. The strong reactive control must included with the first run of specimens for each kit.

i. Carefully cover the tray and incubate on the rotator overnight at room temperature. The rotator should be at approximately 50 RPM's.

DAY 2

j. Remove blotting buffer and conjugate 1 from the refrigerator and allow to come to room temperature before use.

k. Prepare working blotting buffer (enough to use with conjugate 1 and 2)

# STRIPS	BLOTTING BUFFER	+	DI WATER	+	BLOTTING POWDER
1	0.4ml		3.6ml		0.2G
2	0.8		7.2		0.4
3	1.2		10.8		0.6
4	1.6		14.4		0.8
5	2.0		18.0		1.0
6	2.4		21.6		1.2
7	2.8		25.2		1.4
8	3.2		28.8		1.6
9	3.6		32.4		1.8

l. Mix and allow to dissolve. Divide into two tubes (one for conjugate 1 and the other for conjugate 2).

m. Very carefully uncover the tray to avoid splashing or mixing of specimens. Remove condensation on the incubation tray lid by wiping with absorbent towels.

n. Aspirate the mixture from the wells into a trap. Rinse aspirator tip with water first and then diluted wash buffer between samples to avoid cross contamination.

o. Rinse each strip once with 2.0 ml of diluted wash buffer and immediately remove buffer by aspiration.

p. Wash each strip with 2.0 ml of diluted wash buffer and rotate for 5 minutes before aspirating the wash buffer

q. Repeat step p.

r. Add 2.0 ml of working conjugate 1 solution (prepared as directed in supplemental instruction sheet that comes with each package insert - NOTE: the amount of conjugate concentrate may change with each new kit). Incubate for 60 minutes at room temperature on the rotator.

s. Aspirate the conjugate from the wells. Wash each strip 3 times for 5 minutes using 2.0 ml of diluted wash buffer for each wash. Aspirate the buffer from the wells between washes (rinsing the tip each time with D.I. water and then buffer).

t. Add 2.0 ml of working conjugate 2 solution (prepared as directed in supplemental instructions sheet of the package insert) to each well. Incubate for 60 minutes at room temperature on the rotator. Remove substrate A and substrate B from the refrigerator to allow to warm to room temperature.

u. Aspirate the conjugate from the wells and wash each strip 3 times as in step s above. Prepare the working substrate during the last wash incubation by adding equal volume of A & B. The volume of A & B to use is equal to the number of strips set up (Example: 9 strips = 9 ml Substrate A + 9 ml Substrate B)

v. Add 2.0 ml of working substrate solution to each well and incubate for 10-15 minutes at room temperature on the

kit. The interpretation process requires two (2) steps.

a. Each band which appears on the test strip must be assigned a molecular weight based on its position: P17, P24, P 31, GP41, P51, P55, P66, GP120, GP160. Use the Strongly Reactive Control as a reference for position.

b. Each band is assigned a reactivity score based on its intensity: -, +/-, +,2+. Use the Weakly (P24) Reactive Control as a reference for intensity.

Intensity of Band	Reactivity Score	Report As
Absent	-	Not Detected
Less than the intensity of p24 on the Weakly Reactive Control	+/-	Detected
At least as intense as p24 on the Weakly Reactive Control but less intense than p24 on the Strongly Reactive Control	+	Detected
Greater than or equal to the intensity of p24 on the Strongly Reactive Control	2+	Detected

Record the reactivity score on the worksheet. These will be reported in letter form as indicated above.

10 INTERPRETATION OF RESULTS

a. The following statement issued by the CDC will be used for interpretation:

"According to the Center for Disease Control (CDC) Detection of Antibody to Glycoprotein 41 and/or protein 24 is indicative of the presence of antibodies to HIV."

b. As an alternative, the manufacturer recommends interpreting results as "Negative", "Indeterminate", or "Positive" based on the pattern which is present, according to the following table:

Pattern	Interpretation
No bands present	NEGATIVE
Any bands present but pattern does not meet criteria for POSITIVE	INDETERMINATE
A band is present at P24, P31, and either gp41 or gp160. Each band has a reactivity score of + or greater. Commonly, the band at gp41 or gp160 is diffuse.	POSITIVE

11 COMMENTS

a. Results that are not characteristic of typically reactive samples may require a comment such as:

1. LIMITED QUANTITIES OF PROTEIN WERE SEEN IN THE P_____ BAND AREA. THE SIGNIFICANCE OF THIS OBSERVATION IS CONTINGENT ON THE CLINICAL SITUATION OF THIS PATIENT. IF THE CLINICAL SITUATION IS INDICATIVE OF POTENTIAL HIV INFECTION, IT IS SUGGESTED THAT AN ADDITIONAL SAMPLE BE COLLECTED AND TESTED IN 3 TO 6 MONTHS FOR PROPER FOLLOW-UP.

2. LIMITED QUANTITY OF PROTEIN WAS SEEN IN THE P_____ BAND. IT IS SUGGESTED THAT AN ADDITIONAL SAMPLE BE COLLECTED AND TESTED IN 3 AND 6 MONTH INTERVALS FOR PROPER FOLLOW-UP.

3. LIMITED QUANTITIES OF PROTEIN WERE SEEN IN THE GP_____ BAND AREA, AND THE PROTEINS SEEN WERE NOT COMPLETELY CHARACTERISTIC OF THIS BAND. DUE TO THE FACT GP120 AND GP160 WERE NOT DETECTED, FOLLOW-UP SAMPLES ARE RECOMMENDED IN 3 AND 6 MONTHS INTERVALS.

To reference a comment, place an "*" next to the band results. Example: P24: *DETECTED or P24: *NOT DETECTED.

b. Indeterminate interpretations may require a comment such as: "It is known that persons who have recently seroconverted may display incomplete patterns but they will develop increased reactivity when followed for a period of 4-6 months. Therefore, follow up testing is suggested.

12 SIGNATURES

The technologist performing the test and the laboratory director should review and sign all completed report forms.

13 SENDING OUT RESULTS

Results are sent out on Blood Center letterhead and are delivered by the Hospital Services Department or regular US mail.

APPENDIX 6

ACTG VIROLOGY MANUAL

Version 6

Nov 6, 1989

INTRODUCTION

This manual is a compilation of laboratory procedures, laboratory certification standards, guidelines, technical recommendations and policy decisions recommended by the virology technical subcommittee and approved by the virology core committee of the ACTG.

It will be continuously revised and updated as new procedures are introduced. Updated versions will be available on the VAX system as they are prepared. Proposed changes and additions will be sent out for comments and suggestions to Virology Laboratory Directors and Principal Investigators prior to their final adoption in the manual. Suggestions for changes and additional coverage are strongly encouraged.

CONSENSUS CULTURE PROTOCOL
(From the VRL)

This was developed by the VRL from protocols used in reference laboratories. It is mandatory only for labs which have not achieved certification.

1. Processing of patient PBMCs
   a) Centrifuge the anticoagulated blood at 4-800g (room temperature, 20-24°C) for 20-30 minutes and remove most of the plasma.
      1. The plasma should be aliquoted and labelled for storage.
   b) To one part blood cells add one part diluent (saline or PBS), e.g., 8 ml blood to 8 ml diluent.
   c) Add 3 parts lymphocyte separation medium (Organon or Pharmacia) to 4 parts of diluted blood, e.g., 12 ml LSM to 16 ml diluted blood.
   d) Centrifuge at 400 x g, 20-24°C, for 30 min, remove lymphocyte layer, and wash twice in two volumes of PBS or Hanks solution.
2. Processing of donor PBMCs
   a) Stimulation Medium for Donor PBMCs:
      RPMI-1640 with glutamine, 20% FBS (heat inactivated), PHA-P (3 ug/ml, Difco or Sigma) 3-5% IL-2 (Cellular Products, Electro-Nucleonics) penicillin (100 units/ml)/streptocmycin (100 ug/ml) or gentamicin (50 ug/ml).
      Note: IL-2 should be purified human, not recombinant.
      Amphotericin B should be avoided as it may inhibit HIV.
   b) Do not use frozen donor cells or pooled donor PBMCs.
   c) Process random donor buffy coats within 12 hours of collection.
   d) PBMC concentration = 2 million PBMCs/ml.
   e) Incubate 1-3 days before initiating HIV coculture.
   f) Culture donor PBMCs separately to verify absence of HIV infectivity.

3. HIV coculture procedure
   a) Coculture Medium for HIV Isolation:
      RPMI-1640 with glutamine, 20% FBS, 5% IL-2 (Cellular Products, Electro-Nucleonics) penicillin (100 units/ml)/streptocmycin (100 ug/ml) or gentamicin (50 ug/ml).
   b) Coculture patient PBMCs with washed PHA-stimulated donor cells within 8 hours of collection.
   c) Maintain a ratio of donor PBMCs to patient PBMCs of 1:1.
   d) Final PBMC concentration = 2 million PBMCs/ml
   e) Add fresh pelleted PHA-stimulated donor cells resuspended

in coculture medium to HIV coculture once a week.

f) Remove one-half volume of coculture medium every 3-4 days and replace with an equal volume of fresh coculture medium. The removed medium is saved (-30° to -80°) for p24 determination.

g) Assaying medium:
   1) Fresh or frozen medium may be tested
   2) Clarification is optional for thawed samples

1
RECOMMENDATIONS FOR THE COLLECTION AND STORAGE OF SERUM FOR HIV-p24 ANTIGEN DETERMINATION

The success of several ACTG protocols depends in part upon the adequate collection and preservation of aliquotted serum for quantitative HIV-p24 antigen (p24 Ag). Currently, there is uncertainty about the stability of p24 Ag at the typical serum storage conditions of -20° to -80°C, and it is unclear what future serologic testing will be required for the stored serum specimens. It is our goal to collect, catalog and store specimens which will be usable for as wide a range of tests as possible. Therefore the Virology Technical Subcommittee recommends that the following policy concerning serum storage be adopted by the Virology Core Committee and that this then be passed on to the protocol virologists and incorporated as an appendix to existing and future protocols.

These are put forward as general recommendations which can be altered, by the protocol virologist, to fit individual studies.

Serum Storage

The amount of serum to be stored will depend upon the number of anticipated interim analyses to be performed for each protocol. The following generic procedure should encompass most requirements but will need to be reviewed by the protocol virologist during the design of each clinical protocol.

Recommendations for ADULT patients.

1. For each clinic visit requiring a p24 Ag determination a volume sufficient to obtain a minimum of 5 ml of serum should be obtained. This is usually about two-7ml tubes. The use of serum separation tubes should result in a better serum yield from each tube. The serum should be aliquotted (0.5 to 1 ml) and stored at -30° to -70°C.

2. One ml of serum obtained at each sampling interval in (1) should be set aside and stored undisturbed until the completion of the study.

3. The same aliquotting and storage specifications apply to plasma.

4. A log of each specimen with identifier and storage location must be kept to facilitate retrieval. Tubes should be labelled with an identifier, lab number and, possibly specimen date.

The Dataworks Laboratory Management software has a serum storage menu. Laboratory technicians are encouraged to use this facility to record the freezer location of the serum specimens. This will facilitate rapid retrieval of serum specimens for interim analysis as well as providing the protocol virologist with information about the number of serum specimens stored per study patient.

5. Variations from these recommendations should be communicated in writing to the protocol virologist.

PEDIATRIC HIV CULTURE

1. Blood Collection

   a. Two to five milliliters of preservative free heparinized blood should be drawn and immediately transported to the laboratory. The exact volume of blood drawn should be based on reviewing the patients hemoglobin, hematocrit, absolute lymphocyte count and general clinical status. However, in general, the above suggested volume should provide adequate cells for culture.

2. Processing of patient PBMCs

   a. The anticoagulated blood should be centrifuged at 800g for 20-30 minutes to separate most of the plasma.

   b. The plasma should be aliquoted and labeled for storage. Refer to the section on Collection and Storage of Serum in the virology manual for storage requirements.

   c. To one part of separated RBC add one part diluent (PBS or saline) e.g., 5 ml blood to 5 ml diluent.

   d. Add 3 parts lymphocyte separation medium (Pharmacia, Sigma or Organon) to 4 parts of diluted blood, e.g. 9 ml separation medium to 12 ml of diluted blood.

   e. Centrifuge at 400 x g at 20-24°C for 30 minutes. Harvest mononuclear cell layer and wash twice with 5-120 ml of Hanks or PBS salt solution.

3. Processing for donor PBMCs

   a. Stimulation Medium for donor PBMCs:

      RPMI-1640 with glutamine 20% FBS PHA-P (3 µg/ml, Difco or Sigma), 3-5% IL-1 (Cellular Products, Electro-Nucleonics) penicillin (100 units/ml)/streptomycin (100 µg/ml) or gentamicin (50 µg/ml).

   NOTE: IL-2 should be crude or purified human and not recombinant IL-2.

      Amphotericin B should be avoided as it may inhibit HIV.

      Donor MNC should be processed within 12 hours of collection.

MNC should be adjusted to 2 x 10^6/ml.

Feeder cells should be incubated 1-3 days prior to initiating HIV coculture.

Donor MNC should be incubated separately to confirm absence of HIV infectivity.

4. Viral Coculture Procedure

   a. Culture medium for HIV isolation: RPMI-1640 with glutamine 20% FBS, 5% IL-2, penicillin/streptomycin and/or gentamicin.

   b. Coculture patient MNCs with washed PHA-stimulated donor cells within 4-8 hours of collection.

   c. A 1:2 ratio of donor MNCs to patient MNCs should be maintained. Record actual number of patient MNCs which were used to initiate culture.

   d. Concentration of total MNCs should be equal to 1 x 10^6 MNCs/ml.

   e. Fresh pelleted PHA-stimulated donor cells resuspended in coculture medium should be added once a week to HIV culture.

   f. One-half volume of the coculture medium should be removed every 3-4 days and replaced with an equal volume of fresh coculture medium. The removed medium is saved for P24 antigen determination.

FREEZING AND THAWING CELLS

Freezing of peripheral blood cells will be part of studies in the future. These will be studied as new technologies, such as the polymerase chain reaction, are developed.

This method is suggested for laboratories who are not experienced or who are not currently freezing cells.

Freezing: (final cell concentration=$1X10^7$/ml)

Materials:

1. Freezing Media: 20% DMSO + 80% fetal bovine serum, heat inactivated, on ice. DMSO-FBS should be prepared fresh every 3-5 days.

2. Growth Media, containing at least 20% FBS, on ice.

3. Freezing Tray or glass container filled with methanol, -70°C in revco. Alternatively, place the tubes at -70° in a styrofoam box for 12 hours or overnight.

4. Cryo-preserving tubes such as Nunc vials, 1 ml on ice.

5. Ice

Procedure:

1. Centrifuge cells, 4-800g/10 min., room temperature.

2. Resuspend in growth media to $2X10^7$/ml (keep on ice). This is a 2X concentration.

3. Drop by drop, add freezing media (gently swirl to mix). (To 1X concentration).

4. Aliquot 1 ml per nunc vial.

5. Place in methanol in Revco overnight (can leave longer if necessary).

6. Transfer to liquid nitrogen.

THAWING:

Materials:

1. Growth Media, at least 20% FBS, 37°C.
2. 37° water bath.
3. 50 ml sterile centrifuge tube.

Procedure:

1. Thaw rapidly in waterbath.
2. Transfer cells to 50 ml tube, using 1 ml pipette.
3. Drop by drop add warm media, swirling occasionally (gently).
4. After 10 ml have been added, fill tube to top. Mix gently.
5. Let tube sit 10-20 minutes.
6. Centrifuge slowly, 900 RPM/8-10 minutes.
7. Discard supernatant and wash again in 50 ml media.
8. Resuspend pellet in 10 ml media, mixing with pipette motion.
9. Count with hemacytometer (use trypan blue) and dilute appropriately.
10. Transfer to flask.

ACTG AND CGS LABORATORY CERTIFICATION FOR HIV CULTURE AND p24 ANTIGEN DETERMINATION

1. Laboratories will undergo quarterly evaluation for HIV isolation proficiency based on the analysis of four (4) samples per month for a total of twelve (12) samples each quarter.

STATUS OF LABORATORY	TESTING INTERVAL
Certified	Monthly: When certified for two (2) consecutive quarters. The testing interval will be every (3) months.
Provisionally Certified	Monthly
On Probation	Monthly

Laboratories may move to certified status after performing at the certified level for 2 rounds of testing.

2. Only laboratories that are Certified for two (2) consecutive quarters in both HIV culture and p24 antigen assays will be eligible to participate in Program-sponsored projects designed to evaluate new quantitative bioassay procedures.

3. Data from Certified and Provisionally Certified laboratories can be used for virologic endpoints.

4. Laboratories on probation for p24 antigen assay will not be permitted to do p24 testing until they become certified. Specimens from centers using that laboratory will be sent to a certifed laboratory for real time or batch testing.

5. Laboratories that remain On Probation for two (2) consecutive quarters will receive the following formal notification from the Program Office:

   You will not be permitted to participate in any future treatment protocols requiring virologic endpoints UNLESS THERE IS IMPROVEMENT IN YOUR PERFORMANCE DURING THE THIRD CONSECUTIVE QUARTER TO AT LEAST Provisionally Certified STATUS IN THE FOURTH CONSECUTIVE QUARTER.

6. Laboratories On Probation will have their protocols reviewed by the Technical Subcommittee, and recommendations, which may include adopting the consensus protocol, will be made for remedial action. Until these laboratories achieve at least a Provisionally Certified status, all patient samples must be sent to a Certified laboratory for testing.

7. Laboratories that retain a Provisionally Certified status for three (3) consecutive quarters will be placed On Probation.

8. Laboratories which are delinquent in reporting their QC data to RTI will be notified and a copy of this notification will be sent to the Technical Subcommittee and the Program Office.

   Four (4) delinquency notifications to the same laboratory in (1) year, or three (3) if the laboratory is Certified, will AUTOMATICALLY reduce that laboratory to On Probation. This status will be maintained for a minimum of 3 months or until the next quarterly evaluation period is completed, whichever interval is longer.

BATCH TESTING POLICY

The protocol virologist will assure that the times and frequencies of testing of stored specimens are indicated in each protocol. The protocol virologist will make recommendations about the type of kit to be used. VRL standards will be used in all assays. Laboratories must be certified as described in the section on "Laboratory Certification for HIV Culture and p24 Antigen Determination." Except when a test is a criteria for patient recruitment and therefore must be done in real time, i.e. at the time of entry into the study, all testing of stored specimens will be done by batch testing unless and until it can be shown that real time testing is equivalent.

Batch testing will be done at the completion of the protocol or, at intervals specified in the protocol. Specimens from the same patient will be identified and assayed in the same assay run. Interim batch testing in protocols will generally consist of tests on specimens collected since the previous analysis, not on all specimens collected since the beginning of the study.

A sufficient number of aliquots of serum must be stored so that testing can be performed at specified intervals. This will avoid repeated freezing and thawing. In addition, as specified under "Recommendations for the Collection and Storage of Serum for HIV-p24 Antigen Determination," an aliquot of each serum specimen will be stored for analysis at the end of the study.

CRITERIA FOR p24 FOR ENTRY INTO STUDY

The consensus opinion of the technical subcommittee and VRL at this time is that the minimum HIV concentration required for entry into a study should be >=25 picograms/ml (corrected) of p24 antigen as determined from the VRL standard calibrator curve and the QC sample. It appears at this time that this should be easily measurable in certified laboratories, that most specimens will contain antigen (i.e. the false positivity rate will be low) and that a 50% change or response can be determined from this level. This is an interim recommendation which is based on the performance of certified laboratories with the proficency panels and will be evaluated and periodically adjusted based on the results obtained from proficiency panels and protocols.

A number of factors come into the selection of entry criteria for a study. These include the nature of the study, the importance of virologic endpoints in the study, the effect on recruitment of a high entry value (which few candidates could meet, but which would be associated with a low false positive rate) as opposed to a low entry value (which more candidates could meet, but which might be associated with a higher false positivity rate) and the ability to accurately measure a significant change.

It should be noted that kits from different manufacturers measure different viral antigens and that there is no international p24 antigen standard.

CRITERIA FOR p24 CUTOFF

Based on certified laboratory performance in the first three proficiency panels, using VRL standards, the reactive threshold value will be determined by adding 0.050 to the mean absorbance value of the negative controls. This is equivalent to 4 standard deviations above the means of the negative controls, and also approximates the recommendations of various manufacturers. Absorbance values below this level are negative by these criteria while those above this level are positive. Specimens that fall within +/- 10% of the cutoff level may need to be distinguished in the final analysis andmay require repeat testing to confirm the initial result.

Negative control values:

Individual negative control values which do not fall between 0.5 and 1.5 times the mean negative control value should be discarded. If one value is outside the acceptable range, the value should be discarded and the mean recalculated. If two or more values are outside the established range, the assay should be repeated.

ROSTER OF VIROLOGY LABORATORY DIRECTORS AND CHIEF TECHNOLOGISTS with address, telelphone, VAX and FAX numbers

(in preparation)

ROSTER OF ACTG PROTOCOL VIROLOGISTS

(in preparation)

ROLE OF THE ACTG PROTOCOL VIROLOGIST

(Currently under revision)

Each ACTG protocol must have a protocol virologist. The protocol virologist for current protocols must be identified. New protocols must have a protocol virologist. Preferably, this individual should be involved from the time the concept sheet is developed. At the latest, the protocol virologist should be involved from the time that a concept sheet has been approved for protocol development. This means that the operations office should be instructed to alert the virology committee about the need to select protocol virologists at the latest when a concept sheet has been approved for protocol development.

For protocols which do not currently have a virologist, one will be assigned from the ACTG or CGS laboratory virologists by the virology committee in conjunction with the protocol chairman.

One or two virology committee members should have for one year the responsibility for selecting the protocol virologist with the Protocol Chairman.

Duties of the Protocol Virologist

1. The protocol virologist is designated by the virology committee in consultation with the protocol chairman. All protocols must have a designated virologist.

2. The protocol virologist participates in protocol development from inception. Following technical sub-committee recommendations, when appropriate, he:

   a. Recommends virologic tests and manufacturer to be used in the protocol.

   b. If virologic tests are to be entry criteria for a study, he or she recommends the procedure for establishing entry criteria. In general, these tests will have to be done in real time, with specimens saved, for batch testing at the end of the study.

   c. Similarly, the protocol virologist recommends criteria for determining responses when virologic endpoints are used in the study.

   d. The protocol virologist assures that the times of analysis are specified in the protocol so that necessary testing will be performed on time and results transmitted.

e. Further, it is the responsibility of the protocol virologist to be sure that virologic test results are performed, transmitted and appropriately analyzed for interim and final analyses.

3. The protocol virologist is responsible for assuring that centers in the study adhere to conditions of specimen collection, volume or cell count, aliquoting, cataloguing, storage, retrieval, timely testing, and data transmission. Exceptions to policy are reviewed and approved by him.

INSTRUCTIONS FOR TRANSMISSION OF DATA TO RTI USING THE DATAWORKS LABORATORY MANAGEMENT SOFTWARE

1. Select the End of Month Procedures Menu from the Main Menu.

2. Select the appropriate export menu option from the End of Month Procedures Menu.

    a. To export/transmit patient culture results, select the Export Culture Results option.

    b. To export/transmit Quality Assurance culture results, select the Export QA Cultures option.

    c. To export/transmit calibration standards and QC check sample results, select the Export Controls option.

    d. To export/transmit direct p24 "serum" results, select the Export Direct p24 Results option.

3. After selecting the appropriate menu option, the computer will automatically generate a flat ASCII file appropriate for transmission to the CTCC according to the file specifications defined in this manual.

4. Select the appropriate viewing export data option from the End of Month Procedures Menu. You must view the data file after it has been generated in order to verify that it was correctly set up. Accidents do happen and data verification is absolutely essential!

5. After the data have been verified, exit from the Lab Management Program and call up Blast.

6. After you are logged on to the VAX, press <CTRL><V> twice to call up the Blast menu.

7. Select the File Transfer option from the Blast menu.

8. After the File Transfer Menu is loaded, select the Send option.

9. The computer will prompt you to enter the local file name:

    Enter \rtiexprt\rtiXXXXX.vir where XXXXX is the appropriate name for the file you are sending. The patient culture file name is rticult.vir. The QA Culture file is rtiqacul.vir. The calibration standards file is rtivrl.vir. The direct p24 "serum" results file is rtisero.vir.

10. The computer will prompt you to enter the remote file name:

    Enter a % sign by itself and the computer will use the same filename entered in step 9.

11. The computer will prompt you to enter options (T for Text, O for Overwrite, A for Append). Press <ENTER> without selecting any of these options. This will transmit the file in binary format.

12. After the computer has successfully transmitted the file(s), press <ESC> to exit from the File Transfer Menu to the Blast Menu.

13. At the Blast Menu, select the Terminal option.

14. At the $ prompt, enter the command "notify". This will let the computer staff at the CTCC know that you have transferred data files to your account which need to be collected. After copies of your files have been made and checked against th originals in your account, the originals will be deleted to prevent their interference in future data file transfers. Then you will be sent a mail message containing information on the status of the transmission, including the names of the files which were collected.

Data should be exported and transmitted once a month, preferably during the first five working days. However, additional transmissions should be performed if you need to meet a deadline (e.g. QA culture data, proficiency panel results, data requested by protocol virologists for interim analyses, etc.).

To enable your account for using the notification procedure, you must first setup your login.com file by executing the following command at the $ prompt:

```
@ disk17:[ses.setup]virsetup
```

This command should be executed only once. In addition to supplying the notification procedure, executing this command will give you access to ACTG distribution lists that are used for sending electronic mail to groups, such as @virlist for all ACTG virologists and @actulist for all ACTG clinics.

DATA BACKUP

Because of the real problems of disc crashes resulting in lost data, laboratories should back up their data frequently, on a regular schedule using the backup feature of the Dataworks software, the DOS backup command or a backup program such as Fastback.

SPECIFICATIONS FOR THE TRANSMISSION OF DATA TO RTI

The following 5 FILESPECS indicate the format in which data must be prepared for transmission to RTI. Note that users of the Dataworks Laboratory Management Software do not have to concern themselves with this as their data is automatically formatted for transmission.

FILE FOR TRANSMISSION OF p24 CONTROL VALUES

Controls File

File Specs for \RTIEXPRT\RTIVRL.VIR

Field no.	Columns	Field name
1	1-2	Lab ID Number
2	3-8	Date of Export
3	9-14	Assay Run Date
4	15	Run Letter
5	16	Run Type (C,D)
6	17	Kit Manufacturer
7	18-26	Kit Lot Number
8	27-33	Slope
9	34-40	Y-Intercept
10	41-46	Mean Squared Error
11	47-53	Correction factor
12	54-58	Cutoff
13	59	Type of Control
14	60-66	Expected Value (pg/mL)
15	67-72	Absorbance
16	73-79	pg/mL
17	80	Type of Control
18	81-87	Expected Value (pg/mL)
19	88-93	Absorbance
20	94-100	pg/mL
21	101	Type of Control
22	102-108	Expected Value (pg/mL)
23	109-114	Absorbance
24	115-121	pg/mL
25	122	Type of Control
26	123-129	Expected Value (pg/mL)
27	130-135	Absorbance
28	136-142	pg/mL
29	143	Type of Control
30	144-150	Expected Value (pg/mL)
31	151-156	Absorbance
32	157-163	pg/mL
33	164	Type of Control
34	165-171	Expected Value (pg/mL)

35	172-177	Absorbance
36	178-184	pg/mL

37	185	Type of Control
38	186-192	Expected Value (pg/mL)
39	193-198	Absorbance
40	199-205	pg/mL
41	206	Type of Control
42	207-213	Expected Value (pg/mL)
43	214-219	Absorbance
44	220-226	pg/mL
45	227	Type of Control
46	228-234	Expected Value (pg/mL)
47	235-240	Absorbance
48	241-247	pg/mL
49	248	Type of Control
50	249-255	Expected Value (pg/mL)
51	256-261	Absorbance
52	262-268	pg/mL
53	269	Type of Control
54	270-276	Expected Value (pg/mL)
55	277-282	Absorbance
56	283-289	pg/mL
57	290	Type of Control
58	291-297	Expected Value (pg/mL)
59	298-303	Absorbance
60	304-310	pg/mL
61	311	Type of Control
62	312-318	Expected Value (pg/mL)
63	319-324	Absorbance
64	325-331	pg/mL
65	332	Type of Control
66	333-339	Expected Value (pg/mL)
67	340-345	Absorbance
68	346-352	pg/mL
69	353	Type of Control
70	354-360	Expected Value (pg/mL)
71	361-366	Absorbance
72	367-373	pg/mL
73	374	Type of Control
74	375-381	Expected Value (pg/mL)
75	382-387	Absorbance
76	388-394	pg/mL
77	395	Type of Control
78	396-402	Expected Value (pg/mL)

79	403-408	Absorbance
80	409-415	pg/mL

81	416	Type of Control
82	417-423	Expected Value (pg/mL)
83	424-429	Absorbance
84	430-436	pg/mL
85	437	Type of Control
86	438-444	Expected Value (pg/mL)
87	445-450	Absorbance
88	451-457	pg/mL
89	458-459	CR/LF

FILE FOR TRANSMISSION OF HIV CULTURES

FILE SPECS FOR RTIEXPRT\RTICULT.VIR

Field no.	Columns	Field name	
1	1-2	Lab ID Number	
2	3-8	Date of Export	
3	9-15	PID	
4	16-23	SID	
5	24-29	Specimen Date	
6	30-35	Set-up Date	
7	36	Specimen Type	(1=SER 2=PLA 3=BLD 4=CSF 5=SEM 6=TIS 7=MAC 8=SAL 0=Other)
8	37-39	Specimen Type - other	

9	40-45	Assay Date #1	51	280-285	Assay Date #7
10	46	Run Letter	52	286	Run Letter
11	47-53	pg/mL	53	287-293	pg/mL
12	54-59	Absorbance	54	294-299	Absorbance
13	60-66	Corrected pg/mL	55	300-306	Corrected pg/mL
14	67-72	Sample Date	56	307-312	Sample Date
15	73-79	Kit pg/mL	57	313-319	Kit pg/mL
16	80-85	Assay Date #2	58	320-325	Assay Date #8
17	86	Run Letter	59	326	Run Letter
18	87-93	pg/mL	60	327-333	pg/mL
19	94-99	Absorbance	61	334-339	Absorbance
20	100-106	Corrected pg/mL	62	340-346	Corrected pg/mL
21	107-112	Sample Date	63	347-352	Sample Date
22	113-119	Kit pg/mL	64	353-359	Kit pg/mL
23	120-125	Assay Date #3	65	360-365	Assay Date #9
24	126	Run Letter	66	366	Run Letter
25	127-133	pg/mL	67	367-373	pg/mL
26	134-139	Absorbance	68	374-379	Absorbance
27	140-146	Corrected pg/mL	69	380-386	Corrected pg/mL
28	147-152	Sample Date	70	387-392	Sample Date
29	153-159	Kit pg/mL	71	393-399	Kit pg/mL
30	160-165	Assay Date #4	72	400-405	Assay Date #10
31	166	Run Letter	73	406	Run Letter
32	167-173	pg/mL	74	407-413	pg/mL

33	174-179	Absorbance
34	180-186	Corrected pg/mL
35	187-192	Sample Date
36	193-199	Kit pg/mL
37	200-205	Assay Date #5
38	206	Run Letter
39	207-213	pg/mL
40	214-219	Absorbance
41	220-226	Corrected pg/mL
42	227-232	Sample Date
43	233-239	pg/mL
44	240-245	Assay Date #6
45	246	Run Letter
46	247-253	pg/mL
47	254-259	Absorbance
48	260-266	Corrected pg/mL
49	267-272	Sample Date
50	273-279	pg/mL
75	414-419	Absorbance
76	420-426	Corrected pg/mL
77	427-432	Sample Date
78	433-439	Kit pg/mL
79	440-445	Assay Date #11
80	446	Run Letter
81	447-453	pg/mL
82	454-459	Absorbance
83	460-466	Corrected pg/mL
84	467-472	Sample Date
85	473-479	pg/mL
86	480-485	Assay Date #12
87	486	Run Letter
88	487-493	pg/mL
89	494-499	Absorbance
90	500-506	Corrected pg/mL
91	507-512	Sample Date
92	513-519	pg/mL
93	520-521	CR/LF

FILE FOR TRANSMISSION OF p24 VALUES

Direct p24

FILE SPECS FOR RTIEXPRT\RTISERO.VIR

Field no.	Columns	Field name
1	1-2	Lab ID Number
2	3-8	Date of Export
3	9-15	PID
4	16-23	SID
5	24-29	Specimen Date
6	30	Specimen Type (1=SER 2=PLA 3=BLD 4=CSF 5=SEM 6=TIS 7=MAC 8=SAL 0=OTHER)
7	31-33	Specimen Type - other
8	34-39	Assay Run Date
9	40	Run Letter
10	41-46	Dilution Factor
11	47-53	pg/mL
12	54-59	Absorbance
13	60-66	Corrected pg/mL
14	67-73	Kit pg/mL
15	74-75	CR/LF

FILE FOR TRANSMISSION OF QA CULTURES

FILE SPECS FOR RTIEXPRT\RTIQACUL.VIR

Field no.	Columns	Field name
1	1-2	Lab ID Number
2	3-8	Date of Export
3	9-12	Specimen ID
4	13-18	Date Received
5	19-24	Set-up Date
6	25-28	Total Lymphocyte Count (x 10 6 per mm3)
7	29-31	Viability (%)
8	32	Specimen Type (1=SER 2=PLA 3=BLD 4=CSF 5=SEM 6=TIS 7=MAC 8=SAL 0=Other)
9	33-35	Specimen Type - other

10	36-41	Assay Date #1	52	276-281	Assay Date #7
11	42	Run Letter	53	282	Run Letter
12	43-49	pg/mL	54	283-289	pg/mL
13	50-55	Absorbance	55	290-295	Absorbance
14	56-62	Corrected pg/mL	56	296-302	Corrected pg/mL
15	63-68	Sample Date	57	303-308	Sample Date
16	69-75	Kit pg/mL	58	309-315	Kit pg/mL
17	76-81	Assay Date #2	59	316-321	Assay Date #8
18	82	Run Letter	60	322	Run Letter
19	83-89	pg/mL	61	323-329	pg/mL
20	90-95	Absorbance	62	330-335	Absorbance
21	96-102	Corrected pg/mL	63	336-342	Corrected pg/mL
22	103-108	Sample Date	64	343-348	Sample Date
23	109-115	Kit pg/mL	65	349-355	Kit pg/mL
24	116-121	Assay Date #3	66	356-361	Assay Date #9
25	122	Run Letter	67	362	Run Letter
26	123-129	pg/mL	68	363-369	pg/mL
27	130-135	Absorbance	69	370-375	Absorbance
28	136-142	Corrected pg/mL	70	376-382	Corrected pg/mL
29	143-148	Sample Date	71	383-388	Sample Date
30	149-155	Kit pg/mL	72	389-395	Kit pg/mL
31	156-161	Assay Date #4	73	396-401	Assay Date #10
32	162	Run Letter	74	402	Run Letter

33	163-169	pg/mL
34	170-175	Absorbance
35	176-182	Corrected pg/mL
36	183-188	Sample Date
37	189-195	pg/mL
38	196-201	Assay Date #5
39	202	Run Letter
40	203-209	pg/mL
41	210-215	Absorbance
42	216-222	Corrected pg/mL
43	223-228	Sample Date
44	229-235	pg/mL
45	236-241	Assay Date #6
46	242	Run Letter
47	243-249	pg/mL
48	250-255	Absorbance
49	256-262	Corrected pg/mL
50	263-268	Sample Date
51	269-275	pg/mL
75	403-409	pg/mL
76	410-415	Absorbance
77	416-422	Corrected pg/mL
78	423-428	Sample Date
79	429-435	pg/mL
80	436-441	Assay Date #11
81	442	Run Letter
82	443-449	pg/mL
83	450-455	Absorbance
84	456-462	Corrected pg/mL
85	463-468	Sample Date
86	469-475	pg/mL
87	476-481	Assay Date #12
88	482	Run Letter
89	483-489	pg/mL
90	490-495	Absorbance
91	496-502	Corrected pg/mL
92	503-508	Sample Date
93	509-515	pg/mL
94	516-517	CR/LF

FILE FOR TRANSMISSION OF PROFICIENCY PANEL RESULTS

FILE SPECS FOR RTIEXPRT\RTIPRPAN.VIR

Field no.	Columns	Field name
1	1-2	Lab ID Number
2	3-8	Date of Export (MMDDYY format)
3	9-14	Date Panel Received (MMDDYY format)
4	15-20	Date of Assay (MMDDYY format)
5	21	Run Letter (used to identify different runs on same day)
6	22	Kit Manufacturer (A, D, C, P)
7	23	Specimen Type (S or M)
8	24-30	Sample Number (PS 4.01, PM 4.01, AB-50, etc.)
9	31-37	Sample Concentration in pg/mL (two digits after decimal)
10	38-43	Absorbance (O.D. three digits after decimal)
11	44-50	Corrected pg/mL (using 100 QC check sample)
12	51-55	Dilution Factor (if any)
13	56-57	Carriage Return/Line Feed

There will be a separate record for each sample run with all samples (both serum and media) in the same export file.

The controls for the run will be sent in the standard format RTIVRL.VIR format and can be linked up at RTI for analysis.

APPENDIX 7

FLOW CYTOMETRY REPORT
ACTG IMMUNOLOGY COMMITTEE
October 6,1988

BACKGROUND

One of the first charges to the ACTG Immunology Committee was to determine whether the T-cell subset numbers being generated by the laboratories at the medical centers involved in the clinical trials were equivalent and could be pooled. The potential variables in performing flow cytometric evaluation of T-cell subsets are many (e.g. flow cytometer itself, method of preparation of cells, anticoagulant, etc.,). It was decided at an early meeting that the best way to evaluate and compare the testing at the laboratories was to have all the laboratories analyze the same specimen at regular intervals. Thus the quality control program of the ACTG was developed. The first specimens were sent out by Fred Valentine (N.Y.U.) subsequent specimens have been sent out by Helene Paxton (Maryland Medical Labs).

Through August, 1988, 18 quality control specimens have been sent to the laboratories performing T-cell subset testing for the ACTG protocols. The Immunology Committee began sending quality control specimens in September, 1987. In December, 1987, additional centers were added to the original 14 participating in the clinical trials. Those new centers began receiving quality control specimens beginning with specimen #004 (in some cases, beginning with #006).

Analysis of the results returned on the quality control specimens has led to the following requirements and recommendations:

[Please note that this report is not meant to be a comprehensive procedure manual. The National Committee for Clinical Laboratory Standards (NCCLS) will soon issue a report with specific detailed methods and accepted standards for flow cytometry.]

1. WBC and differential
   a. The WBC and lymphocyte count should be performed on an automated instrument. If the specimen is rejected or flagged by the machine, then a manual differential (optimally, counting 500 cells) may be performed.
   b. Specimens should be collected in EDTA and should be run within 6 hours of collection.
   c. It is the responsibility of the ACTG immunologist to decide where the WBC and differential counts will be performed and determine that appropriate quality control programs are being utilized and that the laboratory participates in proficiency testing.

2. Anticoagulant for Lymphocyte Subset Testing
EDTA is strongly recommended for sample collection. This recommendation is based on the observation in data from the quality control specimens that the cell populations appear to be more cleanly separated in older EDTA specimens than in the heparin specimens. Use of EDTA has the additional advantage that a WBC and automated differential can be performed on the same tube that is being used for lymphocyte subsets.

3. Handling of Specimens
Specimens should be kept at room temperature (18-22° C) and transported to the laboratory as soon as possible. Blood should be stained for analysis as soon as possible, definitely within 24 hours of collection. If fixation is used, laboratories should establish a protocol based on maintaining the integrity of the specimens (no loss of fluorescence intensity due to fixation or storage), and specimens should be analyzed according to that protocol. Specimens should be identified as to date and time of collection.

4. Preparation of Cells
a. Proper biohazard precautions should be observed in the preparation of cells. (See CDC recommendations in MMWR vol.36, 2S, Aug.,1987, and vol 37, S-4, April, 1988.)
b. The whole blood lysis (stain and lyse) method is mandatory. Ficoll separation of cells is not an acceptable method for preparation of cells. This recommendation is based the findings in the results of quality control specimens that indicated that a significant number of CD8 positive cells were lost in the ficoll process. The whole blood lysis method has the additional benefit over the ficoll method of requiring much less blood from the patient, an important factor for patients on research protocols.
c. Laboratories may choose their own protocol for whole blood lysis.
d. If fixation of cells is not used, then appropriate biohazard precautions must be observed and analysis should be completed within 2 hours of staining.

5. Flow Cytometer
a. The instrument must have 90 degree light scatter capability.
b. Laser based instrumentation is required.
c. Biohazard precautions should be observed. (See CDC reference above, #4a.)
d. Standardization of the instrument:
i. Grade 1 full bright fluorescent beads should be run daily. The CV and mean peak channel fluorescence of the standardization particle should be recorded. Log fluourescence rather than linear should be used.
ii. A standard substraction/compensation protocol should be established for dual analysis using FITC/PE bead standards and/or appropriate mutually exclusive dual antibody stained samples.

iii. A daily QC maintenance log must be maintained for the flow cytometer being used for ACTG samples.

6. Antibodies to be Used
   CD45
   CD13 or CD14 (Currently most labs use CD14 antibodies which react with monocytes. CD13 is found on the surface of neutrophils as well as monocytes and would identify neutrophils, particularly in older specimens, that have fallen into the lymphocyte gated population. We may have to wait for the commercial antibody producing companies to catch up with us on this.)
   CD3
   CD4
   CD8
   CD20 (CD20 is recommended over CD19 because it gives brighter fluorescence, and, therefore, the positive cells are more easily separated from the negative.)
   isotype control

7. One Color Vs. Two Color Analysis
   Dual color analysis is preferred. Single color analysis is acceptable.
   Recommendation for dual color combinations:
   CD45 vs CD13
   CD3 vs CD8
   CD4 vs CD20
   isotype control matched for fluorochromes and antibody concentration

8. Gating
   a. Gating should be performed on 90 degree vs forward angle light scatter (SSC vs. FSC)
   b. An attempt should be made to gate around the lymphocyte population. If the CD45 is <85%, then the gating should be redrawn. If the CD45 is still <85%, an aliquot of the same specimen should be restained (if the specimen is less than 24 hours old). If, in the restained specimen, the CD45 is still <85%, the specimen should be rejected and the data should not be used for the study. The patient should be redrawn if possible.
   c. If CD13 is >5%, then the bitmap should be redrawn in an attempt to decrease the percentage of CD13 positive cells included in the bitmap. If the CD13 is still >5%, correction should be made (see "Data Reporting", #10) or the specimen rejected depending on the judgement of the laboratory director.

9. Data Analysis
   The cursor should be set so that between 0.5% and 2% of the negative cells as defined by the isotype control fall in the positive region (i.e. to the right of the cursor). No substraction should be done. If the specimen is not optimal

and clearly defined clusters of cells fall outside the established cursors, you should restain (re-setup) the specimen if is <24 hours old, try to obtain another specimen on the patient, or report the data as ANR (assay not reliable).

10. Data Reporting

a. Correction for CD45 percent

CD45 % should be ideally >95%. If CD45 is <85%, the specimen should be restained or a new specimen requested. (See "Gating", #8) Correction should be made for CD45 >85% and <100% and for the CD13% as follows:

raw CD4 % = 60%
CD13 (myeloid/monos) = 3%
CD45 = 94%
Corrected CD4 = 60% /(.94-.03) = 66% (really 65.9%)

b. The % and absolute number of CD3, CD4, CD8, and CD20, corrected for CD45 should be reported to R.T.I. (CD45 should be reported as soon as a new form which includes a place to report this result is available from R.T.I.) Uncorrected CD45 and CD13 should also be reported.

QUALITY CONTROL

1. ACTG Quality Control Program

a. Laboratories performing testing for the ACTG program must participate in the quality control program. Failure to return results within the designated period of time or notify the quality control program of a sample problem is considered non-acceptable performance.

b. At this time only percentage numbers will be evaluated for performance (although absolute numbers will still be reported). The acceptable range will be those values falling within the "lower fence" and the "upper fence". The "lower fence" is defined as the lower quartile minus 1.5 times the interquartile range, and the "upper fence" is the upper quartile plus 1.5 times the interquartile range. The interquartile range is the distance between the 25th and 75th percentile. The interquartile range is the estimate of spread and variability on the middle 50% of labs rather than all labs. (Statistics courtesy of Dr. Rebecca Gelman, Harvard.)

c. Outlier values

The laboratory should attempt to identify the cause of the outlier value, review their recent performance with the technical staff to try to identify reasons for the outlier value, and document in writing any reasons why the outlier may have occurred.

d. Erratic outlier values on multiple QC specimens

If flow cytometry test results of a particular laboratory are frequently and sporadically outside the acceptable range without any definite pattern, it can be assumed that the laboratory either has a problem with their flow cytometric procedures or frequently receives blood that has been handled poorly.

It is the responsibility of the flow cytometry laboratory that is generating the outlier values to try to identify the cause for their problem. They should:

i. review their procedures for sample processing and staining;

ii. consult with the manufacturer of their flow cytometer;

iii. contact the quality control contractor (or one of the ACTG consulting laboratories) for assistance;

iv. document what they believe are the reasons why the outliers may be occurring. This documentation will be useful in trying to resolve problems and discrepancies as the quality control program continues to take shape.

e. Outlier with consistent trends

Some laboratories may consistently get outlier values that are too high or too low compared with the median value. The same actions outlined in "d" above should be followed. However, it is possible that if a laboratory consistently obtains high or low values on QC samples that that laboratories data may be "normalized" with respect to the other laboratories.

f. It is anticipated that the ACTG immunologist will receive monthly reports on the performance of the laboratory on the quality control samples from the quality control contractor.

g. Quarterly reports on the laboratory's performance on QC samples will be sent to the ACTG program office, the principal investigator involved, and the ACTG immunologist.

2. Recommended individual lab QC

a. Reproducibility studies

The ACTG immunologist should arrange for the principal investigator, or other ACTG clinician, to send replicate samples (5 samples from the same patient or combinations of 2 samples from one patient and 3 from another, etc.,) once a month. Replicate sample values should be within 5% of each other. The results of these studies should be reported to Dr. Fred Valentine, chairperson of the Immunology Committee.

b. Establishment of normal laboratory performance range

c. A normal fresh specimen should be run each day. Data from these normals can be used to establish the normal laboratory performance range.

d. Instrument performance standards should include daily running of fluorescent labelled beads, and a log should be maintained with daily results (see page 2 under "Flow Cytometer").

ACTG Immunology Committee, Subcommittee on Flow Cytometry

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Janis Giorgi, Ph.D.
Pam Kidd, M.D.
Alan Landay, Ph.D.
Helene Paxton, M.S.
Ed Walker, Ph.D.

APPENDIX 8

FSI

FASI Systems, Inc.
211 Perry Parkway
Gaithersburg, MD 20877
(301) 977-6536

NIAID AIDS PROGRAM
PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

SUMMARY REPORT
OCTOBER 1989

Texas Children's Hospital
Department of Immunology
6621 Fannin
Houston, TX 77030

(713) 798-1319

NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

LABORATORY: Texas Children's Hospital

OCTOBER 1989

HEMATOLOGY RESULTS VERIFICATION

INSTRUMENT/METHOD USED FOR WBC: Technicon H-1

INSTRUMENT/METHOD USED FOR DIFFERENTIAL: Technicon H-1

SPECIMEN	891011	891012	891013
ABSOLUTE WHITE COUNT	5500	8300	2600
PERCENT LYMPHOCYTES	31	26	50
ABSOLUTE LYMPHOCYTE COUNT	1705	2158	1300

FSI FAST Systems, Inc.

NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

LABORATORY: Texas Children's Hospital

OCTOBER 1989

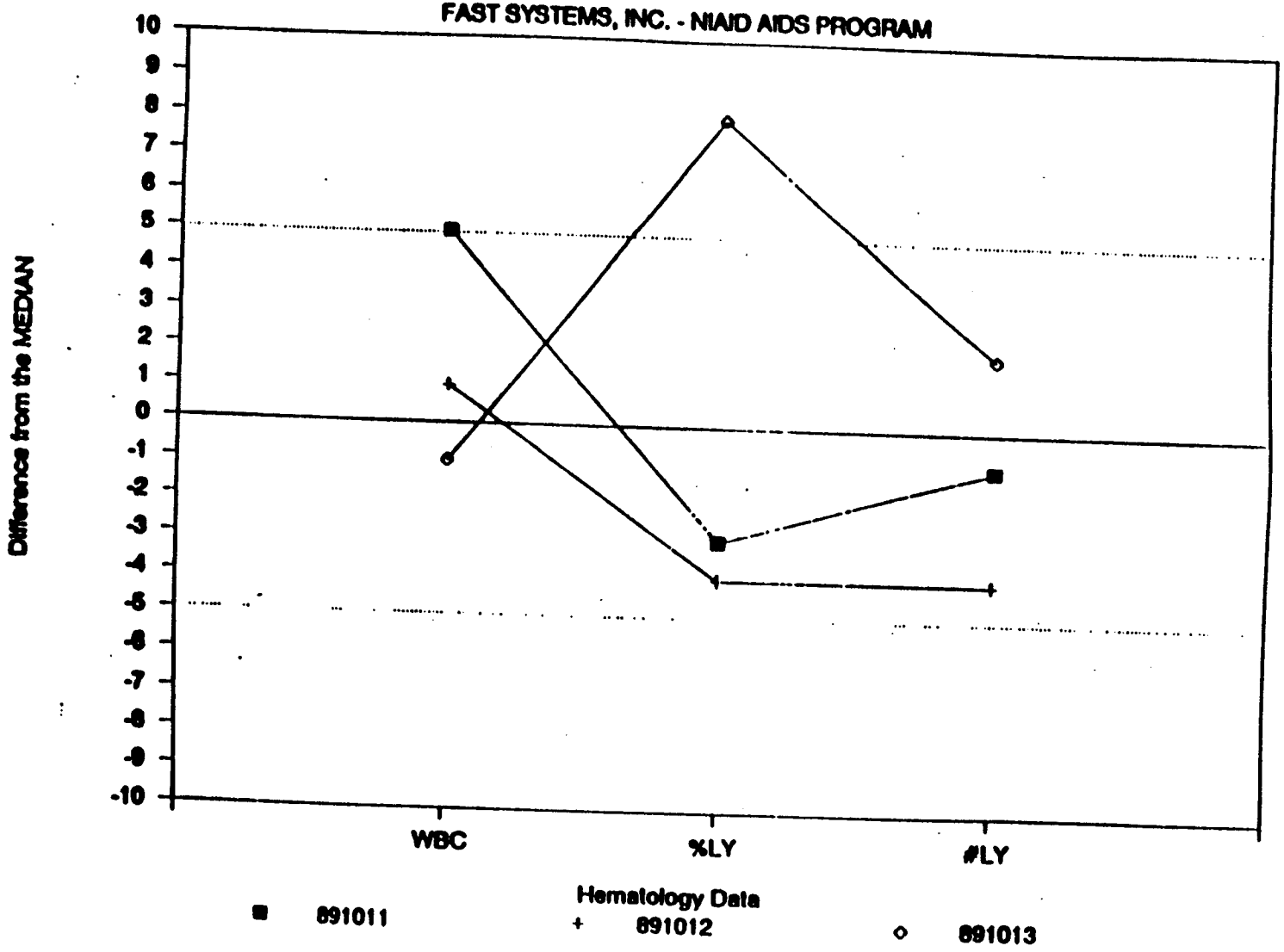
HEMATOLOGY
DIFFERENCES FROM THE MEDIAN VALUE

Specimen 891011	WBC	%LY	#LY
MEDIAN	5000	34	1764
Reported	5500	31	1705
Difference	5	-3	-1

Specimen 891012	WBC	%LY	#LY
MEDIAN	8200	30	2511
Reported	8300	26	2158
Difference	1	-4	-4

Specimen 891013	WBC	%LY	#LY
MEDIAN	2700	42	1134
Reported	2600	50	1300
Difference	-1	8	2

Texas Children's Hospital



FSI FAST Systems, Inc.

NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

LABORATORY: Texas Children's Hospital

OCTOBER 1989

LEUKOCYTE PHENOTYPE RESULTS VERIFICATION

METHOD OF CELL PREPARATION: Isolyte-Prep (Q-Prep)

MODEL OF FLOW CYTOMETER: EPICS Profile

PHENOTYPE	MONOCLONAL ANTIBODY	SPECIMEN 891011	SPECIMEN 891012	SPECIMEN 891013
CD45	KC56-FITC	100	100	100
CD14	Mo2-RD1	0	0	0
CD2	T11-RD1	86	86	79
CD3	T3-FITC	73	79	66
CD4	T4-RD1	57	49	5
CD8	T8-RD1	25	36	71
CD3*CD8	T3-FITC*T8-RD1	20	32	61
CD19	B4-FITC	10	9	14
CD20	B1-FITC	11	8	13
CD56				
CD16				

NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

LABORATORY: Texas Children's Hospital

OCTOBER 1989

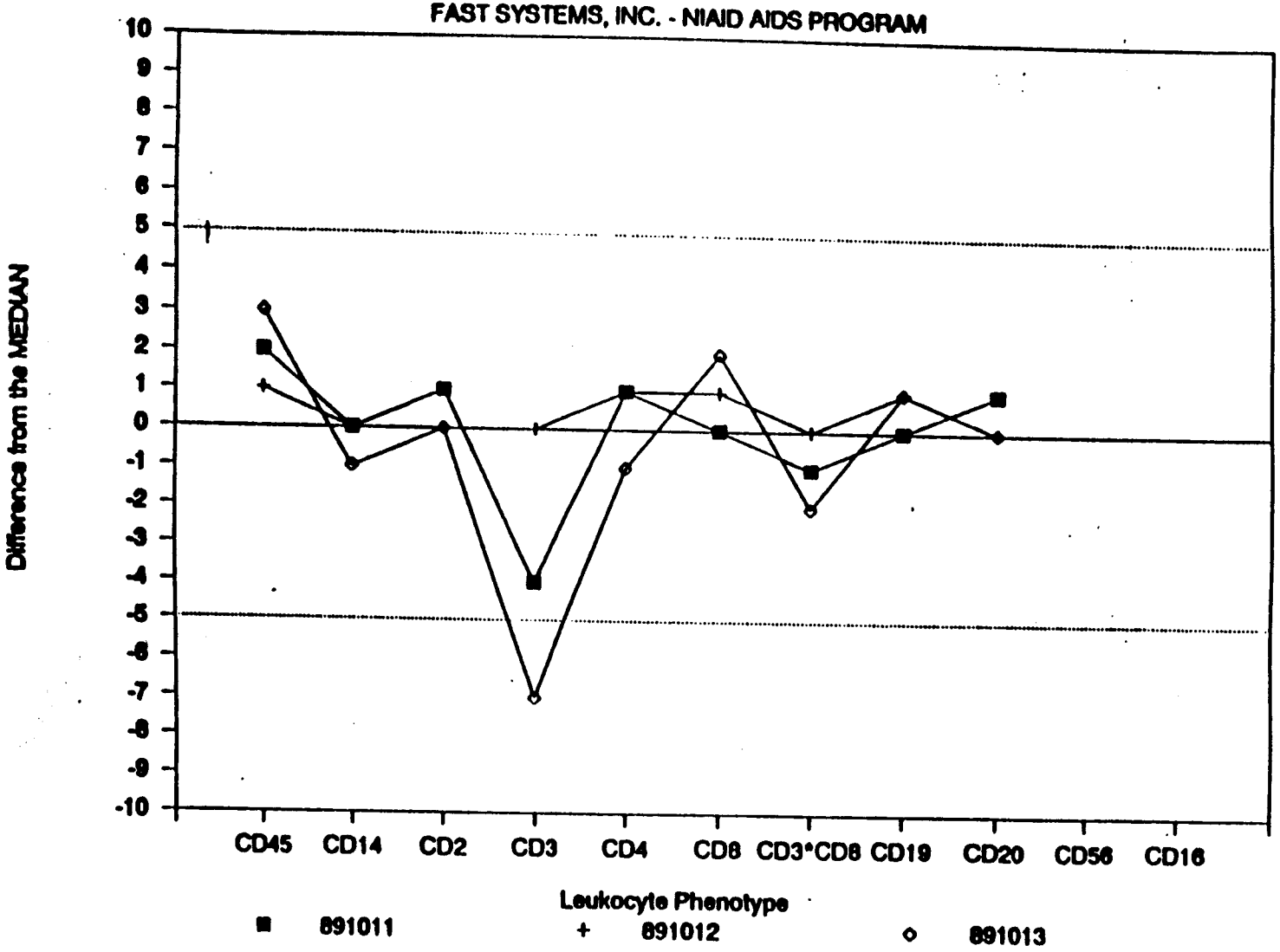
LEUKOCYTE PHENOTYPES
DIFFERENCES FROM THE MEDIAN VALUE

Specimen 891011	CD45	CD14	CD2	CD3	CD4	CD8	CD3*CD8	CD19	CD20	CD56	CD16
MEDIAN	98	0	85	77	56	25	21	10	10		
Reported	100	0	86	73	57	25	20	10	11		
Difference	2	0	1	-4	1	0	-1	0	1		

Specimen 891012	CD45	CD14	CD2	CD3	CD4	CD8	CD3*CD8	CD19	CD20	CD56	CD16
MEDIAN	99	0	86	79	48	35	32	8	8		
Reported	100	0	86	79	49	36	32	9	8		
Difference	1	0	0	0	1	1	0	1	0		

Specimen 891013	CD45	CD14	CD2	CD3	CD4	CD8	CD3*CD8	CD19	CD20	CD56	CD16
MEDIAN	97	1	79	73	6	69	63	13	13		
Reported	100	0	79	66	5	71	61	14	13		
Difference	3	-1	0	-7	-1	2	-2	1	0		

Texas Children's Hospital



NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

SPECIMEN 891014
STAINED AND FIXED

Specimen 891014 was composed of 9 populations of beads. The populations were mixed equally. One of the populations did not contain any fluorochrome. Four populations were covalently linked to FITC. The remaining four populations were covalently linked to PE. The four green fluorescent populations should have been readily resolved to baseline by all instruments. The resolution in orange/red was more difficult. No laboratory resolved the four orange/red peaks to baseline. However, most laboratories returned histograms which discerned the orange/red populations.

The particular goal of this specimen was to evaluate the resolution of closely spaced fluorescence peaks in both the green and orange/red regions of the spectrum. Many laboratories did not resolve the duller of the nine populations because their brightest green and orange/red peaks were at mid-scale. These laboratories did resolve at least two green and two orange/red populations.

FSI FAST Systems, Inc.

NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

LABORATORY: Texas Children's Hospital

OCTOBER 1989

STAINED AND FIXED SPECIMEN RESULTS VERIFICATION
SPECIMEN 890914

SINGLE COLOR DATA

	PERCENT POSITIVE FOR	
	GREEN	ORANGE/RED
NON-FLUORESCENT	68	80
PEAK 1	11	11
PEAK 2	9	9
PEAK 3	11	
PEAK 4		

DUAL COLOR DATA

	PERCENT
GREEN UNSTAINED * ORANGE/RED UNSTAINED	48
GREEN STAINED * ORANGE/RED UNSTAINED	32
GREEN UNSTAINED * ORANGE/RED STAINED	20
GREEN STAINED * ORANGE/RED STAINED	0

COMMENTS FROM THE FSI STAFF ON YOUR INSTRUMENT PERFORMANCE:

NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

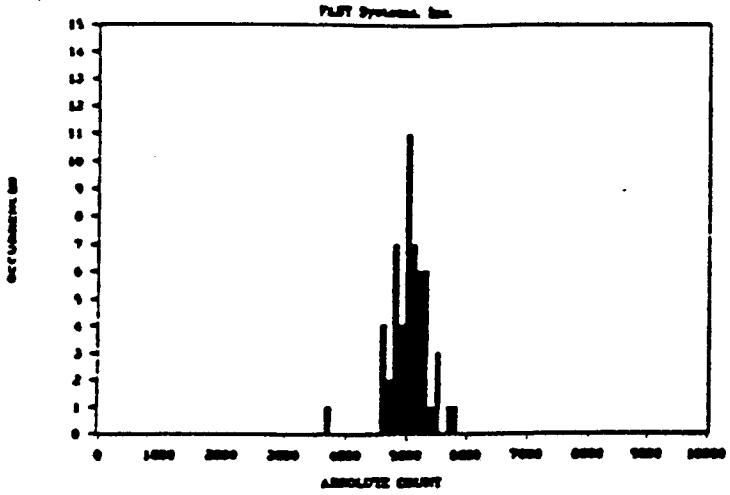
Hematology and Leukocyte Phenotype Percent Statistics

Specimen 891011	WBC	%LY	#LY	CD45	CD14	CD2	CD3	CD4	CD8	CD3-8	CD19	CD20	CD56	CD16
Group N	54	53	53	55	54	22	56	56	56	28	36	32	20	13
Maximum	5800	54	4080	100	5	92	84	62	33	23	15	15	18	11
Upper Quartile	5200	36	1855	99	1	88	78	58	27	22	11	11	13	10
MEDIAN	5000	34	1764	98	0	85	77	56	25	21	10	10	10	9
Lower Quartile	4800	32	1610	98	0	84	75	54	23	20	8	9	8	8
Minimum	3700	18	1380	92	0	72	66	41	18	13	4	7	6	2
IQR	300	3	184	1	1	3	2	3	3	2	2	2	3	2
High Fence	5650	41	2131	100	2	93	81	61	32	25	14	13	18	12
Low Fence	4350	28	1334	97	-1	80	72	50	19	17	5	7	3	6

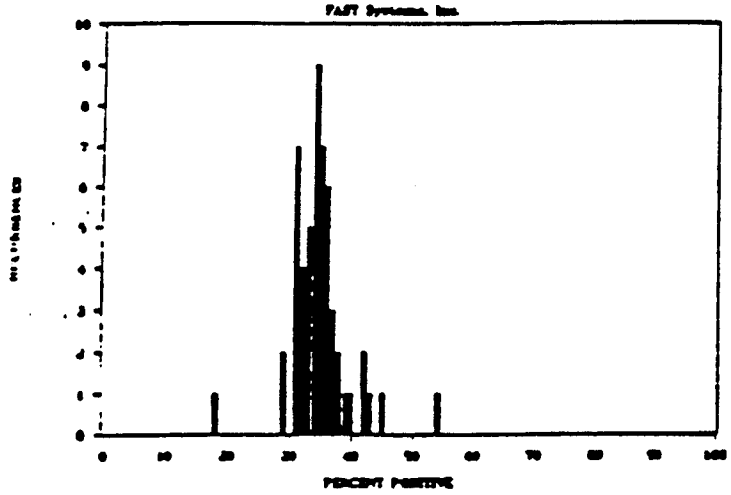
Specimen 891012	WBC	%LY	#LY	CD45	CD14	CD2	CD3	CD4	CD8	CD3-8	CD19	CD20	CD56	CD16
Group N	54	53	53	55	54	22	56	56	56	28	36	32	19	13
Maximum	9000	63	5166	100	8	90	87	62	50	37	18	13	27	10
Upper Quartile	8300	35	2886	99	1	87	82	50	38	34	9	9	23	8
MEDIAN	8200	30	2511	99	0	86	79	48	35	32	8	8	16	7
Lower Quartile	7900	28	2291	97	0	84	78	46	32	28	7	8	14	
Minimum	5100	24	1423	91	0	76	68	40	22	16	5	6	6	
IQR	300	5	446	2	1	2	3	3	4	5	2	1	7	2
High Fence	8750	43	3555	101	2	90	86	55	44	41	11	10	33	10
Low Fence	7450	20	1622	95	-1	81	73	40	26	20	5	7	4	4

Specimen 891013	WBC	%LY	#LY	CD45	CD14	CD2	CD3	CD4	CD8	CD3-8	CD19	CD20	CD56	CD16
Group N	54	53	53	55	54	22	56	56	56	28	36	32	20	13
Maximum	7500	69	4050	100	9	85	91	25	86	79	18	17	8	19
Upper Quartile	2720	46	1274	99	1	81	78	7	73	67	15	14	7	8
MEDIAN	2700	42	1134	97	1	79	73	6	69	63	13	13	6	7
Lower Quartile	2600	38	999	95	0	75	70	5	66	59	11	11	4	4
Minimum	2300	25	600	90	0	69	47	3	56	41	4	6	3	2
IQR	90	6	206	3	1	5	6	2	5	6	3	2	2	3
High Fence	2855	55	1583	104	2	86	87	9	80	76	18	17	9	13
Low Fence	2465	29	690	91	-1	68	61	3	59	50	7	8	1	-1

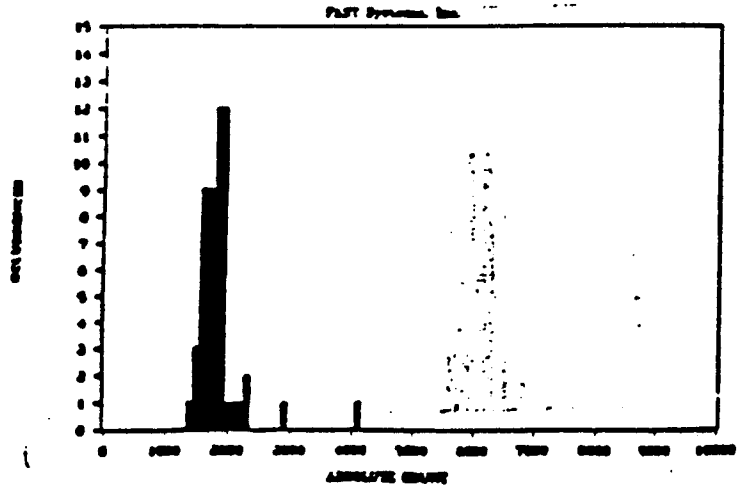
891011 AIDS PROGRAM WBC

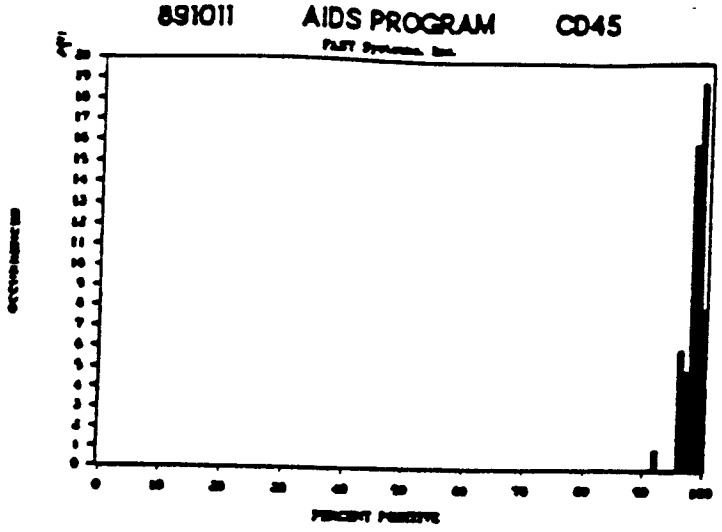


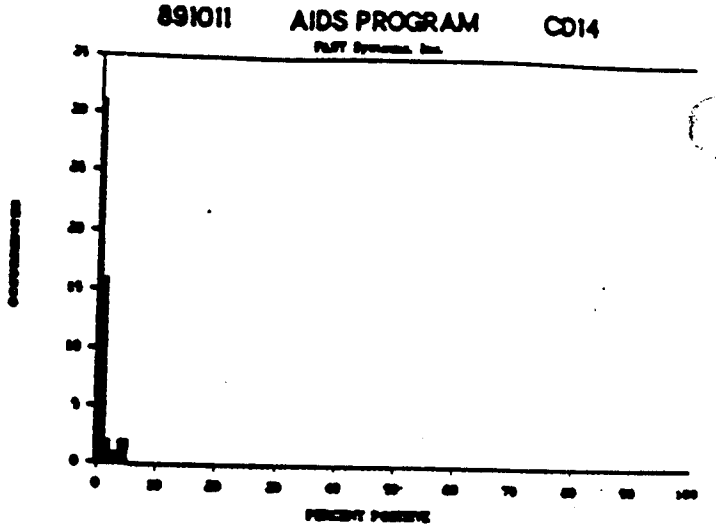
891011 AIDS PROGRAM % LYMPHS

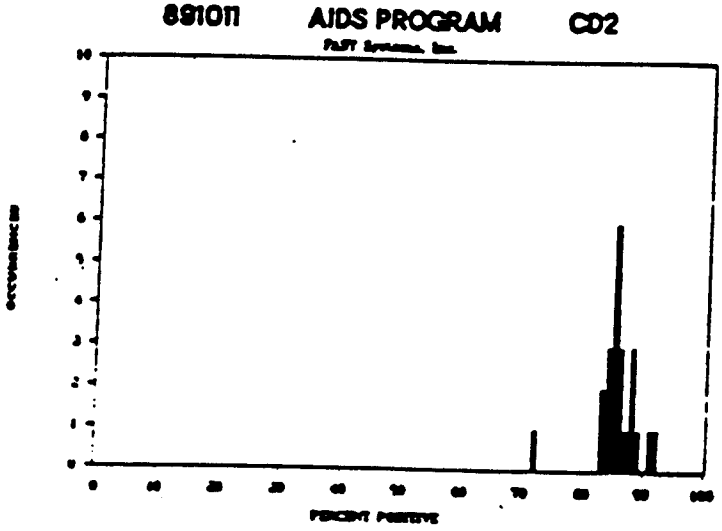


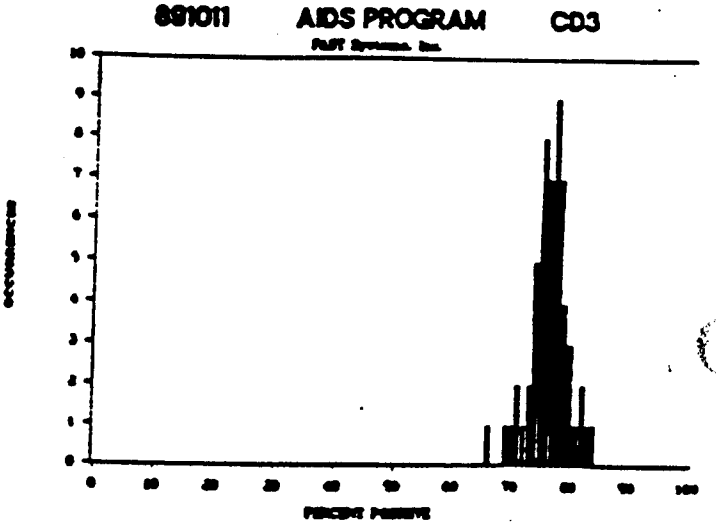
891011 AIDS PROGRAM # LYMPHS

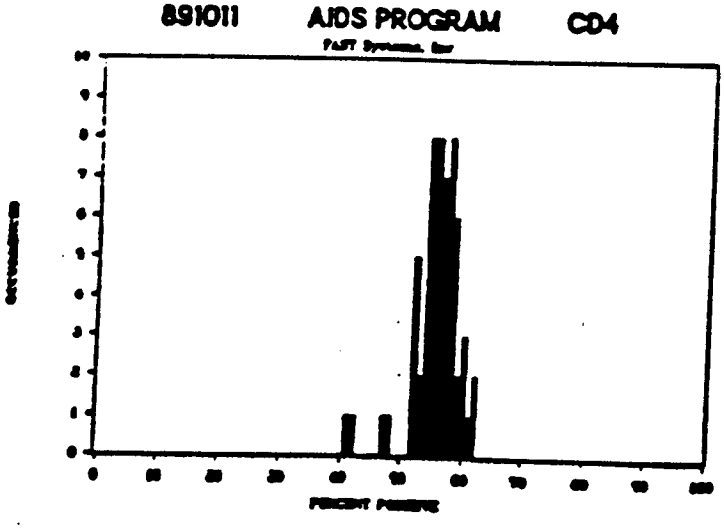


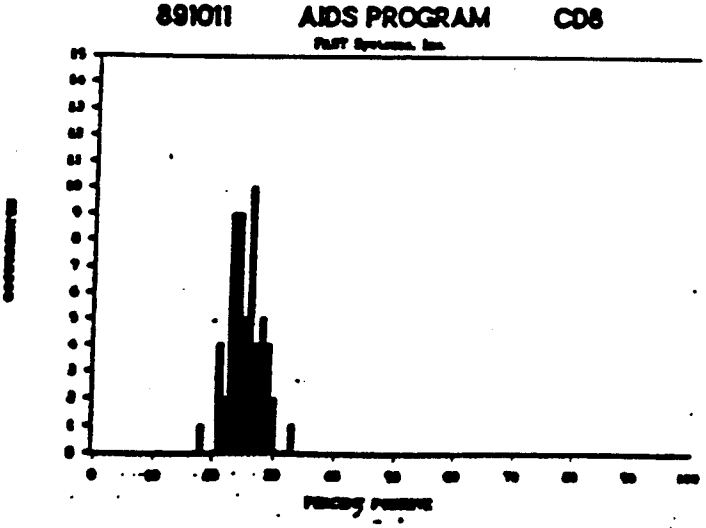

891011 AIDS PROGRAM CD45

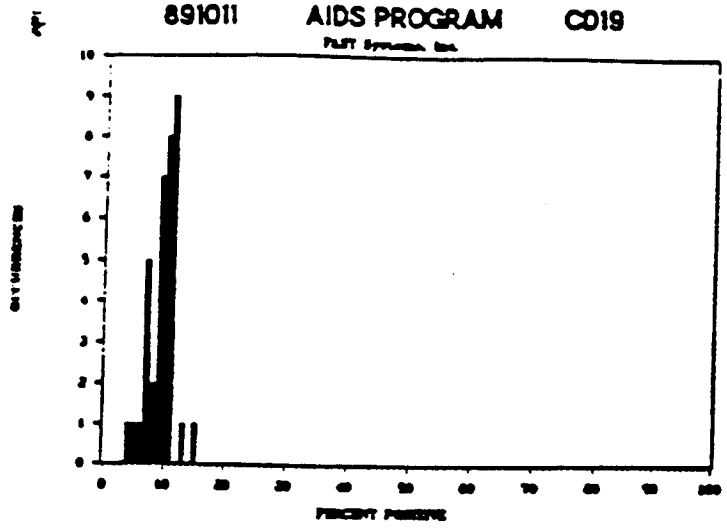

891011 AIDS PROGRAM CD14


891011 AIDS PROGRAM CD2


891011 AIDS PROGRAM CD3


891011 AIDS PROGRAM CD4


891011 AIDS PROGRAM CD8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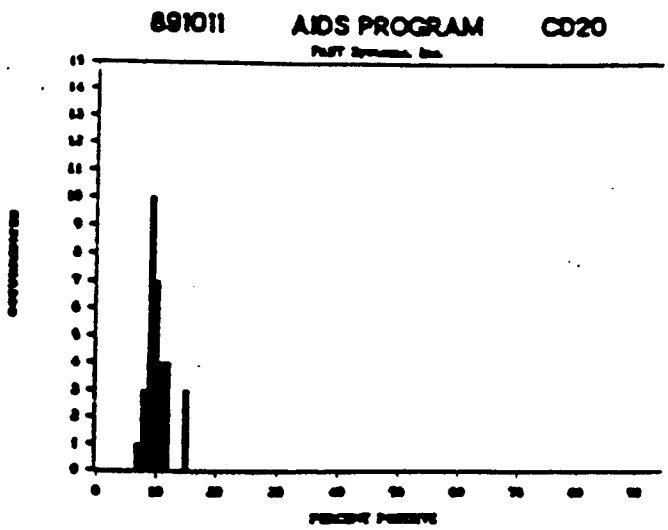
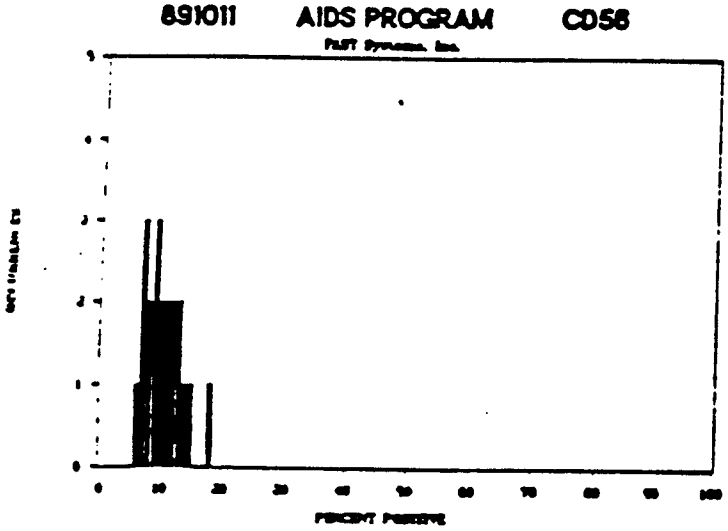

891011 AIDS PROGRAM CD20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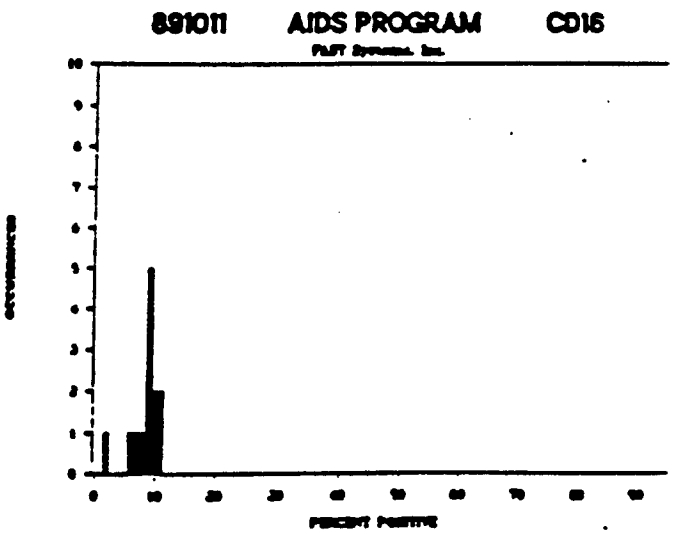
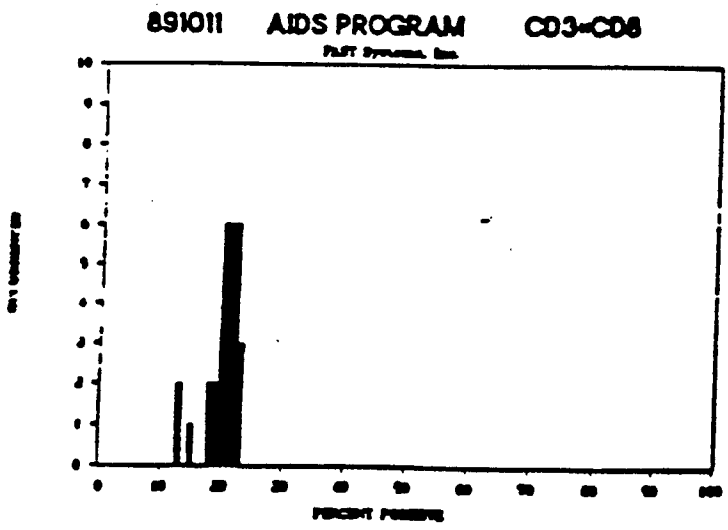
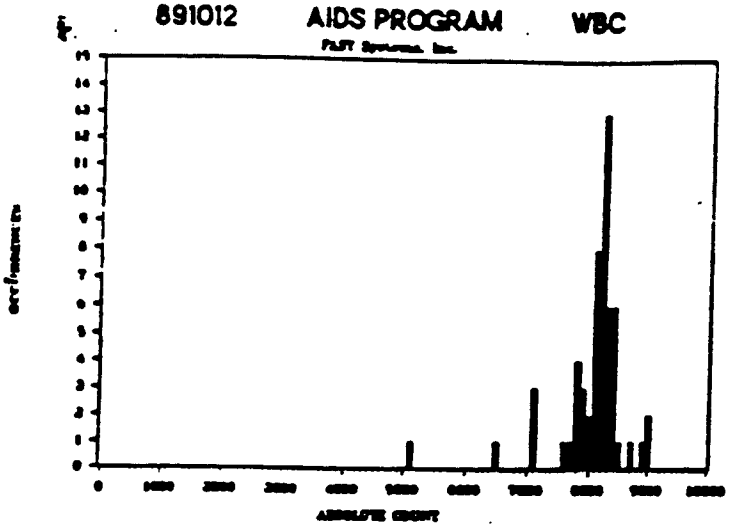
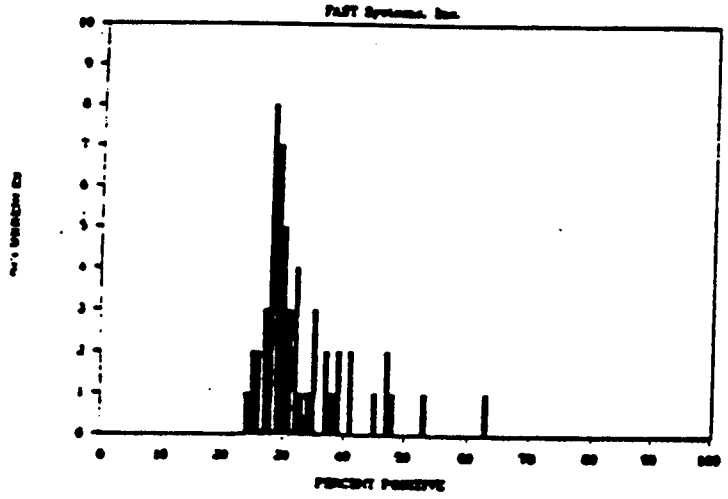

891011 AIDS PROGRAM CD16


891011 AIDS PROGRAM CD3+CD8

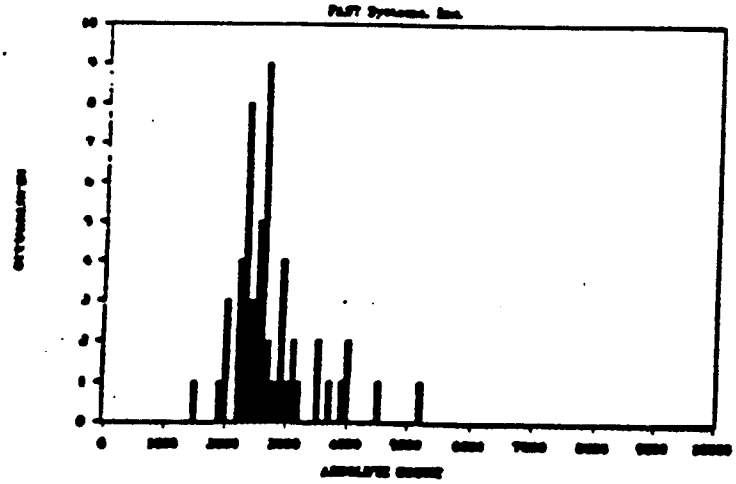
891012 AIDS PROGRAM WBC

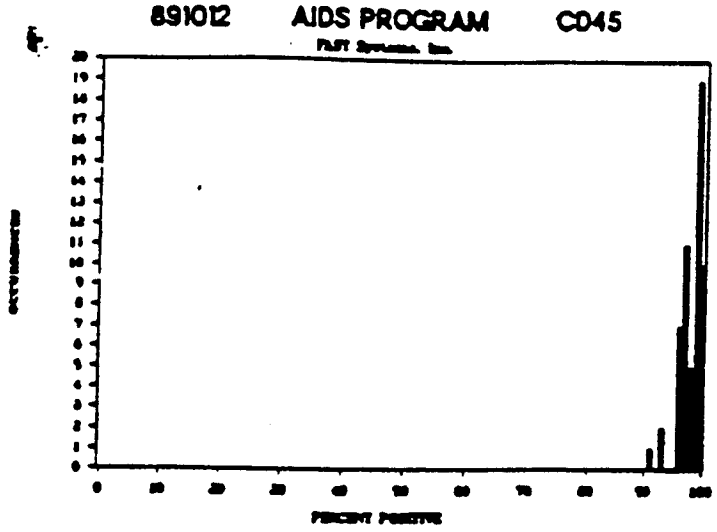


891012 AIDS PROGRAM % LYMPHS



891012 AIDS PROGRAM # LYMPHS

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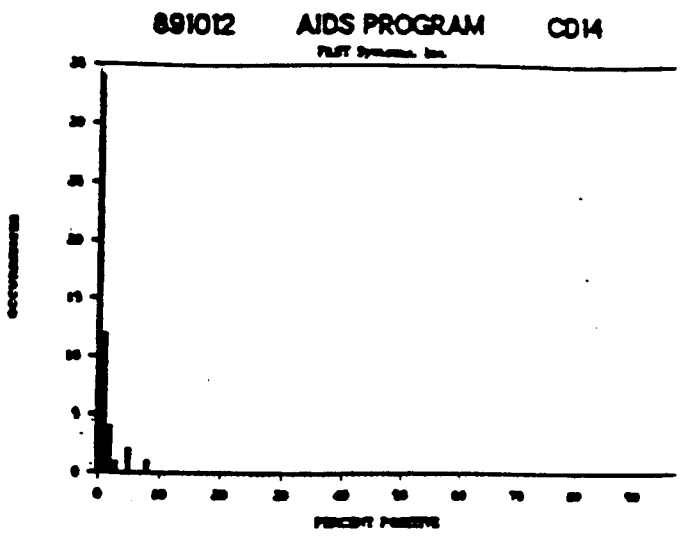
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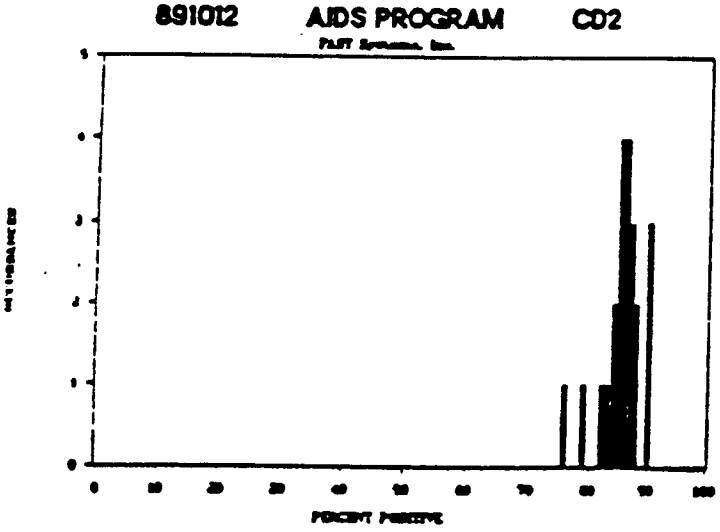
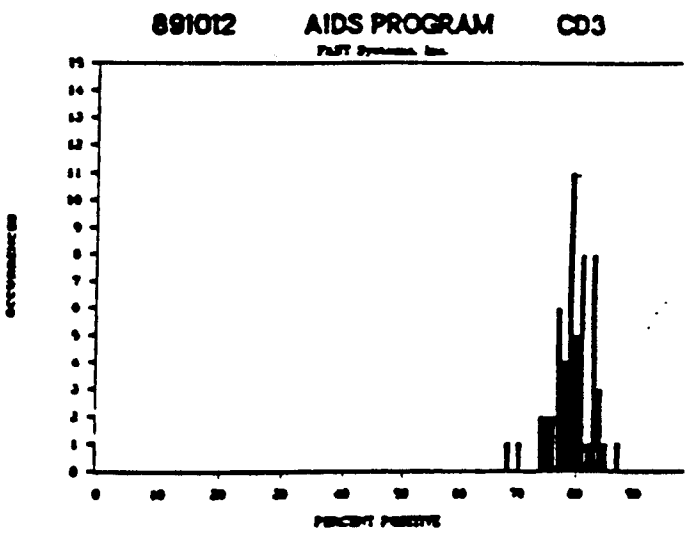

891012 AIDS PROGRAM CD2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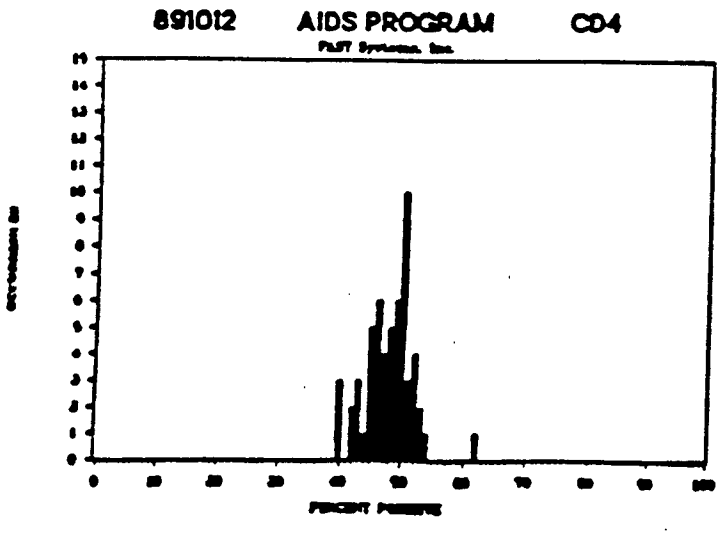
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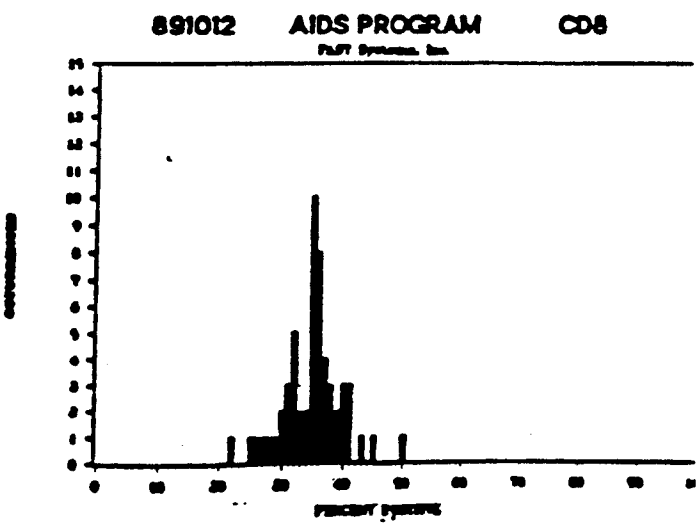
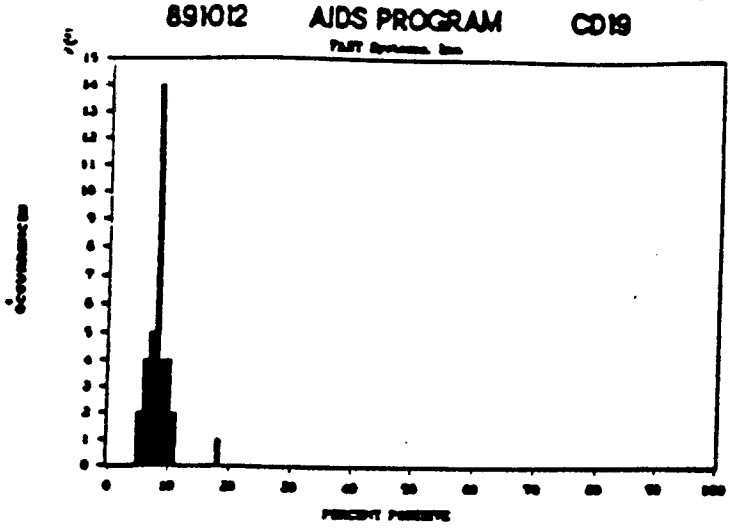

891012 AIDS PROGRAM CD8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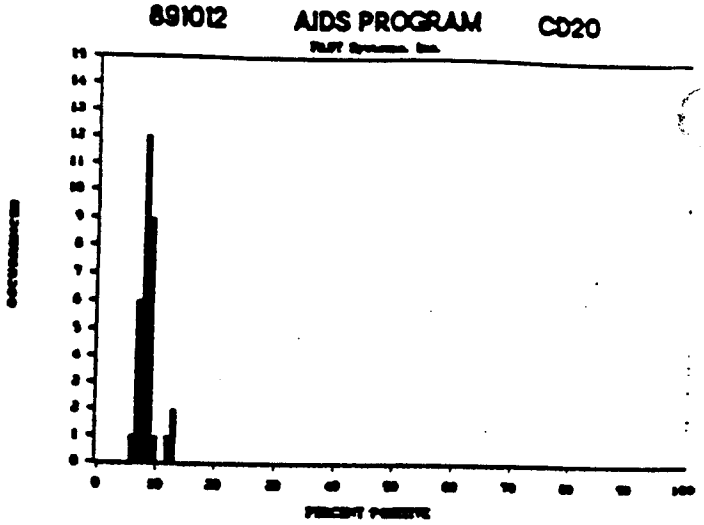
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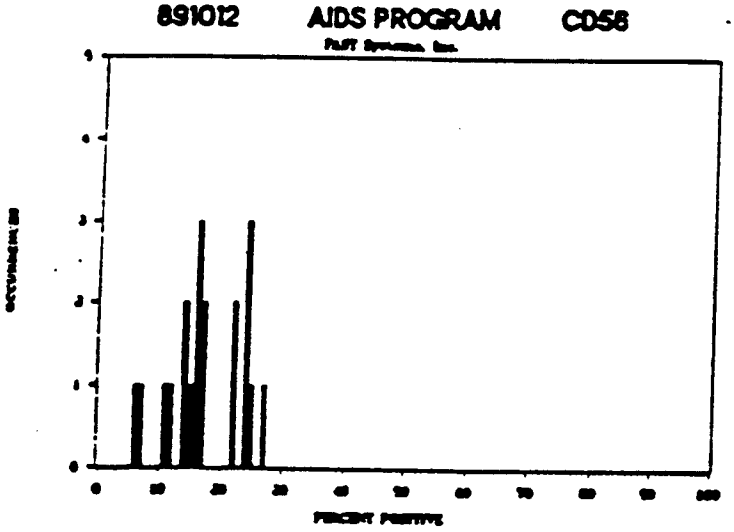
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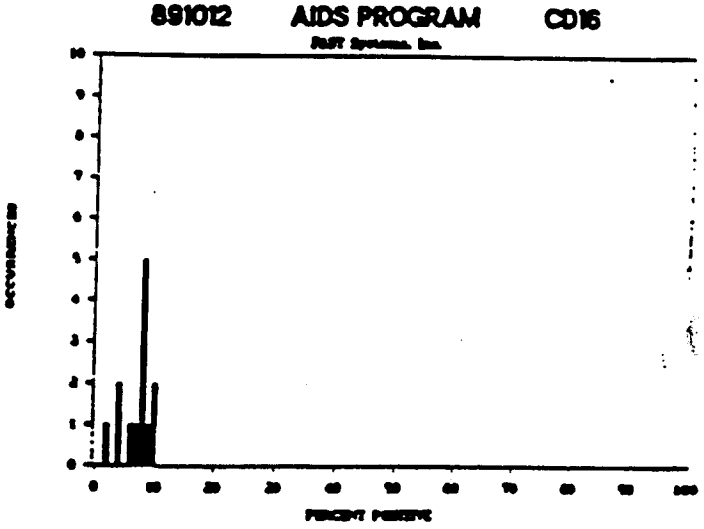
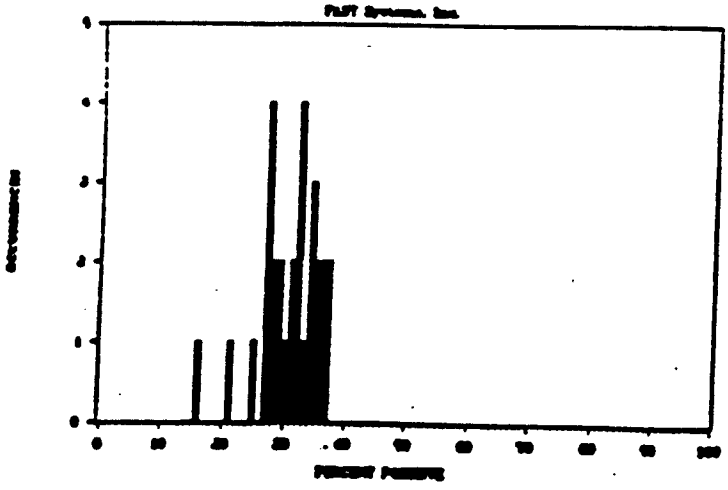
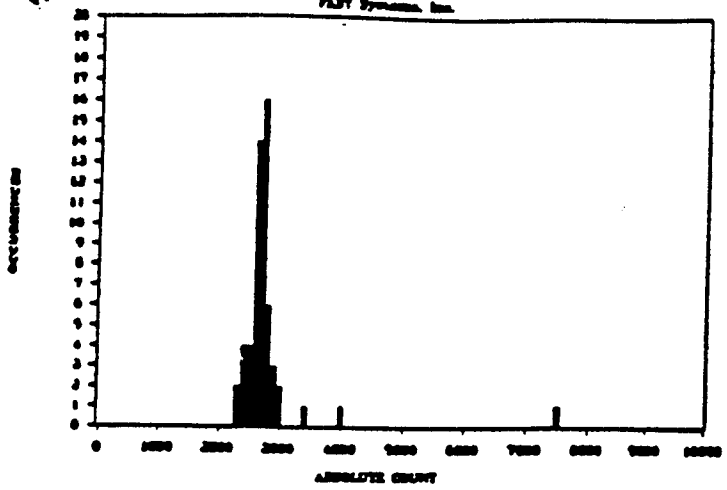
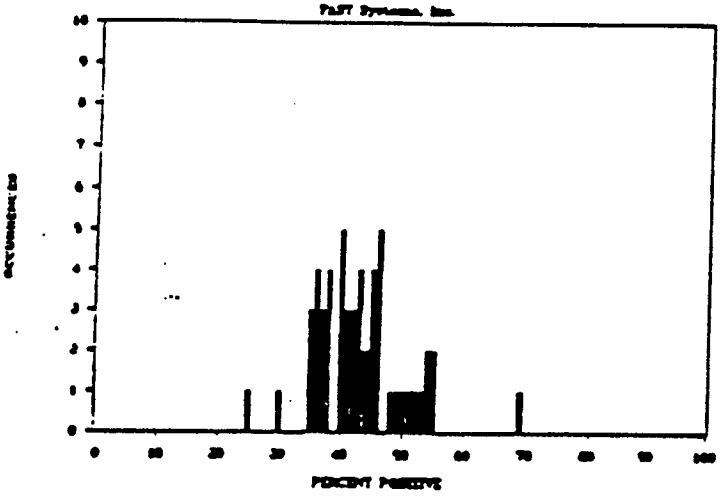

891012 AIDS PROGRAM CD16


891012 AIDS PROGRAM CD3+CD8

891013 AIDS PROGRAM WBC



891013 AIDS PROGRAM % LYMPHS



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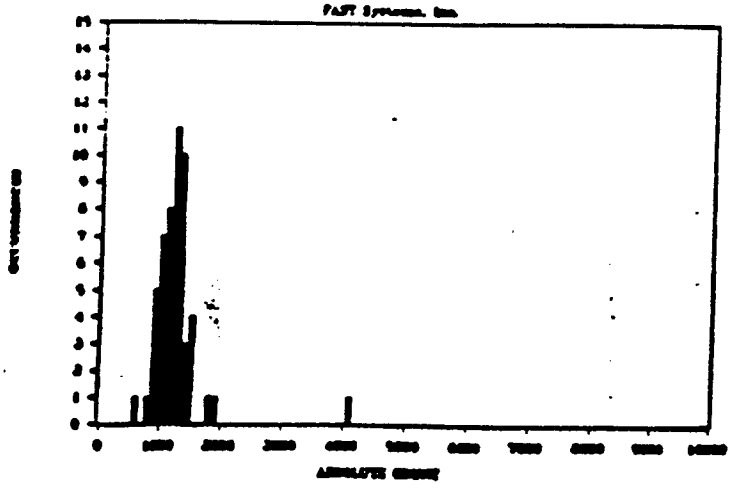

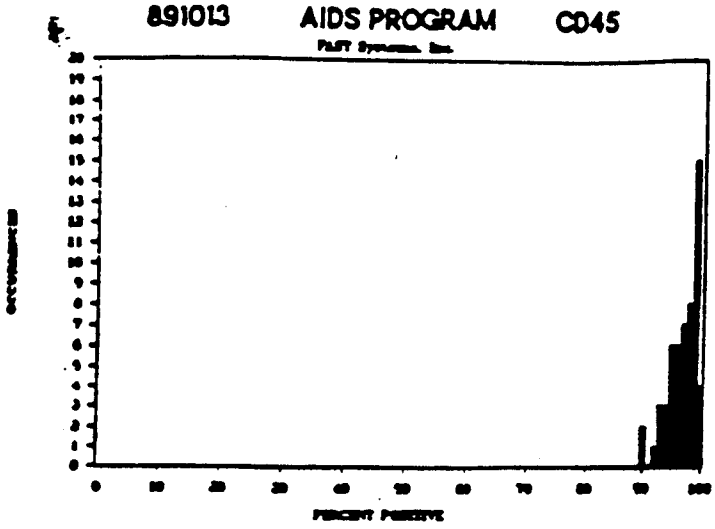
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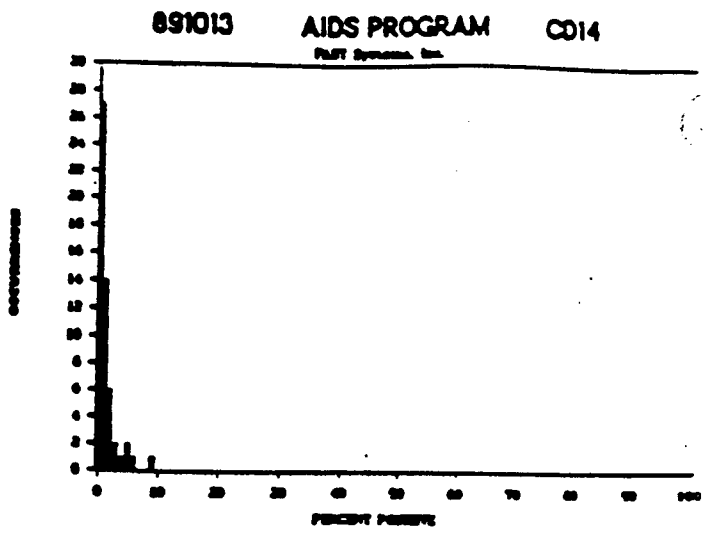
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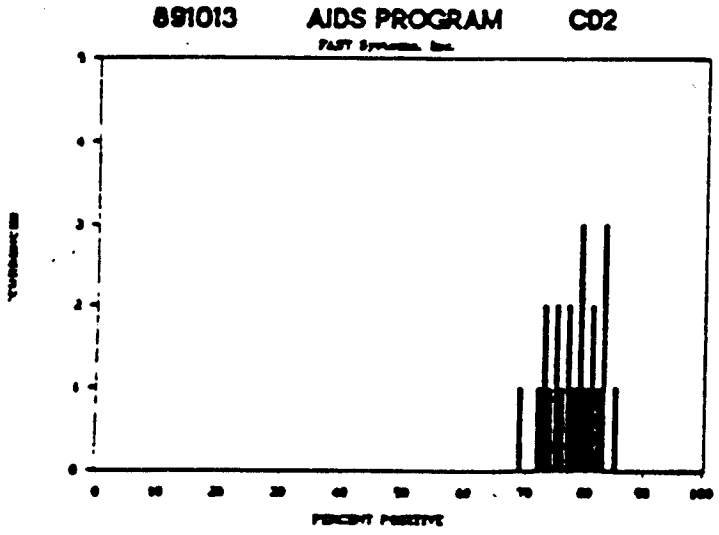
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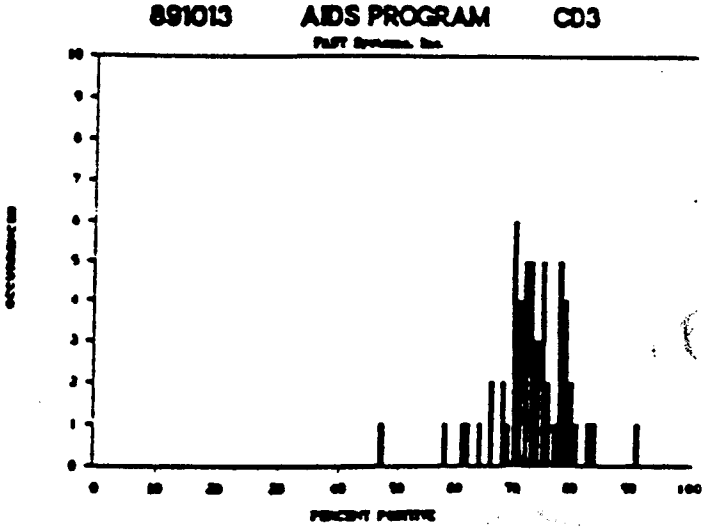
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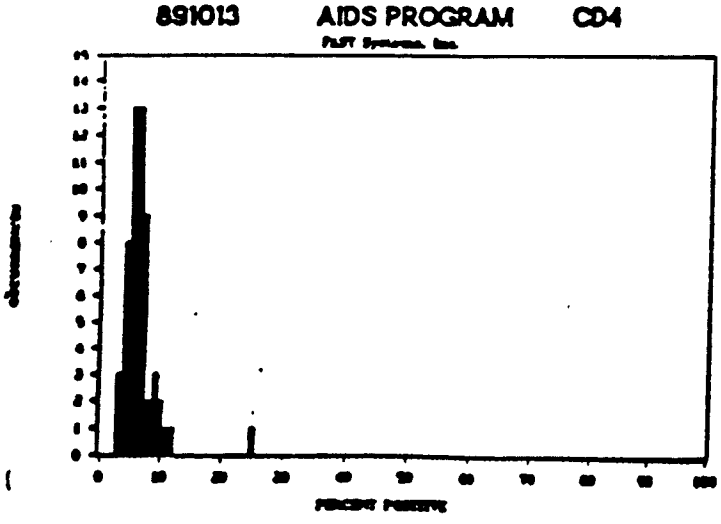
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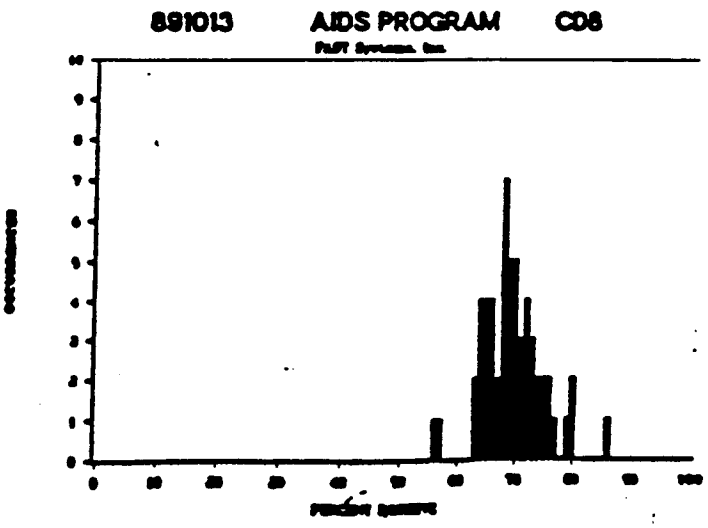

891013 AIDS PROGRAM CD8

FSI *FAST Systems, Inc.*

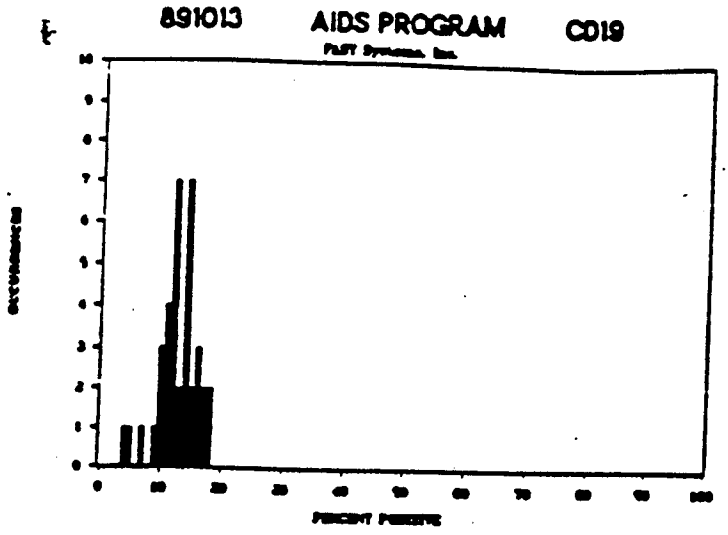
NIAID AIDS PROGRAM - PROFICIENCY TESTING FOR LYMPHOCYTE SUBSETS

Absolute Leukocyte Phenotype Count Statistics

Specimen 891011	CD45	CD14	CD2	CD3	CD4	CD8	CD3+8	CD19	CD20	CD56	CD16
Group N	52	49	19	53	53	53	24	32	29	16	12
Maximum	5346	83	1803	1677	1230	594	470	235	285	335	223
Upper Quartile	1852	18	1591	1406	1045	482	395	175	196	219	168
MEDIAN	1750	0	1527	1343	972	428	359	160	173	187	156
Lower Quartile	1603	0	1470	1202	870	391	323	132	159	142	132
Minimum	1325	0	1292	980	649	330	204	68	105	107	35
IQR	186	14	91	153	131	66	84	33	28	57	27
High Fence	2131	38	1727	1636	1242	584	476	224	238	305	209
Low Fence	1323	-20	1334	973	673	289	242	83	117	56	82

Specimen 891012	CD45	CD14	CD2	CD3	CD4	CD8	CD3+8	CD19	CD20	CD56	CD16
Group N	52	49	19	53	53	53	24	32	29	15	12
Maximum	8700	193	3829	3606	2315	1845	1113	516	356	775	230
Upper Quartile	2897	26	2508	2229	1383	1016	881	231	252	537	217
MEDIAN	2485	0	2250	1971	1204	842	773	204	226	432	185
Lower Quartile	2259	0	1985	1804	1074	777	700	164	202	333	132
Minimum	1806	0	1851	1432	830	541	367	120	145	201	58
IQR	479	20	392	319	232	179	136	50	38	153	64
High Fence	3615	55	3096	2707	1731	1285	1084	306	308	765	313
Low Fence	1540	-29	1396	1326	726	508	496	88	146	104	36

Specimen 891013	CD45	CD14	CD2	CD3	CD4	CD8	CD3+8	CD19	CD20	CD56	CD16
Group N	52	49	19	53	53	53	24	32	29	16	12
Maximum	9702	108	1360	1394	140	1305	1155	723	247	149	191
Upper Quartile	1248	13	1037	907	84	894	829	181	189	78	110
MEDIAN	1102	6	891	816	65	790	726	155	144	60	72
Lower Quartile	993	0	758	700	50	680	645	128	125	39	43
Minimum	582	0	621	450	24	402	369	30	47	31	20
IQR	190	10	209	185	26	176	138	40	33	29	50
High Fence	1531	28	1350	1140	122	1157	1036	241	219	121	185
Low Fence	707	-15	445	457	12	397	438	68	76	-5	-33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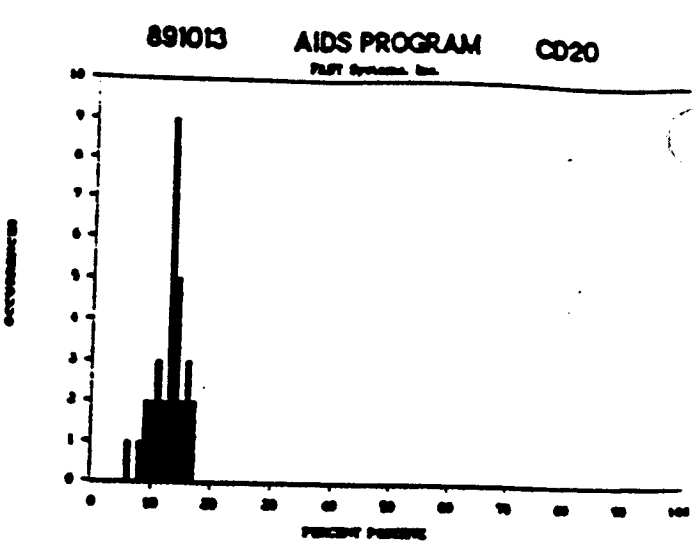
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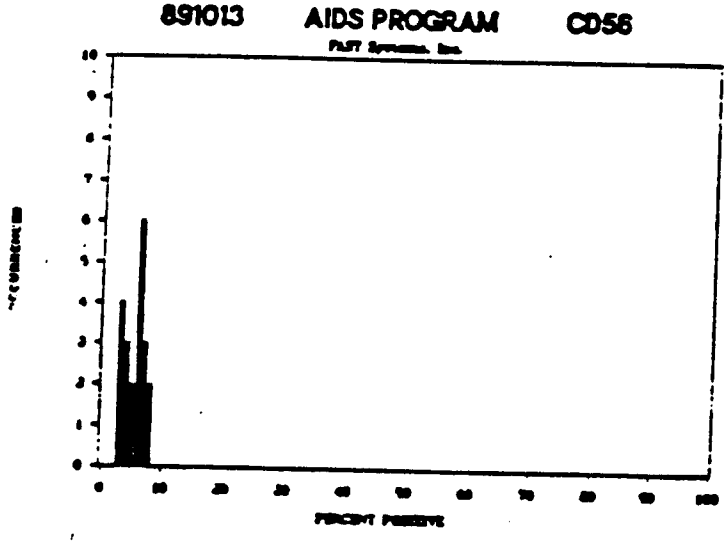
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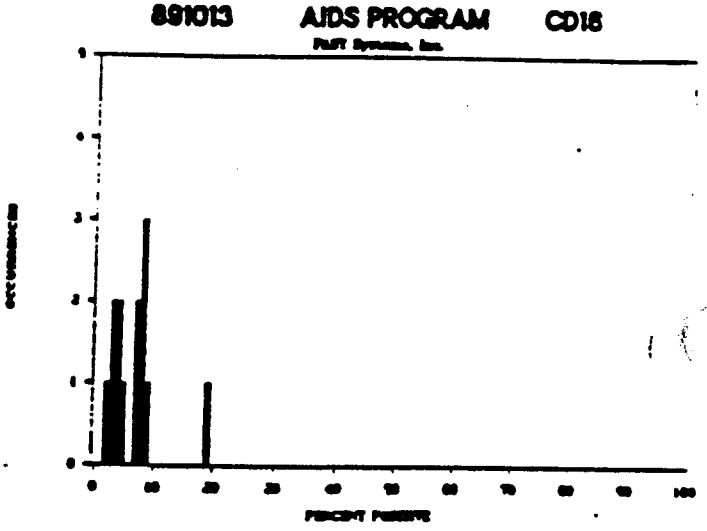
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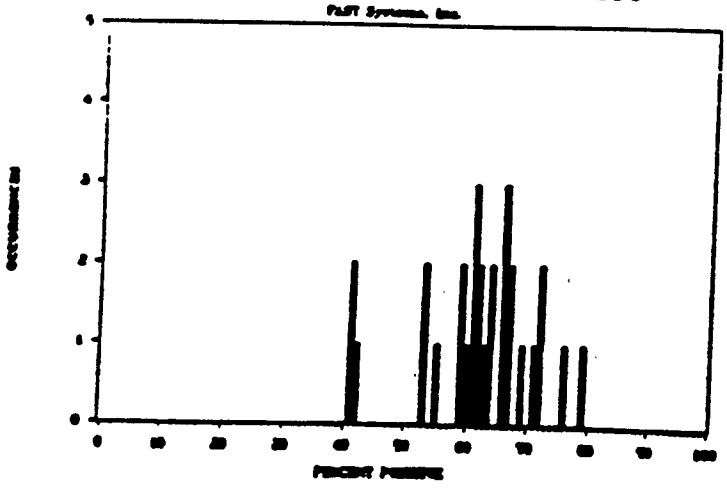
891013 AIDS PROGRAM CD3+CD8

APPENDIX 9

INTENDED USE

This kit is an anticomplement immunofluorescence (ACIF) assay for the detection of antibodies against Epstein-Barr virus (EBV) nuclear antigen (EBNA) that can aid in the diagnosis of infectious mononucleosis (IM).

SUMMARY AND EXPLANATION

Epstein-Barr virus (EBV), discovered by Epstein, Achong and Barr (1) has been identified as the causative agent of infectious mononucleosis (2,3). Other viruses can cause disease syndromes similar to classical acute IM. Separation of these situations can be accomplished by the measurement of the EBV antibody titers. EBNA positive lymphocytes have been demonstrated in the peripheral blood of IM patients (4). Although IgG and IgM antibodies appear and rise very soon after the onset of IM, antibody to EBNA may not appear for 3 weeks to six months (5,6,7). EBNA antibodies then persist for years, probably for life (6). Primary acute mononucleosis can be diagnosed by the determination of high levels of IgG and IgM VCA antibodies in the absence of EBNA antibody in serum. When serum is found to be VCA IgG positive-IgM negative while EBNA positive, this indicates the infection by EBV occuired at some indeterminate time in the past. Antibodies to VCA are measured by standard indirect immunofluorescence (IFA). EBNA can't be detected by IFA. Measuring antibody to EBNA requires a more sensitive assay (ACIF) to amplify the reaction. Reedman and Klein in 1973 reported the first measurement of EBNA using the ACIF assay (8).

This assay kit uses the ACIF assay technique. The major disadvantage is that the complement is very labile and must be handled carefully. The major advantage of this EBNA kit compared with other commercially available kits is that the negative control for antinuclear antibody (ANA) is an integral part of the EBNA slide and therefore a second EBV negative slide is not required. This results in a savings of time to perform the assay and removes the possibility of mismatching wells of positive sera on the EBNA slide and the ANA control from a separate slide.

TEST PRINCIPLE

This device uses the ACIF antibody assay. ACIF detects the binding of human IgG antibody specific for the EBNA antigens found in the nucleus of fixed cells on the antigen substrate slide wells. In the first step human serum dilutions are added to the wells of the antigen substrate slide and EBNA antibody, if present, binds to the EBNA on the slide. The serum dilutions are washed off. In the second step a source of complement (guinea pig serum) is added to each well. This complement will bind to antigen-antibody complexes (EBNA and antiEBNA).

In the third step, fluorescein isothiocyanate (FITC) conjugated antibody specific for guinea pig C3 is added to detect the presence or absence of the complement bound in the nucleus of the cells. If the serum was from a seronegative person or a person that has just been infected by EBV, then the conjugate has no complement to bind to and the test well on the slide shows no specific nuclear fluorescence. When antibodies to EBNA antigens are present in the test serum, the cell nucleus will evidence specific fluorescence. One can determine the titer of the test sample by reading the fluorescence or non-fluorescence of the dilutions on the wells of the slide. The titer is determined by observing the last dilution to evidence specific nuclear fluorescence. A small number of high titer EBNA sera have demonstrated prozone. Therefore, one should test 1:5 AND 1:20 dilutions and a negative must be negative at both 1:5 and 1:20 dilutions.

PRECAUTIONS

1. Avoid splashing or the generation of aerosols
2. Each reagent has been made to perform optimally therefore one should not substitute reagents from other manufacturers or other lot numbers. Dilution or adulteration may adversely affect the results obtained with the kit. Do not use beyond expiration date.
3. For In Vitro Diagnostic Use.
4. Patient specimens and all material coming into contact with them should be handled as if capable of transmitting disease and disposed of as biohazardous waste.
5. Never pipette by mouth.
6. Limit exposure of reagents to strong light.
7. Reusable glassware must be thoroughly washed and rinsed to avoid soap carryover.
8. Use of time and temperatures other than those listed here may give unreliable results.
9. Process the slides timely to avoid having the wells dry out between steps of the assay. Drying of the wells between reagent steps may lead to unreliable results.

STORAGE CONDITIONS

All kit reagents should be stored at 2-8 degrees centigrade when not being used. Since incubation is to be at 37 C it is not necessary to allow the reagents to come to room temperature before performing the assay. The reconstituted complement should be stored tightly capped at 2-8 C at all times. The use of a crushed ice bath is suggested for interim storage of the complement vial during the performance of the assay. DO NOT freeze and

thaw the complement after reconstitution. If the environment is extremely humid it is recommended that slides be used within 1 hour of removing them from their pouch. Each of the 3 vials of complement contains enough reagent to add 10 uL per well to all 5 slides in the kit.

SIGNS OF DETERIORATION

Should any reagent become cloudy or turbid, suggesting microbial contamination, they may not give reliable performance and should be discarded. The positive and negative controls should meet expected values for the assay to be considered valid.

SPECIMEN COLLECTION AND PREPARATION

Blood obtained by venipuncture is allowed to clot. The serum is removed aseptically and placed in a sterile tube. If the serum is not to be used immediately it should be frozen and stored at or lower than -20 C in a non-self defrosting freezer. Avoid freeze/thawing. A one ml sample of blood should yield enough serum to perform the assay. Samples that are highly lipemic, hemolysed, or contaminated may not be suitable for analysis. Under these conditions, request a new sample for analysis.

PROCEDURE

A. MATERIALS SUPPLIED

1. EBV-EBNA + NEGATIVE CELL SLIDES-individually packaged in foil packets. 5 slides with 12 wells each. Each well contains EBNA positive and EBNA negative cells in approximately equal proportions. Stable for one year at 2-8 C.

2. GUINEA PIG COMPLEMENT-Lyophilized 0.1 ml after reconstitution with reagent quality water at least 1 hour before use. See storage conditions for special instructions. Dilute 1:7 with complement diluent for use. Stable 3 days if kept 2-8C and handled aseptically. Discard if cloudy.

3. COMPLEMENT DILUENT-Diluent is phosphate buffered saline containing calcium and magnesium. 1-5 ml vial. Stable for one year at 2-8 C. Caution. Contains 0.02% sodium azide[1].

4. FITC CONJUGATED POLYCLONAL ANTI-GUINEA PIG C-3 Lyophilized 1 vial 2 ml upon reconstitution with the accompanying conjugate diluent. Stable for one year at 2-8 C. Contains 0.02% sodium azide[1].

5. CONJUGATE DILUENT-Diluent is phosphate buffered saline with Evans Blue counterstain (0.02%), 2 ml. The entire contents of the diluent is to be quantitatively transferred to the conjugate vial for reconstitution for use.
Caution. Contains 0.02% sodium azide[1]. Write date of reconstitution on the conjugate vial. Stable for 30 days after reconstitution if treated aseptically and stored at 2-8 C.

6. EBNA POSITIVE HUMAN CONTROL-Lyophilized 1 vial 0.5 ml after reconstitution with 0.01M PBS pH 7.2 (see materials not supplied). This control was prepared from a human serum or a pool of human sera containing antibody to EBNA and will evidence a positive reaction at a 1:5 AND 1:20 dilutions. After gentle agitation this solution will be the 1:5 dilution to be used in the assay as the positive control. The reconstituted control is stable for 30 days if treated aseptically and stored at 2-8 C. Do not heat inactivate this control.

Caution Although this control has been found non-reactive for hepatitis B surface antigen and HTLV III antibody by the FDA required tests they must be treated as though they are biohazardous materials[2] because these tests cannot assure the absence of these viruses. This control contains as a preservative 0.02 % sodium azide[1] and must be handled with due care.

7. EBNA NEGATIVE HUMAN CONTROL-Lyophilized 1 vial 0.5 ml after reconstitution with PBS. This control was prepared from a human serum or a pool of human sera negative for EBNA and will evidence a titer of less than 1:5. After gentle agitation this solution will be the 1:5 dilution to be used in the assays as the control. The reconstituted control is stable for 30 days if treated aseptically and stored at 2-8 C. Do not heat inactivate this control.

Caution Although this control has been found non-reactive for hepatitis B surface antigen and HTLV III antibody by the FDA required tests they must be treated as though they are biohazardous materials[2] because these tests cannot assure the absence of these viruses. This control contains as a preservative 0.02 % sodium azide[1] and must be handled with due care.

[1] Sodium azide may form lead or copper azide in plumbing. These azides may be explosive on percussion. Thorough flushing of drains with water during and after disposal of reagents will reduce potential azide formation.

[2] Biohazardous materials must be handled as though capable of transmitting disease. Further, they must be decontaminated (e.g. autoclaved) before disposal as ordinary trash.

B. MATERIALS REQUIRED BUT NOT SUPPLIED

1. PHOSPHATE BUFFERED SALINE (PBS) 0.01M pH 7.2

$Na_2HPO_4.7H_2O$	2.15	gm/L
KH_2PO_4	0.2	gm/L
NaCl	8.0	gm/L
KCl	0.2	gm/L
$CaCl_2.2H_2O$	0.13	gm/L
$MgCl_2.6H_2O$	0.1	gm/L
NaN_3	0.2	gm/L
Tween 20	0.5	ml/L
Reagent Quality Water	1.0	Liter

Store in a clean closed container at room temperature. Discard unused buffer if it becomes cloudy.

2. COVERSLIP MOUNTING FLUID
Glycerol mixed 1:1 (volume to volume) with 0.01M PBS pH 8.0. Discard if cloudy.
3. STYRENE MICROPLATE FOR SERUM DILUTIONS
4. MICROPIPETTOR AND TIPS (MULTICHANNEL, OPTIONAL)
5. MOISTURE CHAMBER (TUPPERWARE TYPE BOX WITH LID)
6. COTTON SWABS OR SLIDE BLOTTERS AND ROLLER
7. CLEAN WASH BOTTLE AND CONTAINER FOR PBS
8. COVERSLIPS 22 X 50 MM OPTICAL GLASS NO. 1
9. DARK FIELD FLUORESCENCE MICROSCOPE, e.g. AMERICAN OPTICAL ONE-TEN WITH VERTICAL FLUORESCENCE. FITC HAS EXCITATION AND EMISSION PEAKS AT 490 AND 520 nm RESPECTIVELY AND THEREFORE MUST HAVE THE PROPER FILTERS
10. INCUBATOR 37 C

C. METHOD

1. Remove the EBNA + NEGATIVE cell antigen substrate slide from the foil packet gently so as not to scratch the cells on the glass surface.

2. Mark the end on each slide with the sample or control designation of the material you intend to apply to it.

3. If only a screening of the patient samples is to be performed one should screen at 1:5 and 1:20 dilutions.

4. The following is an appropriate series to perform for the assay controls and patient samples in a screening assay:

WELL NO.	SAMPLE	DILUTION
1	PBS(conjugate control)	-
2	NEGATIVE CONTROL	1:5
3	POSITIVE CONTROL	1:5
4	POSITIVE CONTROL	1:20
5	Patient Sample A	1:5
6	Patient Sample A	1:20
7	Patient Sample B	1:5
8	Patient Sample B	1:20
9	Patient Sample C	1:5
10	Patient Sample C	1:20
11	Patient Sample D	1:5
12	Patient Sample D	1:20

5. The following is a suggested dilution series for samples being titered for EBNA antibody (optional):

WELL NO.	SAMPLE	DILUTION
1	PBS(conjugate control)	-
2	NEGATIVE CONTROL	1:5
3	POSITIVE CONTROL	1:5
4	POSITIVE CONTROL	1:20
5	Patient a	1:5
6	Patient a	1:20
7	Patient a	1:40
8	Patient a	1:80
9	Patient a	1:160
10	Patient a	1:320
11	Patient a	1:640
12	Patient a	1:1280

6. Place 10 ul of the appropriate dilution of the serum or control on the well of the appropriate slide. Be sure the well is totally covered and the serum is confined only to the well. Cross contamination of wells may give erroneous results.

7. Carefully place the slides in the moisture chamber. Incubate at 37 C (+ OR - 3 C) for 20 minutes. A wet paper towel in the bottom of the chamber usually is enough to keep the slides moist.

8. Carefully remove the slides from the chamber and wash the serum samples off with a gentle stream of PBS from the wash bottle. The wash takes only seconds to perform. Exercise care not to direct the stream directly onto the cells as this may wash them off the glass.

9. Shake the PBS off the slide smartly. If PBS bridges remain between wells carefully remove them with a cotton swab or a slide blotter.

10. Place 10 ul of properly diluted guinea pig complement on all wells of all slides and incubate as in step 7.

11. Process as in steps 8 & 9.

12. Place 10 ul of the conjugate on each well and incubate as in step 7.

13. Wash the slides as in step 8.

14. Mount a coverslip with 20 ul of mounting fluid described previously onto each slide. Avoid air entrapment. High background may result from inadequate washing.

15. Read each slide with the fluorescence microscope within 2 hours to avoid possible quenching. If the slides are to be read later or reread they may be stored for a day or so as long as they are kept dark and if possible cool (2-8 C).

D. QUALITY CONTROL

1. Positive and negative controls should be run each time the assay is performed.

2. It is strongly recommended that the positive and negative controls be read before attempting to evaluate the test samples. This will aid in establishing the positive and negative references needed to interpret the test samples. It may be helpful to refer to these controls periodically when large numbers of test samples are assayed.

3. The negative control at 1:5 and conjugate PBS control well reactions should appear as red counterstained cells with no nuclear fluorescence. If not, the test is invalid and should be repeated. The negative control has been tested and found negative for EBV EBNA and no other claims with respect to any other antigens is made or implied.

4. The positive control reaction at 1:5 and 1:20 dilutions should appear as 40-50% of the cells fluorescing bright apple-green in the nucleus (30-70% is acceptable) with the remainder of the cells counterstained red. The fluorescence should be seen in the nucleus only. If not, the test is invalid and should be repeated.

5. The intensity of the observed fluorescence may vary with the microscope and the filter system used.

RESULTS

A specimen is POSITIVE if its reaction at 1:5 or greater is as described above for the positive control. A positive reaction is indicated by a significant apple-green fluorescence in the nucleus of 40-50% of the cells. The titer is the reciprocal of the last dilution that gives a positive nuclear reaction in 40-50% of the cells on the slide.

The specimen is NEGATIVE if its reaction is as described for the negative control.A negative reaction is defined as negative at a dilution of 1:5 AND 1:20. The nuclei of a negative will appear red counterstained.

Non-specific reactions such as ANA will be seen as significant apple-green fluorescence in the nuclei of nearly 100% of cells rather than in the nuclei of the 40-50% of the EBNA positive cells.

The CLINICAL SIGNIFICANCE of an EBNA negative result is that either the person has not been infected with EBV and may be susceptible to IM or the infection was very recent and measurable EBNA antibody has not yet developed. A positive EBNA result indicates the infection with EBV was at some indeterminate time in the past.

Although IgG and IgM antibodies appear and rise very soon after the onset of IM, antibody to EBNA may not appear for 3 weeks to six months (5,6,7). EBNA antibodies then persist for years, probably for life (6). Primary acute mononucleosis can be diagnosed by the determination of high levels of IgG and IgM VCA antibodies in the absence of EBNA antibody in serum. When serum is found to be VCA IgG positive-IgM negative while EBNA positive, this indicates the infection by EBV occurred at some indeterminate time in the past.

Demonstration of elevated EBV VCA IgG titers in conjunction with EBV VCA IgM and negative or low EBNA antibody titers improves the specificity of the serological diagnosis of IM (4, 5, 6, 7).

LIMITATIONS

1. All results with this device should be correlated with the clinical findings of the patient and other diagnostic tests such as IgG and IgM EBV VCA.

2. The components of this device have been manufactured

to perform optimally with the procedure given. It is not recommended to use the components of this kit with kits of any other manufacturer nor that components from other manufacturers be used with this device.

3. The intensity of the reactions seen will depend on the microscope and filter system used and may affect the titer.

4. Absence of EBNA antibody has been observed in immunosuppressed individuals as well as in early acute IM (9).

5. Antibody to EBNA may be elevated in nasopharyngeal carcinoma(10) and in African Burkitts lymphoma (11).

EXPECTED VALUES

In the USA 80-90% of the adult population is seropositive for EBV VCA (12). Although IgG and IgM antibodies appear and rise very soon after the onset of IM, antibody to EBNA may not appear for 3 weeks to six months (5,6,7). EBNA antibodies then persist for years, probably for life (6). Primary acute mononucleosis can be diagnosed by the determination of high levels of IgG and IgM VCA antibodies in the absence of EBNA antibody in serum. When serum is found to be VCA IgG positive-IgM negative while EBNA positive, this indicates the infection by EBV occurred at some indeterminate time in the past.

SPECIFIC PERFORMANCE CHARACTERISTICS

In a COMPARATIVE STUDY with another commercial ACIF kit no discrepancies were found. The study included 10 samples from normal adult individuals (20-40 years), both EBV negative (2 samples) and EBNA positive (8 samples which were IgG VCA positive, IgM VCA negative) and 16 clinically confirmed IM patients (IgG VCA titers ranging from 640-10,240 and IgM positive) which were EBNA negative.

A REPRODUCIBILITY STUDY has shown that this assay kit performs within the reproducibility specifications of plus or minus one two-fold dilution when used for titrations.

CROSS-REACTIVITY STUDIES indicated that sera positive for other herpesviruses (CMV, HSV and VZV) do not react with EBV specific antigens.

BIBLIOGRAPHY

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APPENDIX 10

Ped Cardiol 1:133–152 (1979/80)

Pediatric Cardiology

Percentile Charts

ECG Standards for Children

André Davignon *et al.*

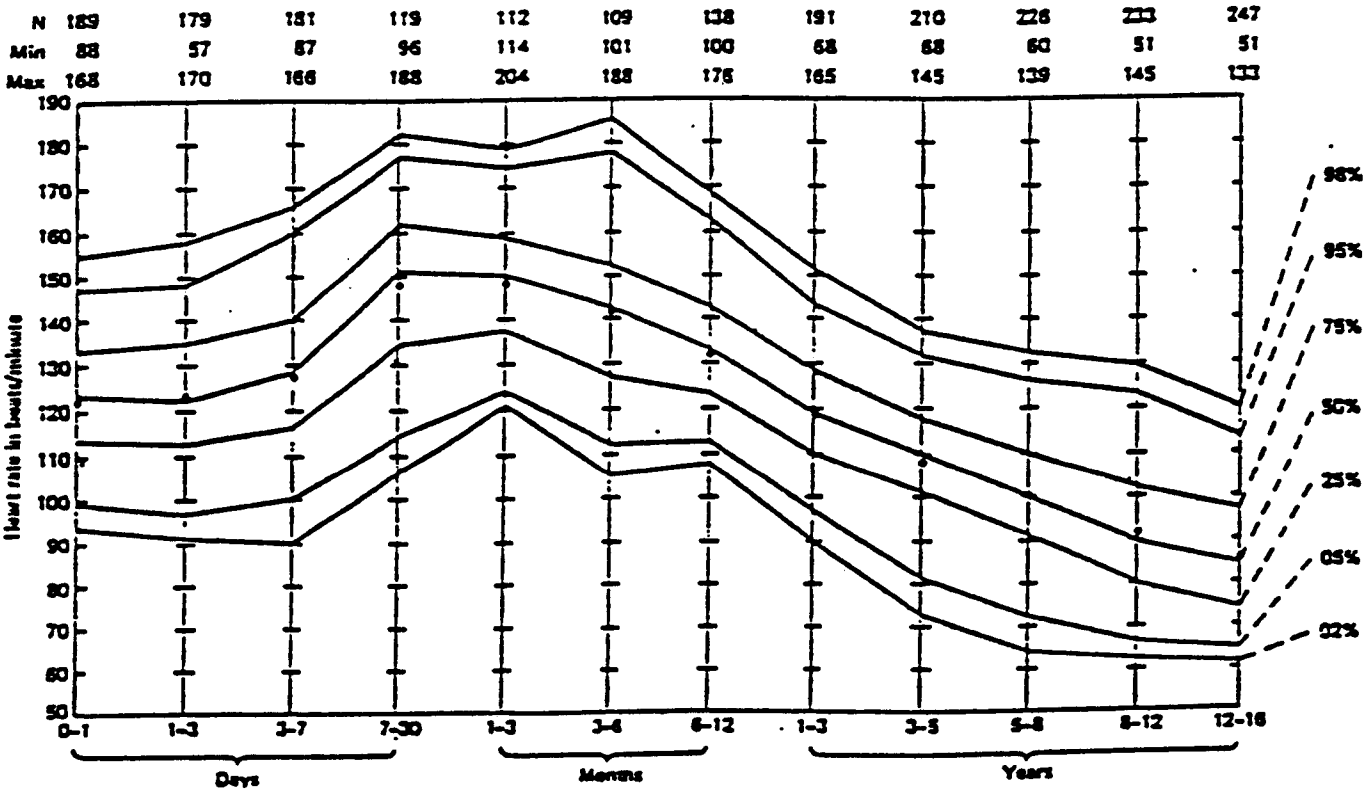

Fig. 1. Heart rate vs. age (● = mean)

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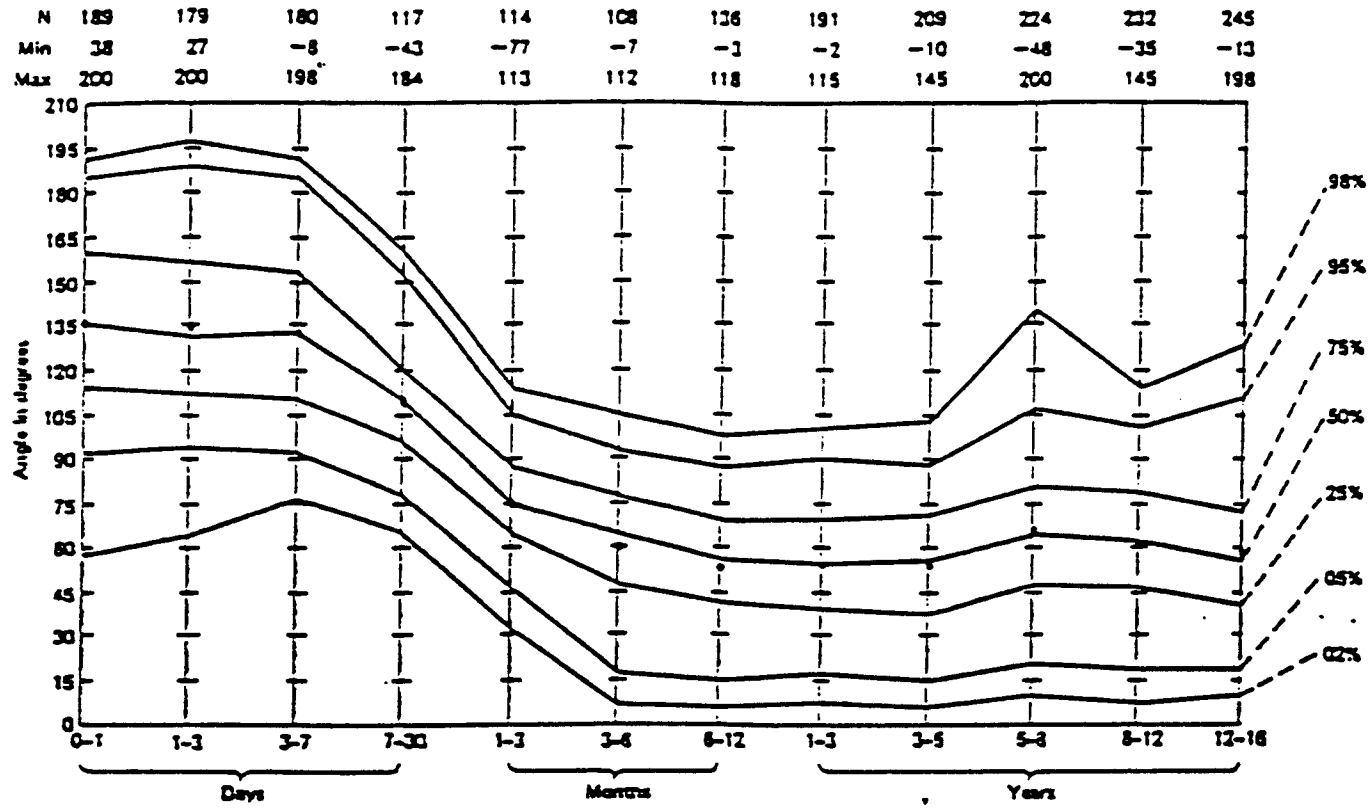


Fig. 2. Frontal plane QRS angle vs. age (● = mean)

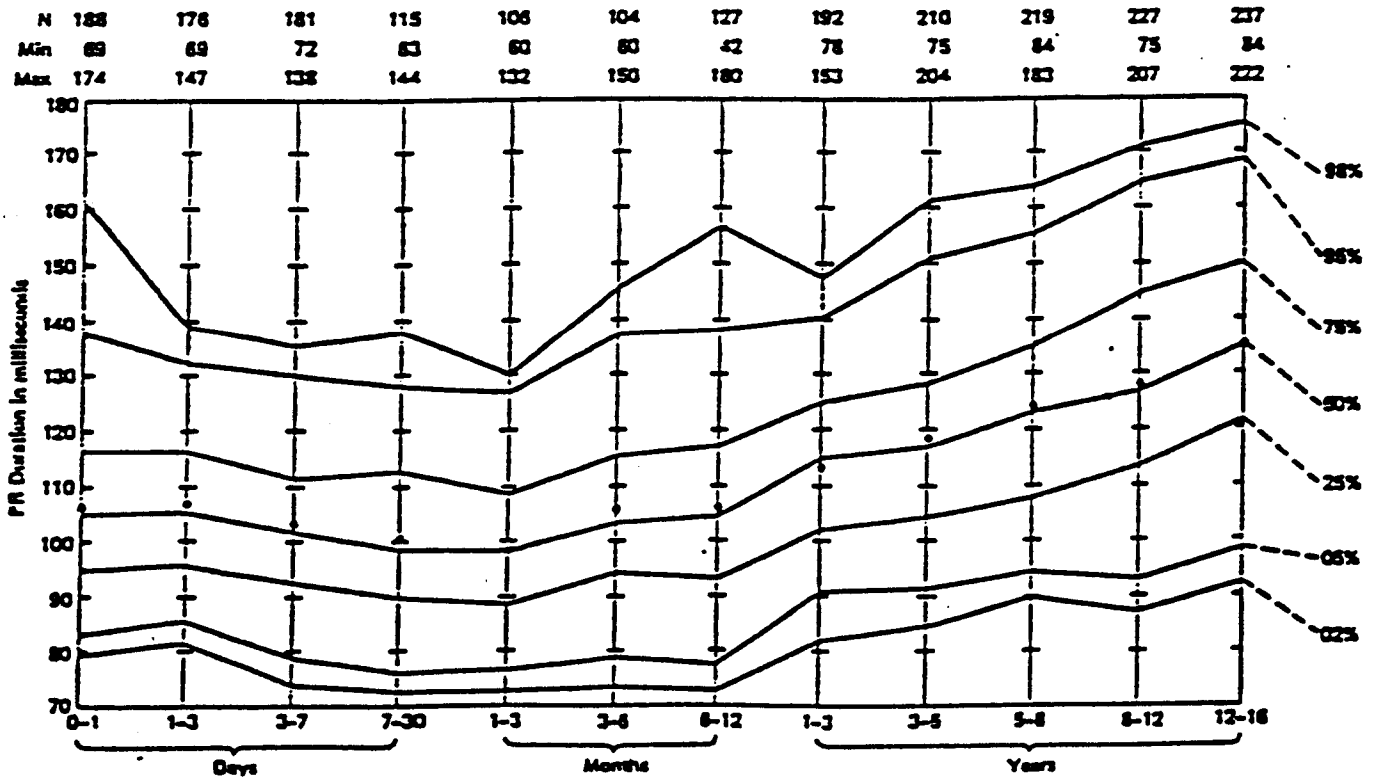


Fig. 3. PR duration vs. age in lead II (● = mean)

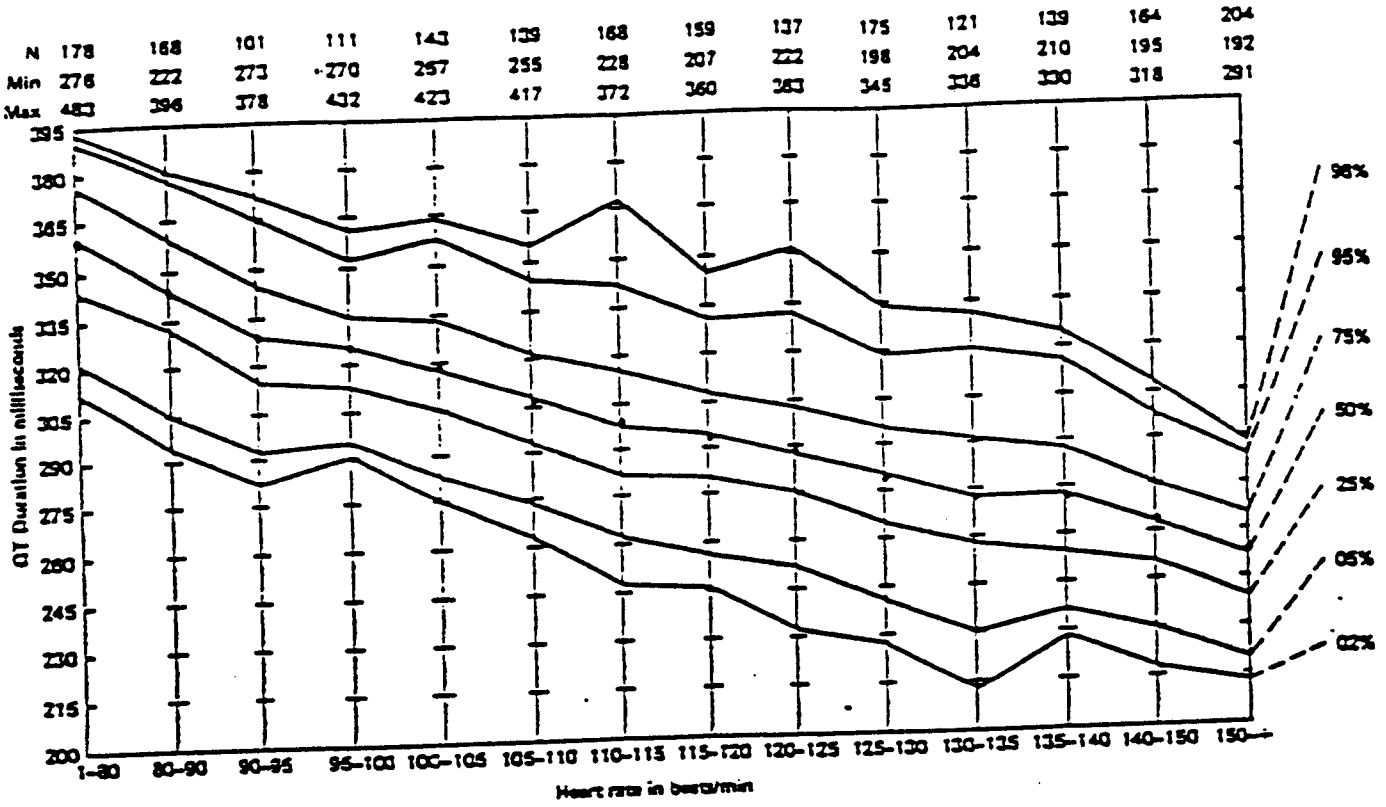


Fig. 6. QT duration vs. heart rate in lead V5 (● = mean)

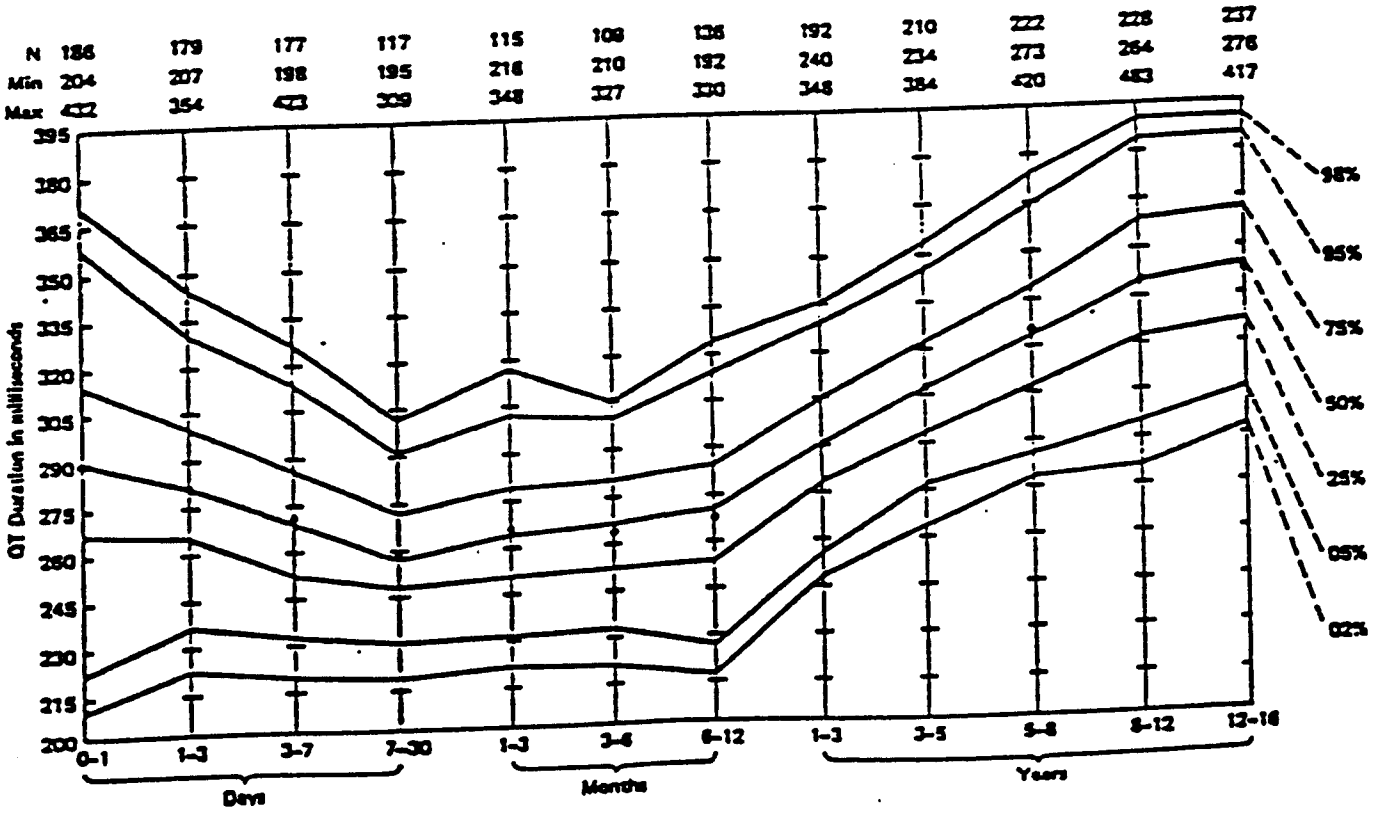


Fig. 7. QT duration vs. age in lead V5 (● = mean)

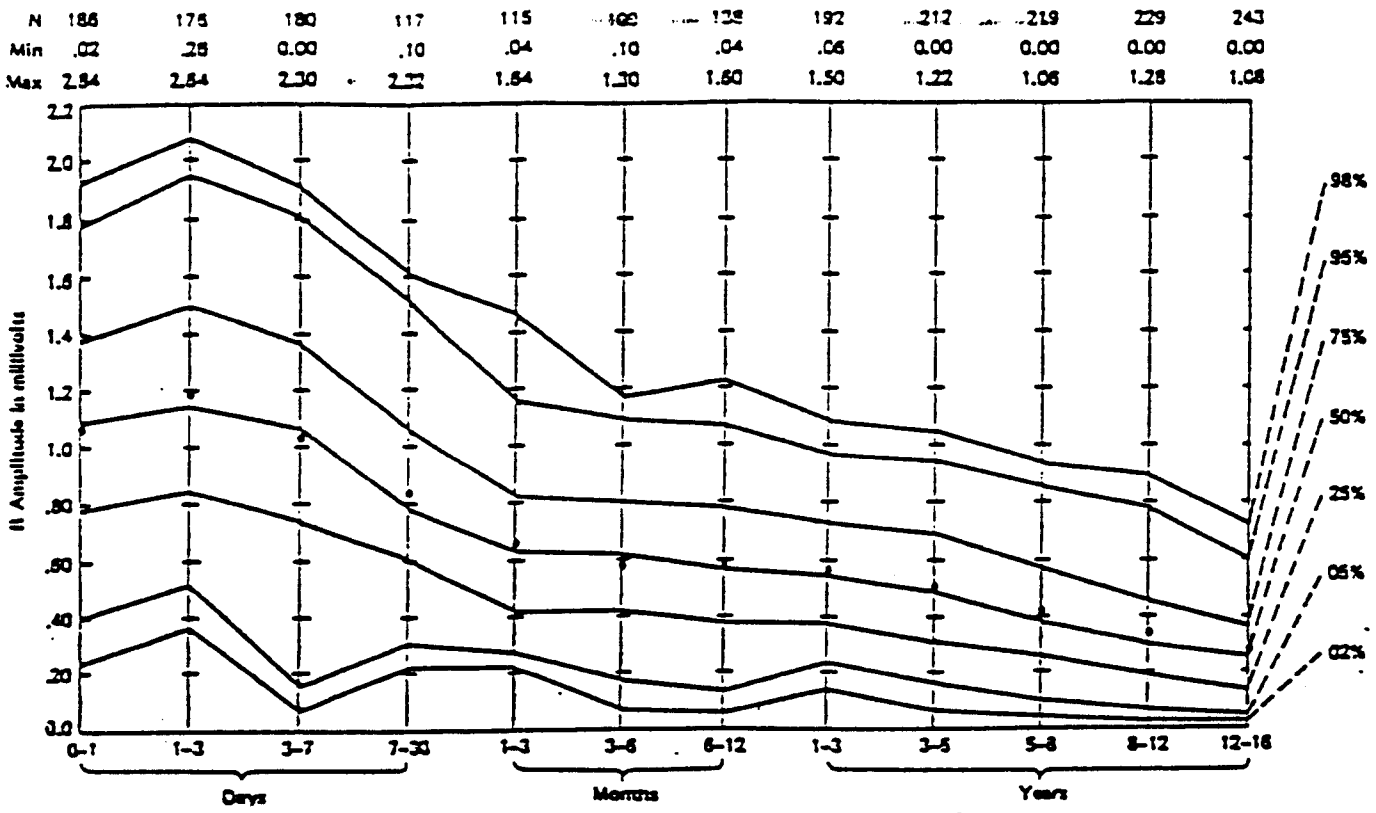


Fig. 14. R amplitude vs. age in lead V3R (● = mean)

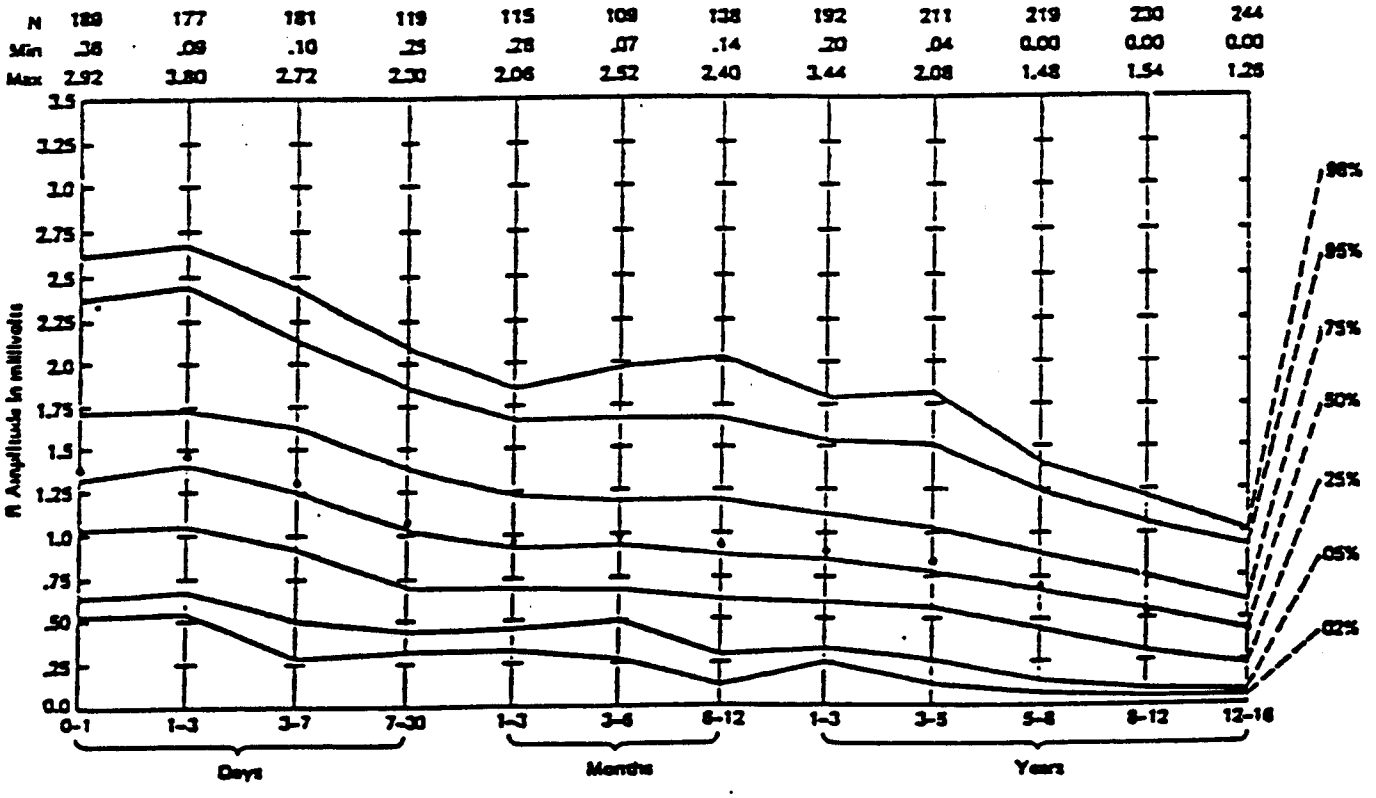


Fig. 15. R amplitude vs. age in lead V1 (● = mean)

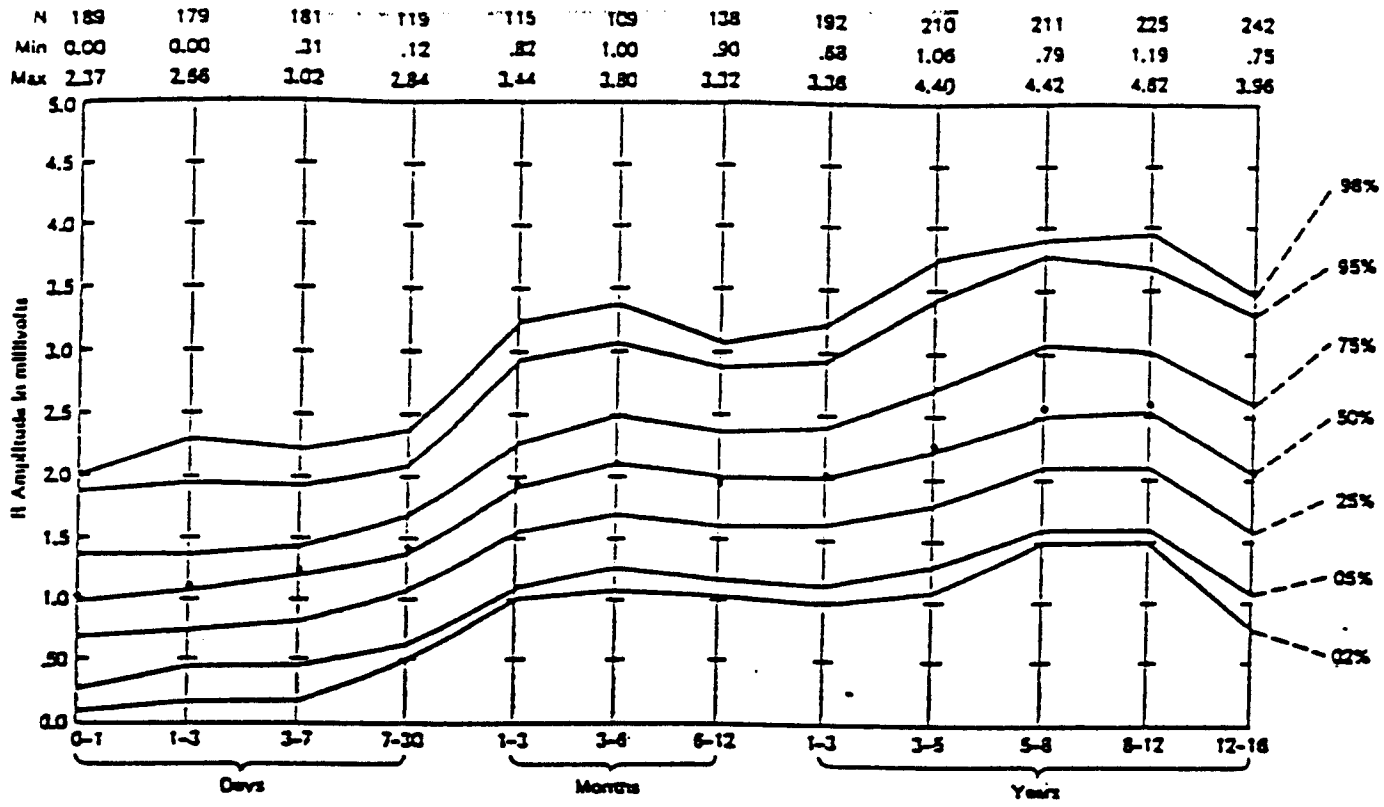


Fig. 18. R amplitude vs. age in lead V5 (● = mean)

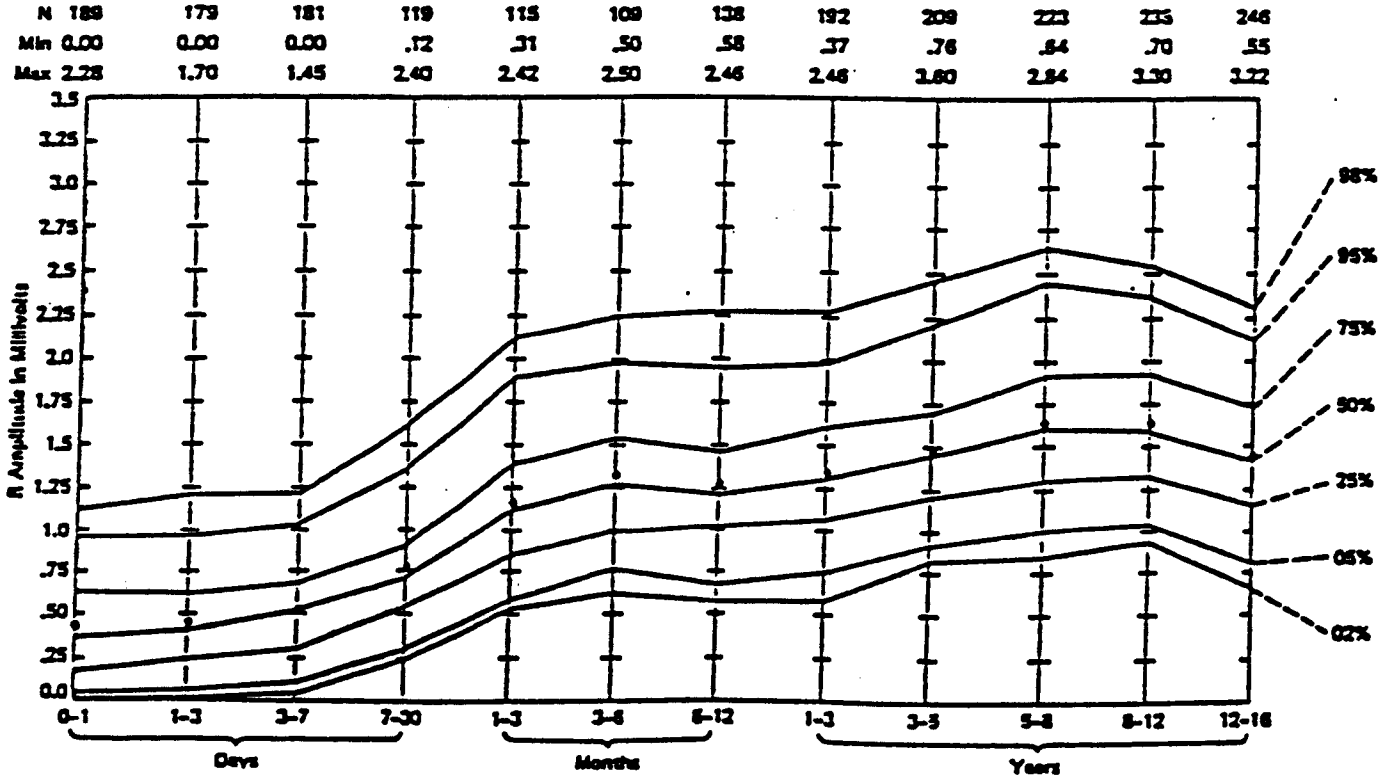


Fig. 19. R amplitude vs. age in lead V6 (● = mean)

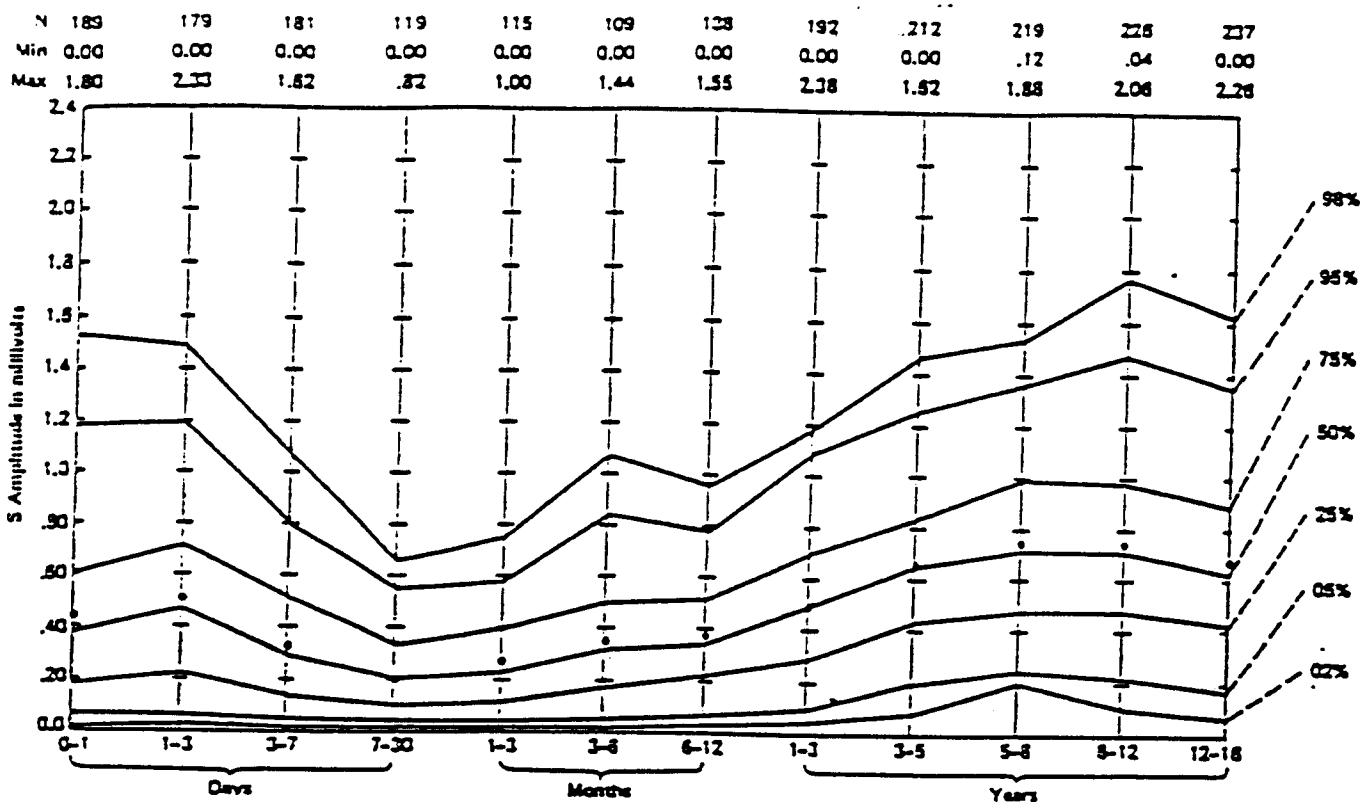


Fig. 20. S amplitude vs. age in lead V3R (● = mean)

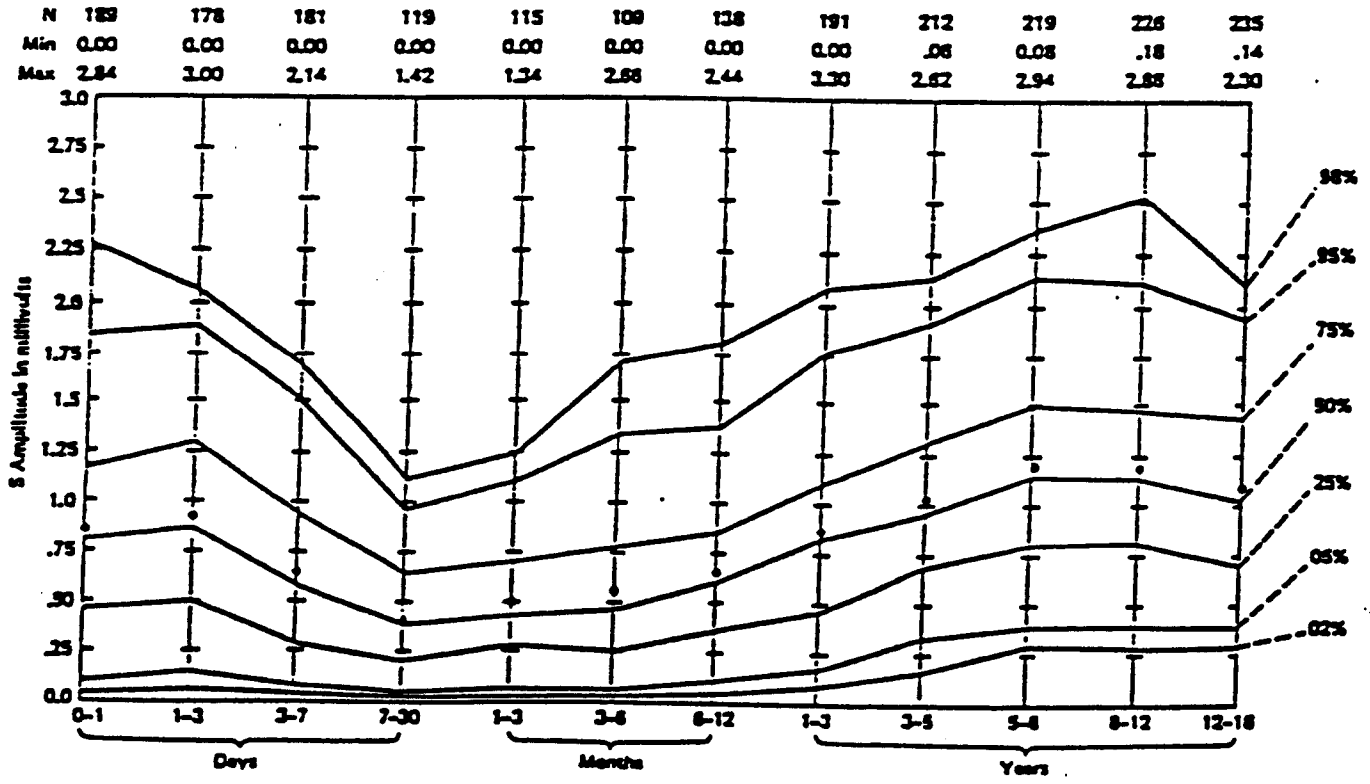


Fig. 21. S amplitude vs. age in lead V1 (● = mean)

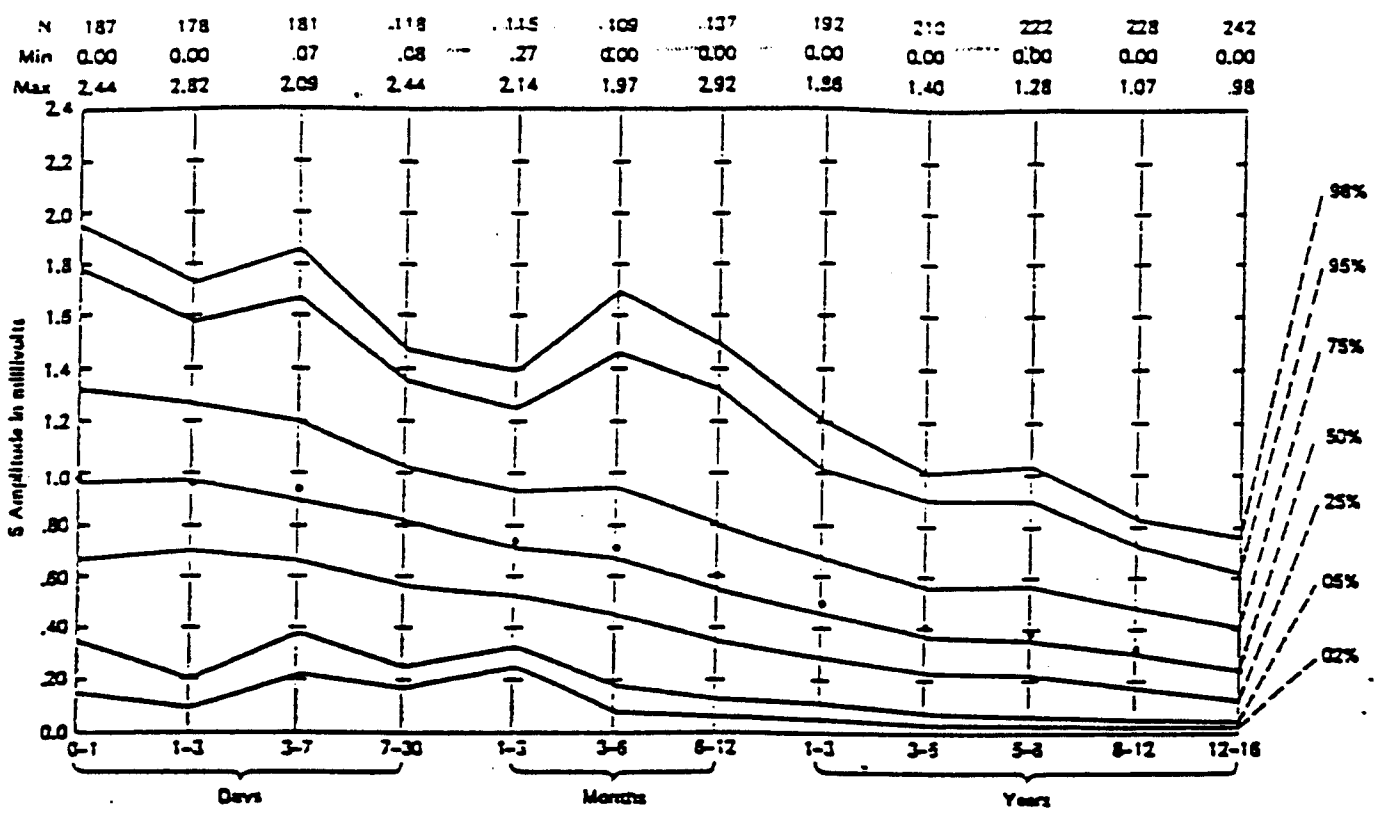


Fig. 24. S amplitude vs. age in lead V5 (● = mean)

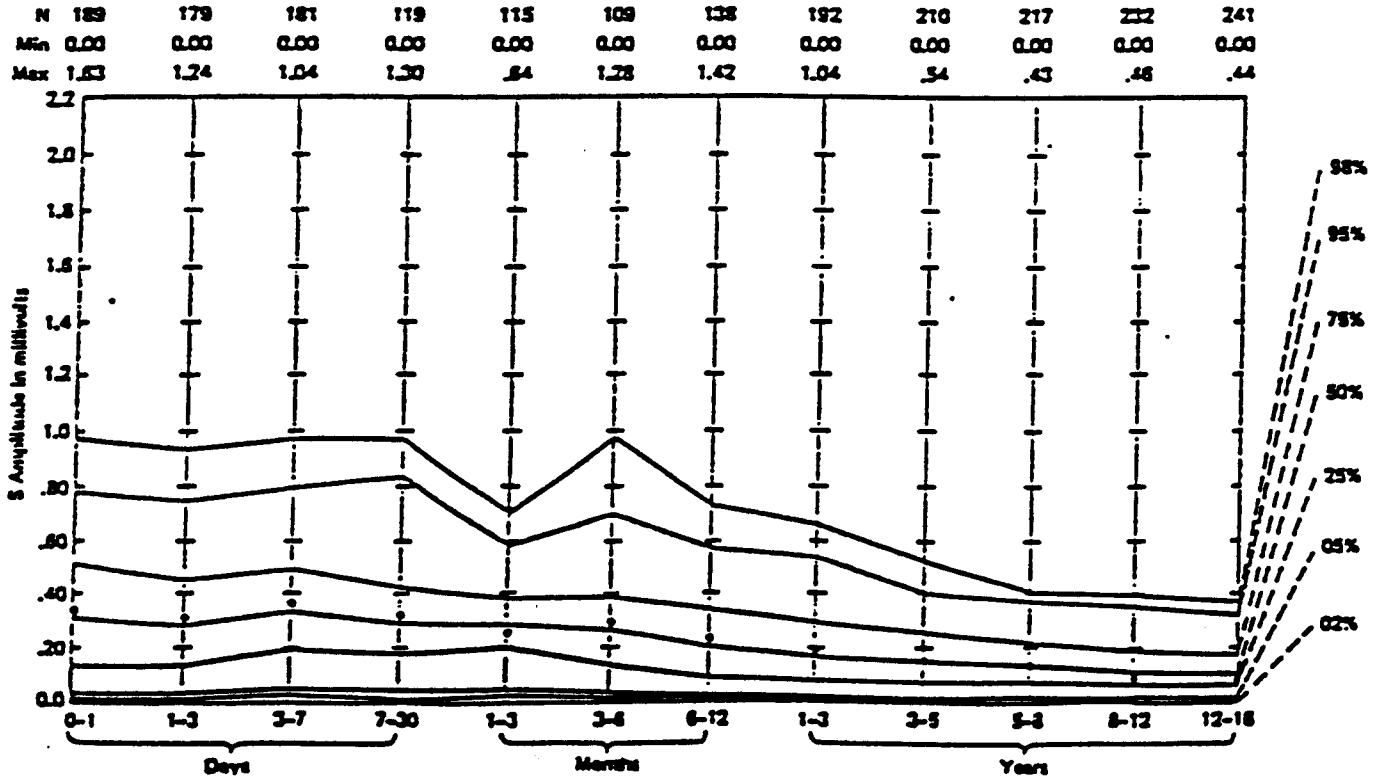


Fig. 25. S amplitude vs. age in lead V6 (● = mean)

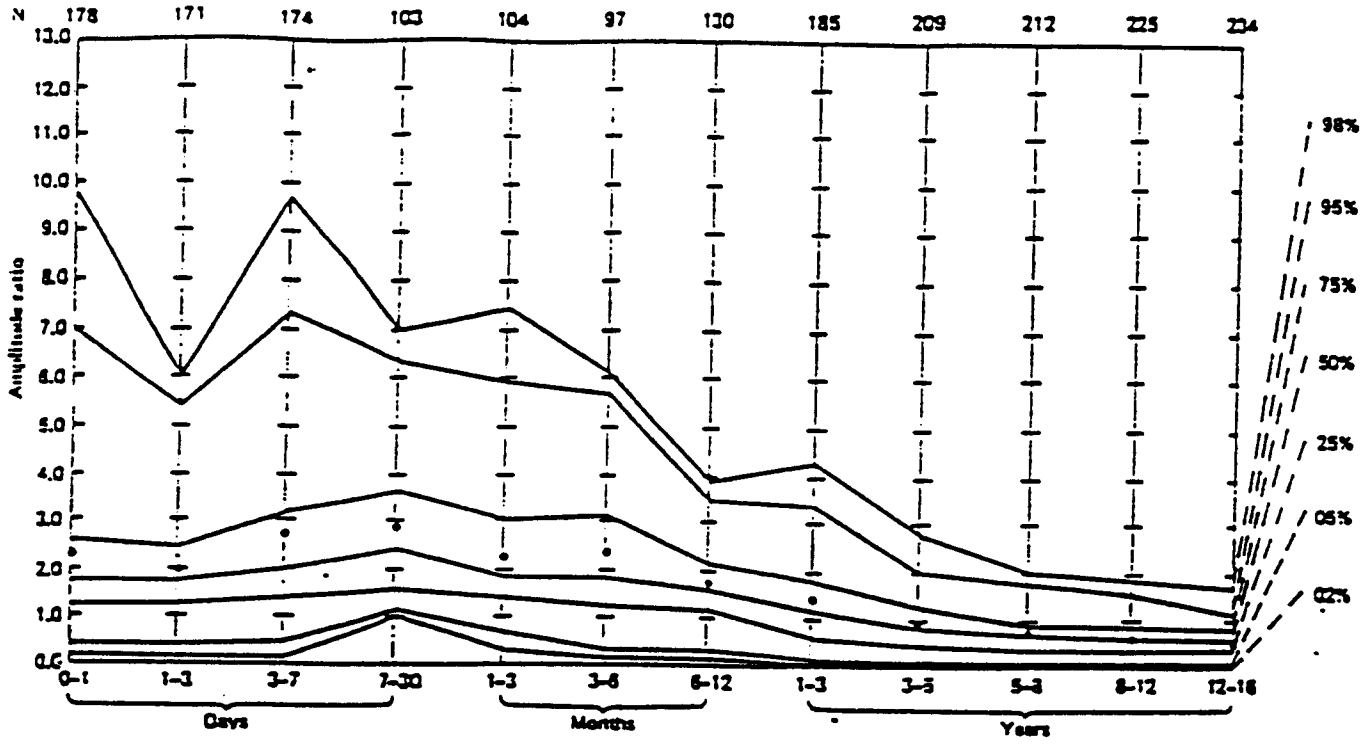


Fig. 32. R/S amplitude ratio vs. age in lead V1 (● = mean)

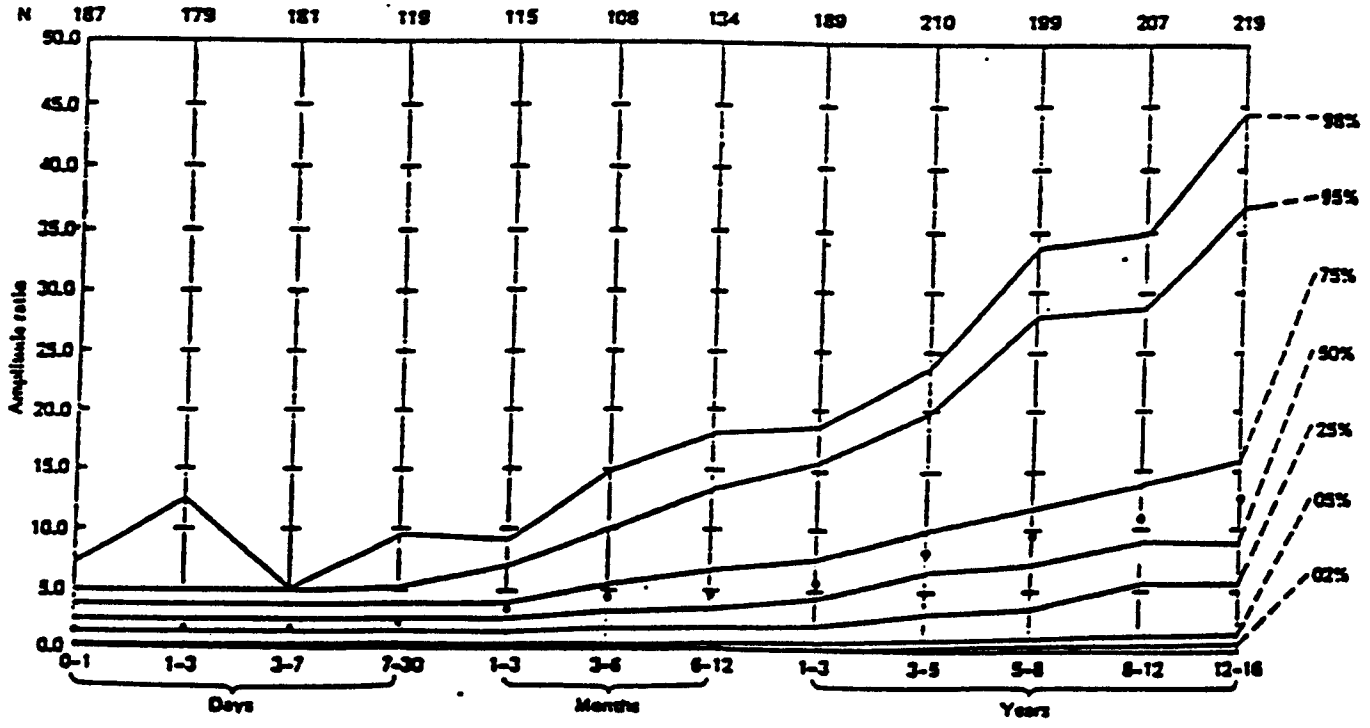


Fig. 33. R/S amplitude ratio vs. age in lead V5 (● = mean)

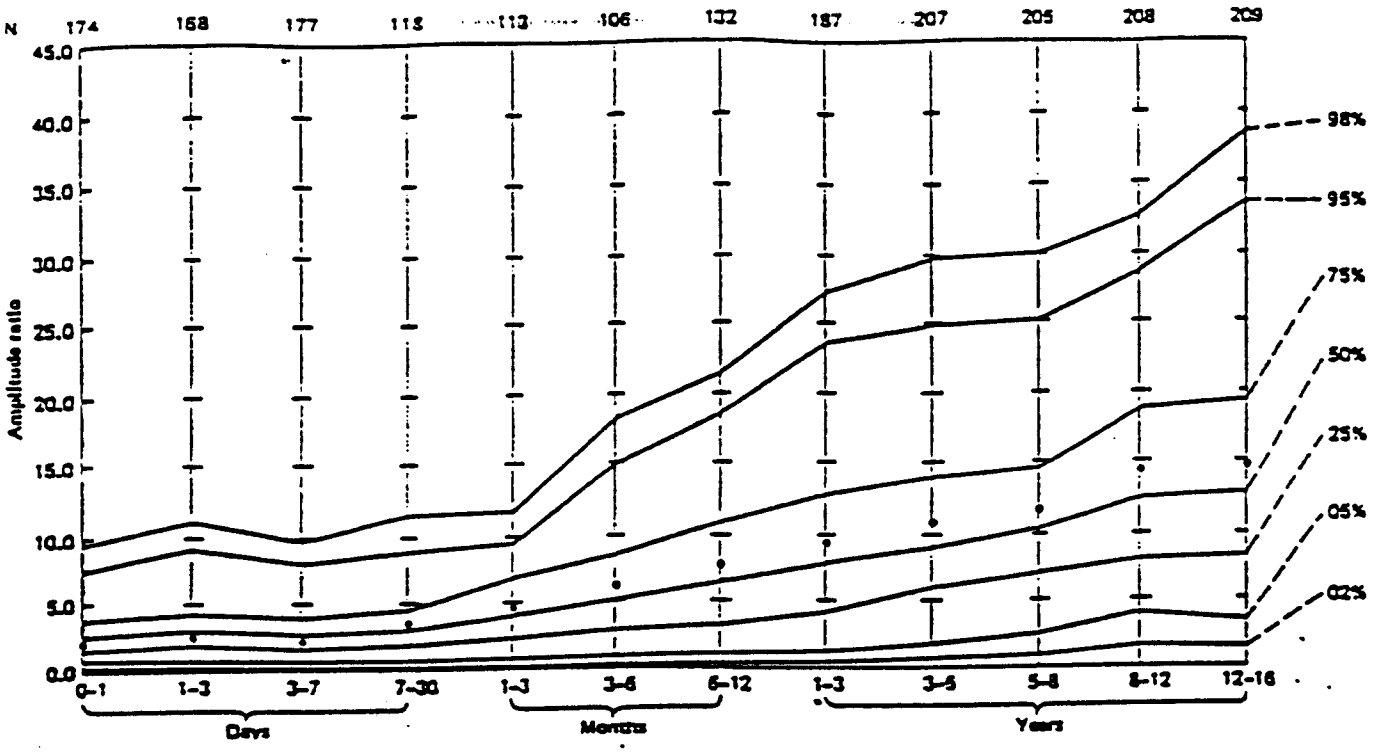


Fig. 34. R/S amplitude ratio vs. age in lead V6 (● = mean)

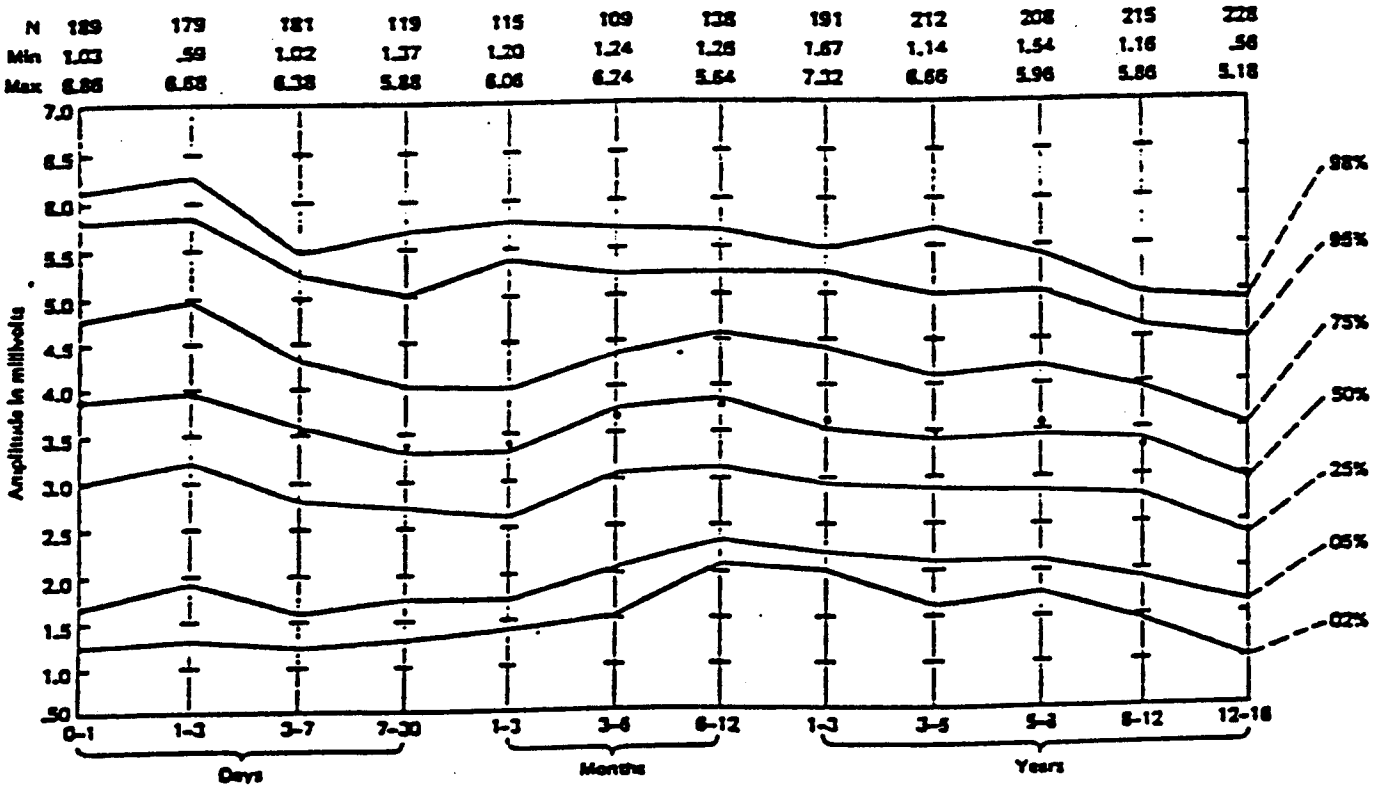


Fig. 35. R+S amplitude vs. age in lead V2 (● = mean)

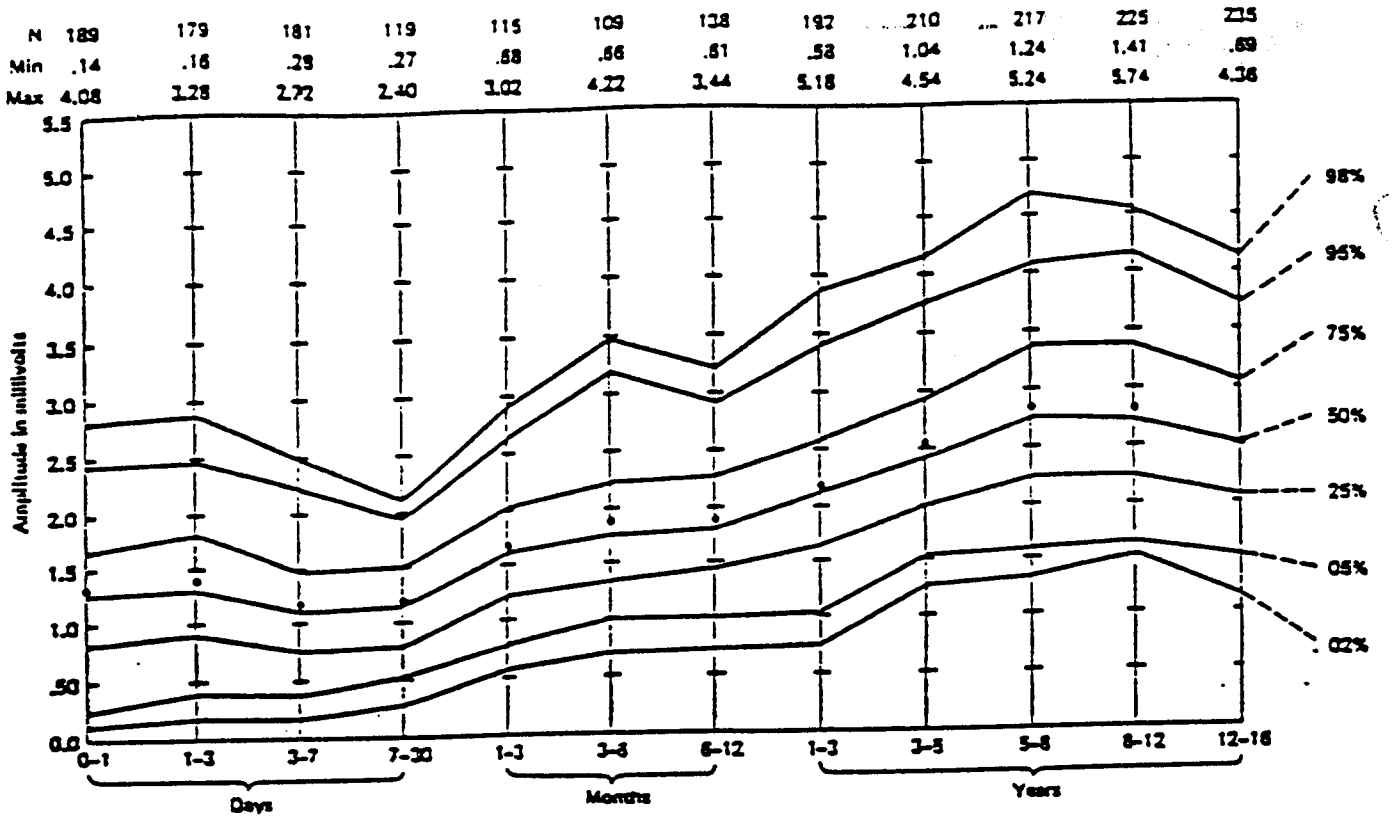


Fig. 38. Amplitude R in lead V6 + S in lead V1 vs. age (● = mean)

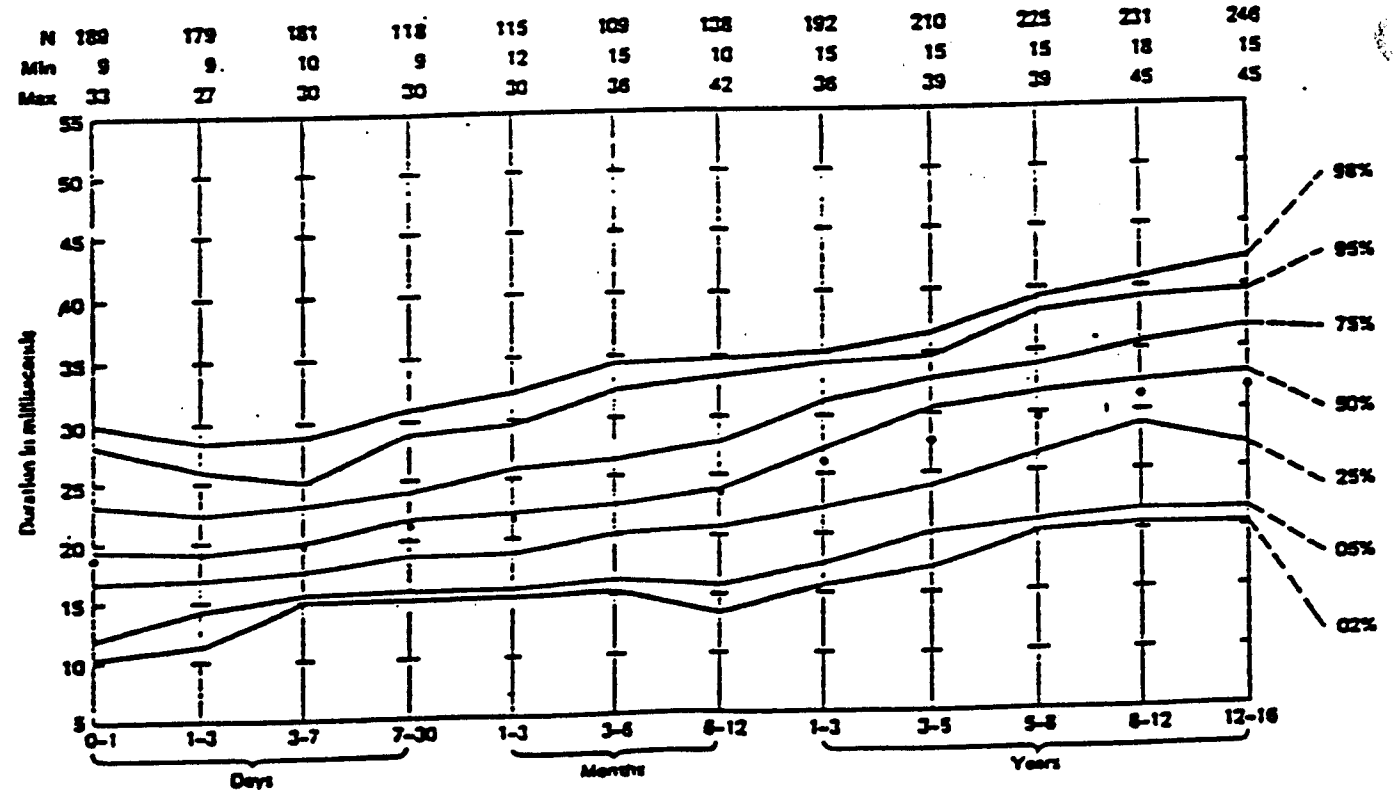


Fig. 39. Ventricular activation time vs. age in lead V5 (● = mean)

APPENDIX 11

BASIC PROCEDURE FOR LUNG CANNULATION

Autopsied lungs should be received in saline only, and there should be as little delay as possible between the time of the autopsy and the time the lungs are put on the lung tank. The larynx, upper trachea, and esophagus, and heart should be removed except in cases of congenital heart disease. Always check the autopsy case number on the specimen to make sure it is correct. Examine the specimen and note anything unusual and any incision made into the lung.

1) Carefully separate the two lungs by cutting down the trachea removing the left main stem bronchus at the carina leaving as much bronchus attached as possible. Rinse off excess blood. Try to remove any mucus from the bronchi by gentle squeezing.

2) Weigh each lung on the Sartorius balance and record weight.

3) Evacuate lungs for several minutes.

4) Cannulate lungs using plastic stopcocks which are inserted into the bronchus. Care should be taken not to push the stopcock too far down the bronchus past the branching point. If this happens, one of the lobes will not inflate.

5) Secure the bronchus around the end of the stopcock with carpet thread (best choice) making sure to tie it on so that the lung does not slip off the end.

6) Tag the lung with its case number.

7) Attach the lung to a stopcock in the lung tank. Make sure both stopcocks are open. Watch the lung to make sure that it appears to be infiltrating properly. Lungs need to remain on the tank a minimum of 24 hours.

8) After at least 24 hours, lungs are removed from the tank and volumed in water.

FIGURE 1. FORMALIN TANK USED FOR DISTENTION & FIXATION OF CHILDREN'S LUNGS

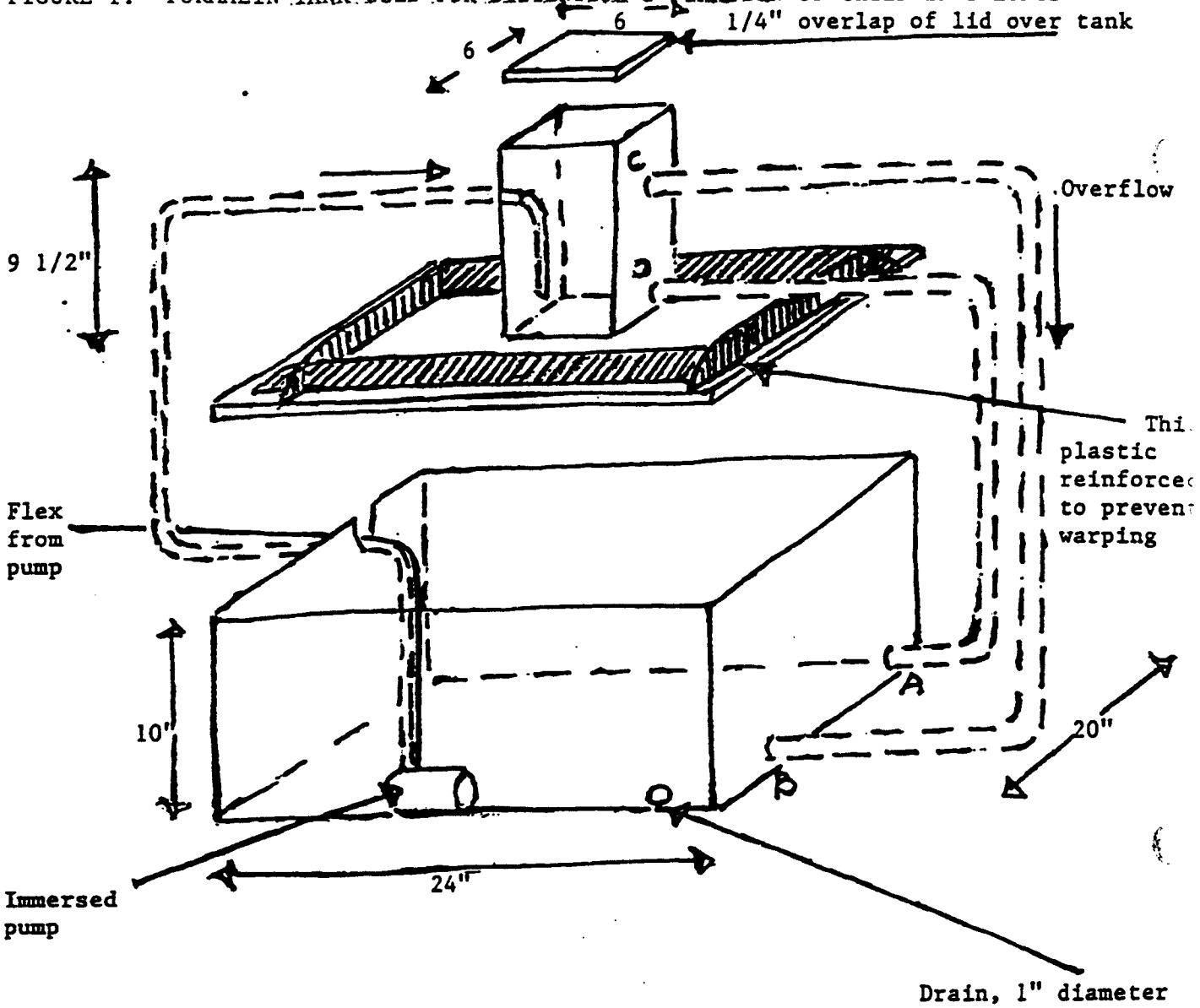


Figure 1: The tank is constructed of 1/2" thick transparent plastic. (We find that plastic thinner than this warps badly in response to formalin vapour.) The lids of the upper and lower tanks have a lip which overhangs the sides of the tanks, reducing escape of formalin vapour.

The upper tank contains formalin up to the base of the overflow tube. The lower tank also contains formalin, and there is a 25 cm. difference in height between the levels of fluid in the upper and lower tanks. Thus, lungs are distended at a constant transpulmonary pressure of 25 cm. water.

Inflow A in the lower tank opens into a manifold from which lead tubes to the lungs. Formalin entering the lower tank via the overflow and via the inflow A is returned to the upper tank by an immersed pump.

Tubes A, B, C, and D are all 1" in diameter. Connecting tubing between upper and lower tanks is tygon tubing.

FIGURE 2 MODE OF SAGITTAL SLICING OF LUNG

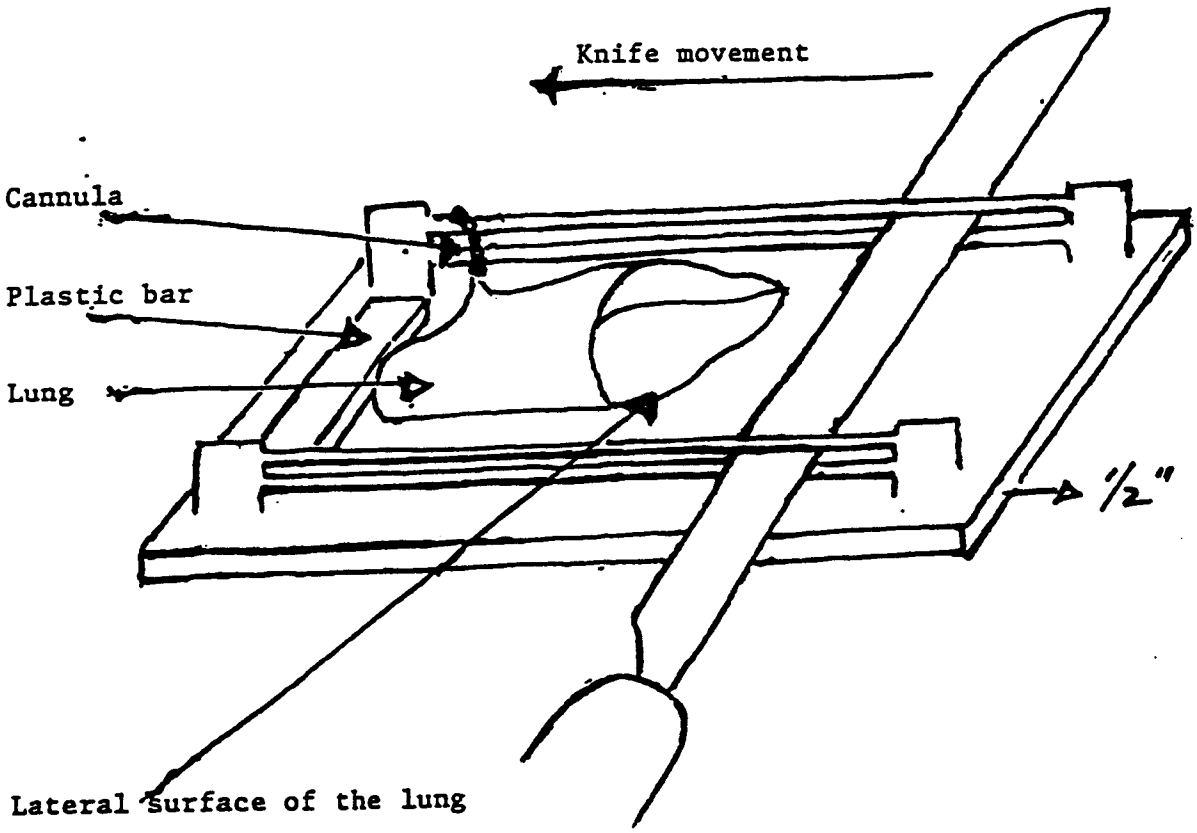
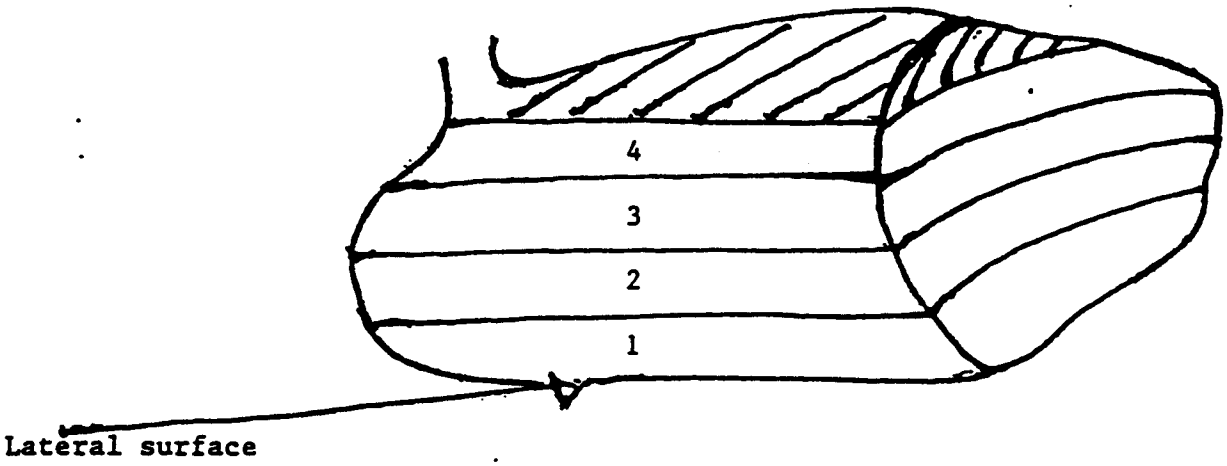
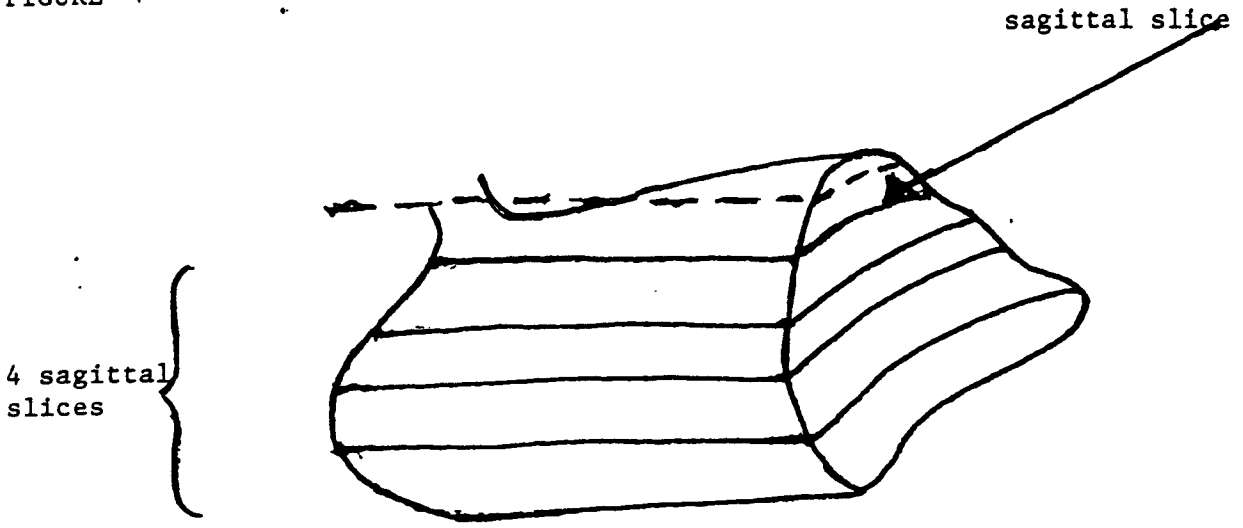


FIGURE 3 Numbering of sagittal lung slices



Medial portion of lung, which is discarded

FIGURE 4



To illustrate the region of the lung where the last sagittal cut is made. The diagram shows a lung divided into 4 sagittal slices. The dotted line shows the hypothetical position of the sagittal cut which would be made in order to produce a 5th sagittal slice of the lung. In the medial surface of the 5th slice, the main bronchus is separated from the rest of the slice. This situation should be avoided.

APPENDIX 12

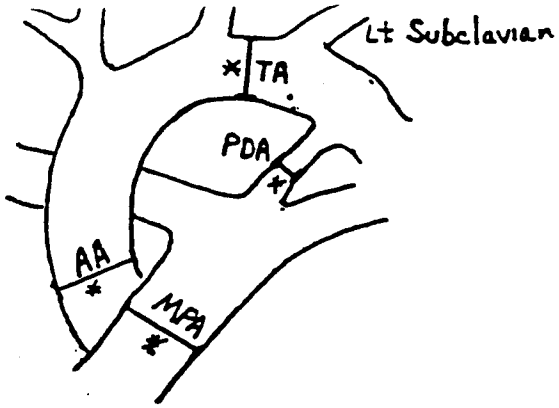


Figure 1: External diameter measurements (*) of aorta, pulmonary artery, and ductus arteriosus. See text for abbreviations.

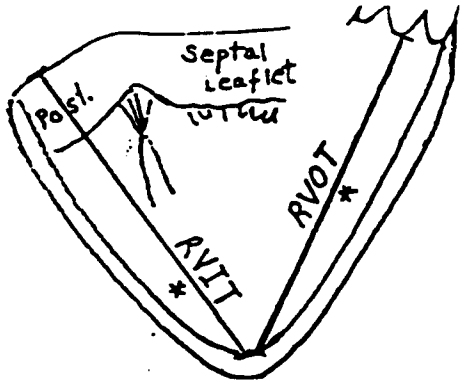


Figure 2: Opened right ventricle. Measured le for the inflow (RVIT) and outflow (RVOT) tra

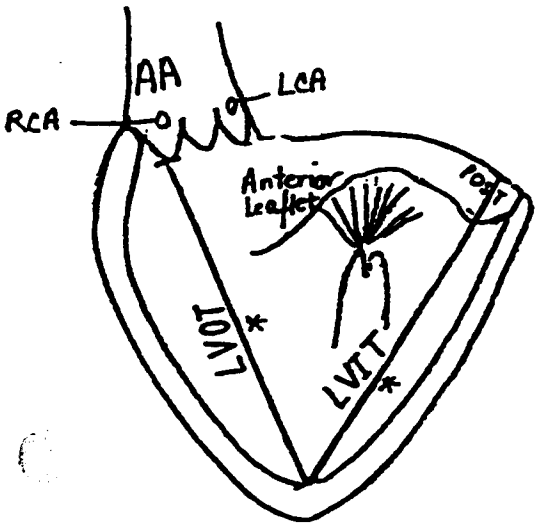


Figure 3: Opened left ventricle. Measured lengths for the inflow (LVIT) and outflow (LVOT) tracts.

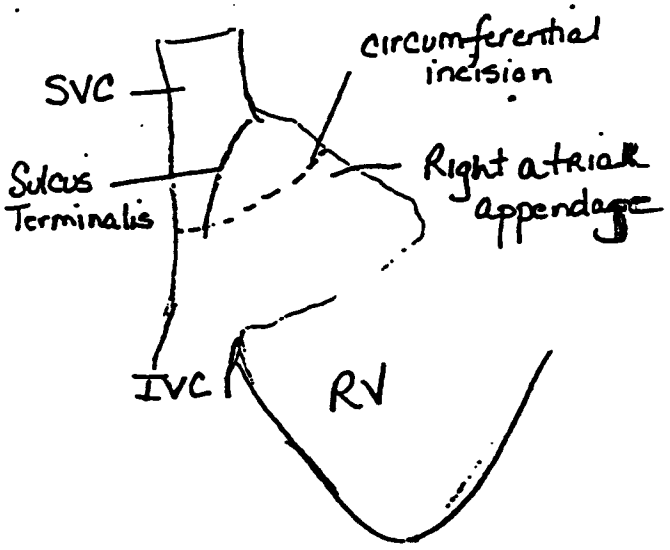


Figure 4: Right atrium. Circumferential incisio excise SA node en bloc.

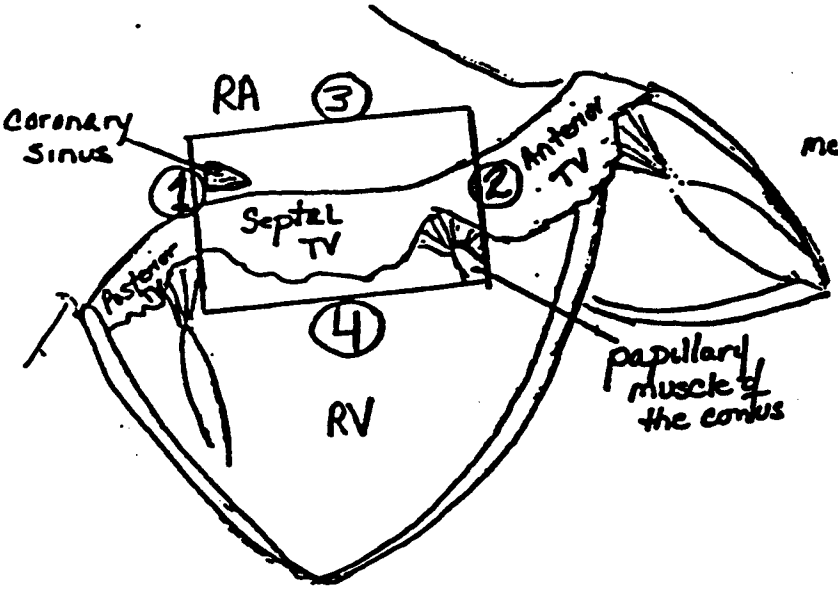


Figure 5: Opened right atrium and right ventricle. Placement of incisions (1-4) for en bloc excision of atrioventricular conduction axis.

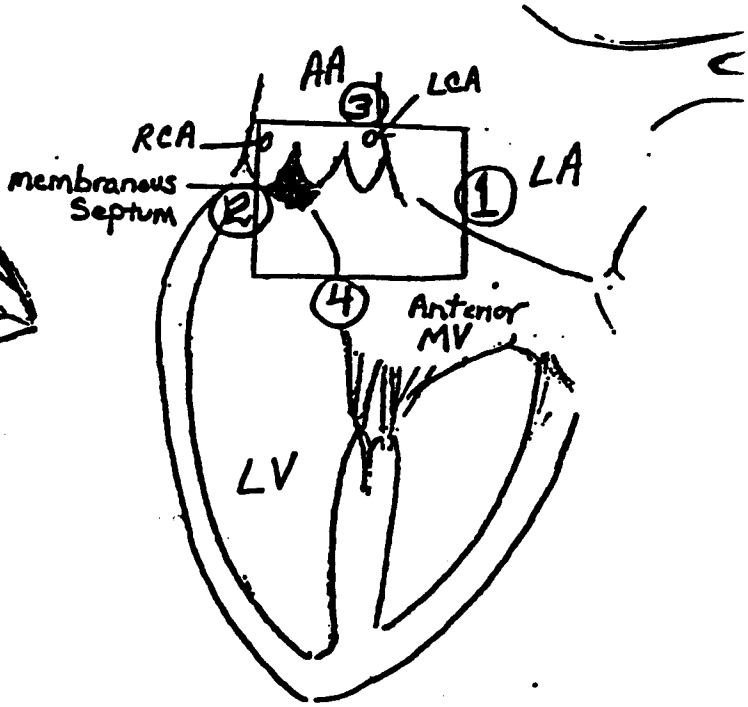


Figure 6: Opened left atrium and left ventricle Placement of incisions (1-4) for en bloc excisio atrioventricular conduction axis.

APPENDIX 13

Gross Heart and Lung Examination Worksheet

Heart:

Heart weight _____

External Measurements:

Maximal cardiac breadth _____

Maximal A-P diameter _____

Base-apex length _____

AA _____

MPA _____

TA _____

PDA-E _____

PDA-I _____

Cardiac Valves:

TV _____

PV _____

MV _____

AV _____

Chamber Dimensions:

RVIT _____

RVOT _____

LVIT _____

LVOT _____

Wall Thickness:

RVI _____

RVO _____

RVM _____

LVI _____

LVO _____

LVM _____

Lung:

Right lung weight _____

Right lung volume _____

Left lung weight _____

Left lung volume _____

RNAase Free Paraformaldehyde	Electron Microscopy	Frozen Tissue
Myocardium:		
RVI	RV x 2	RVI-2
LVI	LV x 2	LVI-2
Coronary Arteries:		
RCA	mid RCA	included with RVI-2
LAD	mid LAD	LAD-2
Systemic Arteries:		
renal arteries (right & left)		
mesentery		mesentery
left carotid artery at arch		
Other Organs:		
renal cortex and medulla	cortex & corticomedullary junction	cortex and medulla
spleen		spleen
lung (right & left)	lung either side	lung, right or left
skeletal muscle	skeletal muscle	skeletal muscle
thymus		thymus
		cells and fluid pleural and pericardial effusions
Brain:		
carotid artery circle of willis		
middle cerebral artery		mid portion middle cerebral artery
frontal cortex with meninges		frontal cortex with meninges
cerebellum with meninges		cerebellum with meninges

Special Cardiovascular Sections in Formalin	
Myocardium:	
RVO	
RVA	
LVO	
LVA	
LVP	
VS	
Coronary Arteries:	
LCA	
LCX	
PD	

APPENDIX 14

Table 5–5. CONVERSION FROM BPD IN MM TO MENSTRUAL AGE IN WEEKS

BPD (mm)	Menstrual Age (weeks)	90% Range	BPD (mm)	Menstrual Age (weeks)	90% Range
22	12.7	12.2–13.2	61	24.2	22.6–25.8
23	13.0	12.4–13.6	62	24.6	23.1–26.1
24	13.2	12.6–13.8	63	24.9	23.4–26.4
25	13.5	12.9–14.1	64	25.3	23.8–26.8
26	13.7	13.1–14.3	65	25.6	24.1–27.1
27	14.0	13.4–14.6	66	26.0	24.5–27.5
28	14.3	13.6–15.0	67	26.4	25.0–27.8
29	14.6	13.9–15.2	68	26.7	25.3–28.1
30	14.8	14.1–15.5	69	27.1	25.8–28.4
31	15.1	14.3–15.9	70	27.5	26.3–28.7
32	15.3	14.5–16.1	71	27.9	26.7–29.1
33	15.6	14.7–16.5	72	28.3	27.2–29.4
34	15.9	15.0–16.8	73	28.7	27.6–29.8
35	16.2	15.2–17.2	74	29.1	28.1–30.1
36	16.4	15.4–17.4	75	29.5	28.5–30.5
37	16.7	15.6–17.8	76	30.0	29.0–31.0
38	17.0	15.9–18.1	77	30.3	29.2–31.4
39	17.3	16.1–18.5	78	30.8	29.6–32.0
40	17.6	16.4–18.8	79	31.2	29.9–32.5
41	17.9	16.5–19.3	80	31.6	30.2–33.0
42	18.2	16.6–19.8	81	32.1	30.7–33.5
43	18.5	16.8–20.2	82	32.6	31.2–34.0
44	18.8	16.9–20.7	83	33.0	31.5–34.5
45	19.1	17.0–21.2	84	33.5	31.9–35.1
46	19.4	17.4–21.4	85	34.0	32.3–35.7
47	19.7	17.8–21.6	86	34.5	32.8–36.2
48	20.0	18.2–21.8	87	35.0	33.4–36.6
49	20.3	18.6–22.0	88	35.5	33.9–37.1
50	20.6	19.0–22.2	89	36.1	34.6–37.6
51	20.9	19.3–22.5	90	36.6	35.1–38.1
52	21.2	19.5–22.9	91	37.2	35.9–38.5
53	21.5	19.8–23.2	92	37.8	36.7–38.9
54	21.9	20.1–23.7	93	38.3	37.3–39.3
55	22.2	20.4–24.0	94	39.0	37.9–40.1
56	22.5	20.7–24.3	95	39.7	38.5–40.9
57	22.8	21.1–24.5	96	40.3	39.1–41.5
58	23.2	21.5–24.9	97	41.0	39.9–42.1
59	23.5	21.9–25.1	98	41.8	40.5–43.1
60	23.9	22.3–25.5			

From Kurtz AB, Wapner RJ, Kurtz RJ, et al: J Clin Ultrasound 8:319, 1980.

Table 5–6. PREDICTED MENSTRUAL AGE FOR FEMUR LENGTHS

Femur Length (mm)	Menstrual Age (weeks)	Femur Length (mm)	Menstrual Age (weeks)
10	12.8	45	24.5
11	13.1	46	24.9
12	13.4	47	25.3
13	13.6	48	25.7
14	13.9	49	26.1
15	14.2	50	26.5
16	14.5	51	27.0
17	14.8	52	27.4
18	15.1	53	27.8
19	15.4	54	28.2
20	15.7	55	28.7
21	16.0	56	29.1
22	16.3	57	29.6
23	16.6	58	30.0
24	16.9	59	30.5
25	17.2	60	30.9
26	17.6	61	31.4
27	17.9	62	31.9
28	18.2	63	32.3
29	18.6	64	32.8
30	18.9	65	33.3
31	19.2	66	33.8
32	19.6	67	34.2
33	19.9	68	34.7
34	20.3	69	35.2
35	20.7	70	35.7
36	21.0	71	36.2
37	21.4	72	36.7
38	21.8	73	37.2
39	22.1	74	37.7
40	22.9	75	38.3
41	22.5	76	38.8
42	23.3	77	39.3
43	23.7	78	39.8
44	24.1	79	40.4

From Hadlock FP, Harrist RB, Deter RL, Park SK: Fetal femur lengths as a predictor of menstrual age: sonographically measured. AJR 138:875, 1982.

EBV METHODOLOGY

I. Serology

Indirect immunofluorescent assays will be performed to detect IgG antibodies to viral capsid antigen (VCA) and early antigen (EA), IgM antibodies to VCA and complement fixing antibodies to EB nuclear antigen (EBNA).

IgM antibodies to EBV capsid antigen will be determined by a modification (1) utilizing smears of P3J-HR1K cells pretreated with tumor-promoting agent. These cells will be incubated with serial dilutions of the patient's serum, which had previously been adsorbed with recombinant Protein G. The smears are then washed with PBS, incubated with affinity purified fluorescein isothiocyanate-conjugated goat antibodies to human IgM (Tago, Burlingame, Calif.), washed again, and mounted onto slides for UV immunofluorescence.

IgG antibodies to EBV capsid antigen will be determined on acetone fixed cell smears with P3J-HR1K cells that have been incubated with the test sera and then affinity purified fluorescein isothiocyanate-conjugated goat antibodies to human IgG (2).

Antibodies to diffuse and restricted components of EBV early antigen will be titrated by a modification of the method described by Henle et al (3) by using Raji cells that had been activated by 5-iododeoxyuridine (4) (Calbiochem, San Diego).

Antibodies to EBV nuclear antigen will be determined by a modification of the method of Reedman and Klein (5). Raji cells are successively overlaid with inactivated test sera, guinea pig complement and, finally, fluorescein isothiocyanate-conjugated antibodies to guinea pig C_3 (Cappel Labs). Molt 4 cell smears were used as a negative antigen control.

The patient's immune status to EBV per the above immunofluorescent reactions will be determined by published serologic criteria (6).

II. Culture

EBV will be isolated from throat wash and tumor using the umbilical cord blood cell transformation assay described by Chang et al (7). Briefly, a gargle specimen of RPMI 1640 with oropharyngeal secretions or aliquot of tumor tissue (cells) is incubated with umbilical cord cells and observed for signs of transformation, i.e. clumping, rapid growth, unusual morphology. Transformed cell cultures are examined for EBV nuclear antigen. If available, the number of EBV-infected cells in the peripheral blood will be estimated by using an endpoint dilution method of Rocchi et al (8).

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GULL

EBV IgM Test Instructions

An Immunofluorescence Test for the Detection of IgM Antibodies to Epstein-Barr Virus

INTRODUCTION

Epstein-Barr virus is perhaps the most common and widely disseminated human virus. Virtually everyone acquires antibodies to EBV with advancing age (1). Responses to primary EBV infection range from subclinical infection in children less than two years to severe and debilitating disease in young adults. The predominant symptomatic disease of EBV infection is infectious mononucleosis (IM). IM has an insidious onset with headache, sore throat, fever, enlarged cervical lymph nodes, splenomegaly, malaise, and some form of pharyngitis. Abnormal liver function is more marked with EBV mononucleosis than CMV mononucleosis and must be considered in the differential diagnosis of hepatitis (2).

Other EBV-induced disease can involve the neurologic, cardiac, ocular, respiratory, hematologic, digestive, and renal systems. Neurological syndromes associated with EBV infection include meningitis, encephalitis, Guillain-Barré syndrome, Bell's palsy, myelitis, cranial nerve neuritis, and psychotic disorders. Bulbar involvement with ensuing respiratory paralysis can be fatal (3). EBV is also associated with Burkitt's lymphoma and nasopharyngeal carcinoma (1).

Many viral antigens are released in EBV infection. Viral capsid antigens (VCA) not only appear first but also develop in all cases of EBV infection. Since IgM to EBV capsid antigen is the acute phase antibody, it is the antibody of choice for detection of active disease. Serology is the only means to identify EBV infection in the absence of typical diagnostic symptoms of IM. Likewise, EBV serology is important in diagnosing infection in young children since they usually do not develop typical IM symptoms or heterophil antibodies (4).

PRINCIPLE

The indirect fluorescent antibody method is used in the EBV IgM Test. Patient serum is reacted with the Burkitt's lymphocytic-cell substrate and if IgM antibodies to EBV are present they will bind to the antigen substrate and not rinse off. Subsequently, when fluoresceinated antihuman IgM is added to the reaction site, it will bind to the IgM antibodies, causing the EBV infected cells to fluoresce when viewed through the fluorescence microscope.

INSTRUCTIONS FOR USE OF KIT

The entire kit should be stored in the refrigerator (5°C). The kit is ready for use after reconstitution of reagents. If reconstituted conjugate and sera are to be stored at -20°C, self-defrosting freezers are not recommended. Individual slide packets should remain sealed until just before use. Reagents and antigen slides should not be used beyond stated expiration dates.

The components of this kit have been tested and standardized as a unit. Use of components from other lots or other manufacturers may yield unsatisfactory results.

PRECAUTIONS

This product is for IN VITRO diagnostic use only.

WARNING—Reagents marked with ** on the label are considered POTENTIAL BIOHAZARDOUS MATERIAL. Each donor unit used in the preparation of this material was tested by an FDA approved method for the presence of antibody to human T-lymphotropic virus type III/lymphadenopathy associated virus (HTLV-III/LAV) as well as hepatitis B surface antigen (HBsAg) and found to be negative. Because no test method can offer complete assurance that infectious agents are absent, all reagents and patients' specimens should be handled at the Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual "Biosafety in Microbiological and Biomedical Laboratories", 1984.

MATERIALS PROVIDED

Preparation, Storage, and Stability

1. EBV Antigen Substrate Slides: Ten 10-well or ten 5-well slides with HR1 Burkitt's lymphocytic cells fixed onto each well. Approximately 5 to 10% of the cells express the viral antigen to permit easy reading and optimal contrast. The cells are rendered noninfectious by the PsoraiSafe™ process. The individually packaged slides are ready for use when removed from the foil packets. Slides stored at 5°C are stable until the date stated on the slide package.
2. Phosphate Buffered Saline (PBS powder), 1 vial: Rehydrate to 1 liter with distilled water. When rehydrated, the PBS is a 0.01 M phosphate buffer with a pH of 7.5 and contains 0.01% merthiolate as a preservative. Rehydrated PBS stored at 5°C is stable indefinitely.
3. Control Sera, 2 vials, lyophilized: Each control serum is reconstituted with 1 ml PBS to give a 1:10 working dilution. The reconstituted sera are stable for 6 weeks at 5°C or 6 months at -20°C.
   a. The Positive Control is human serum containing IgM antibodies to EBV. The titer is stated on the label.
   b. The Negative Control is human serum containing IgG but not IgM antibodies to EBV. The presence of immune IgG antibodies controls for the specificity of the anti IgM conjugate.
4. Conjugate, 1 vial, lyophilized: FITC-labeled antihuman IgM, heavy-chain specific, is reconstituted with 2 ml of PBS. The conjugate contains counterstain and is pretitered for use with each kit. The reconstituted conjugate can be stored up to 4 weeks at 5°C or aliquoted and stored up to 6 months at -20°C. Thawed aliquots should not be refrozen.
5. Mounting Fluid, 1 bottle, 3 ml: The mounting fluid, which is buffered to pH 8.0, is a glycerol-water combination formulated to minimize elution of counterstain. It is ready for use and can be stored at room temperature.

ADDITIONAL MATERIALS REQUIRED

1. Serology laboratory supplies: 12x75 mm test tubes, test tube rack, serological and Pasteur pipets
2. Liter volumetric flask
3. Wash bottle
4. Distilled water
5. Staining dish
6. Cotton-tipped swabs, absorbent towels
7. Moist chamber
8. Cover slips, no. 1, 22x50 mm
9. 37°C Incubator
10. Fluorescence microscope. A FITC blue light excitation filter and a 515 nm barrier filter, or any comparable filter system, is suggested for transmitted-light microscopy using a darkfield condenser and for incident light microscopy using a 500 nm dichroic mirror.

SPECIMEN COLLECTION AND PREPARATION

Blood obtained by venipuncture should be allowed to clot at room temperature and then be centrifuged. The serum should be separated as soon as possible and refrigerated (5°C), or stored frozen (-20°C) if not tested within one week. Self-defrosting freezers are not recommended as storage units. The use of sera exhibiting hemolysis, lipemia or microbial growth is not recommended.

Screening dilutions of 1:10 and 1:40 are recommended. However, if one screening dilution is preferred, a 1:20 dilution has been shown to be sufficiently sensitive for routine testing.

TEST PROCEDURE

1. *Prepare screening dilution(s) of the patient serum with PBS.*
2. *Remove slide from foil packet.*
3. *Using a Pasteur pipet, add just enough appropriately diluted serum (approximately 10 µl) to cover each reaction site.*
4. *Place the slide in a moist chamber and incubate at 37°C for 90 minutes.*
5. *Rinse slide briefly with a gentle stream of PBS (avoid directing PBS at wells) and then immerse in PBS for 5 minutes.*
6. *Air dry the slide by standing on end on absorbent toweling.*
7. *Wipe between wells with a cotton-tipped swab moistened with distilled water.*
8. *Add just enough conjugate (approximately 10 µl) to cover each reaction site.*
9. *Incubate the slide at 37°C in a moist chamber for 30 minutes.*
10. *Rinse and dry the slide as described in steps 5 and 6.*
11. *Just before reading, place a small drop of mounting fluid on each reaction site and cover with a 22x50 mm, no. 1 cover slip.*
12. *Read slide as soon as possible at 150X to 200X magnification.*

QUALITY CONTROL

Each kit contains positive and negative control sera which should be incorporated into each test run. The negative control must be negative at the screening dilution. The positive control serum is titered to provide a standard for checking test sensitivity. It should exhibit a positive reaction at the 1:10 dilution and diminish to a 1+ reaction at its stated titer. A day to day variance of one two-fold dilution on either side of the stated titer is considered acceptable performance.

INTERPRETATION OF RESULTS

The reaction is POSITIVE when 5 to 10% of the cells in each field exhibit a greenish-yellow fluorescence. The remaining cells provide a contrasting red background. A positive reaction at a 1:10 or greater dilution is indicative of a current EBV infection. It is not necessary to titrate positive sera in the EBV IgM Test. (See Test Limitations to rule out false positives due to rheumatoid factor.)

The reaction is NEGATIVE when cells do not fluoresce greenish-yellow but appear red due to the counterstain. A negative reaction at the screening dilution(s) indicates no detectable IgM antibody to EBV.

TEST LIMITATIONS

The presence of rheumatoid factor (RF) in serum may cause a false positive reaction if EBV IgG antibody is also present. Routine RF tests may not detect small amounts of RF which can cause false positive results in the more sensitive IFA technique. Therefore, all sera which demonstrate a positive IFA IgM reaction should be treated by ion-exchange chromatography to eliminate possible RF interference. Treated serum specimens should be retested for specific IgM to EBV.

High titers of immune IgG competing with immune IgM for antigen sites may produce a false negative IgM result at low screening dilutions. A higher screening dilution, such as 1:40, alleviates this problem.

Due to the innate ability of HRI lymphocytes to synthesize IgM in vitro, some lots of slides will exhibit a faint background fluorescence. This staining consists of unevenly distributed fluorescing dots seen within the majority of cells. This stippled staining, when it appears, is easily distinguished from the relatively uniform, whole-cell fluorescence produced by immune IgM to EBV.

IgM anticell antibodies, if present in the serum, may interfere with the EBV IgM Test. However, this interference is easily discerned since the staining produced by these antibodies involves all of the cells, whereas EBV IgM staining involves only 5 to 10% of the cells.

EXPECTED VALUES

EBV capsid IgM is produced in all primary EBV infections, whether symptomatic or asymptomatic. Peak IgM titers in patients with diagnosed EBV infection range from a low of 40 to as high as 1280 with Gull's system. IgM antibodies decline rapidly to undetectable levels in 8-10 weeks(4).

PERFORMANCE CHARACTERISTICS

When performed according to instructions, the EBV IgM Test is a sensitive and rapid method for diagnosing EBV infection. The sensitivity of each kit lot is controlled by testing with reference sera to ensure reproducible results. Clinical studies of the EBV IgM Test showed Gull's test to have 96% sensitivity and 100% specificity for IgM antibody to EBV capsid antigen (5). No cross reactivity has been observed with antibody to other herpes viruses.

Tests for heterophil antibody and IgG antibody to EBV have inherent limitations in the rapid diagnosis of active disease, since they measure antibodies which develop more slowly and persist for longer periods of time. This antibody persistence can create confusion in patients later infected with other agents, especially when the original EBV infection was not diagnosed (6). IgM testing resolves the heterophil false positive responses and the false negative problem in children (4). Because IgG VCA titers are frequently at their peak at the time the first serum specimen is taken, diagnosis of IM by seroconversion or by demonstrating a rise in titer is unlikely. Likewise, diagnosing active disease by the IgG titer of a single serum specimen is difficult since the upper range of persisting convalescent titers overlaps with the lower range of maximal titers obtained in the acute phase of IM (7). Since IgM is present only in current disease, the EBV IgM Test requires only a single serum specimen, positive sera do not need to be titered, and results can be simply reported in terms of presence or absence of immune IgM.

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Product No. EB140 (50 tests)
EB150 (100 tests)

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APPENDIX 15

The Electrocardiogram in Infants and Children"

Arthur Garson, Jr., M.D.

Table A-2*

	Heart Rate (beats/min)					
AGE	Min	2%	Mean	98%	Max	No. Subjects
Less than 1 day	88	93	123	154	168	189
1-2 Days	57	91	123	159	170	179
3-6 Days	87	91	129	166	166	181
1-3 Weeks	96	107	148	182	188	119
1-2 Months	114	121	149	179	204	112
3-5 Months	101	106	141	186	188	109
6-11 Months	100	109	134	169	176	136
1-2 Years	68	89	119	151	165	191
3-4 Years	68	73	108	137	145	210
5-7 Years	60	65	100	133	139	226
8-11 Years	51	62	91	130	145	233
12-15 Years	51	60	85	119	133	237

' The limits of "normal" include the 2nd and 98th percentile.

*This Table corresponds to the Davignon graph for heart rate.
(See Appendix 10)

APPENDIX 16

Tidal Flow-Volume Curves

Collection of tidal flow-volume curves to determine proper head, neck and jaw position for subsequent measurements

Discussion:

Beware of tidal loops that don't close. This was noted to be fairly common. The most common reason is leak on expiration. It was suggested that the use of Parafilm^{R} or a silicone putty could be used to improve the mask seal. A concern was raised regarding excessive pressure against the mask and the potential this would have for changing tidal loops (and, presumably hugs, compliance/resistance measurements).

If a leak can be ruled out, the suspicion for the cause of loops that fail to close must fall on the pneumotach or pneumotach/valve apparatus (see discussion under Compliance/Resistance Calibration Drift). It was noted that the addition of the 2600 valve apparatus to the pneumotach changed the measurement characteristics of the pneumotach alone and makes relinearization a necessity. Flow-volume flutter (predominately inspiratory flutter) has been noted and found to be related to head/neck position (e.g. occasionally correctable with proper jaw positioning).

When it was suggested that we adopt a rule that tidal loops that didn't close be rejected, it was noted that a change in end-expiratory lung volume can occur transiently without pneumotach alinearity or a leak being present. This would indicate an actual change in the end-expiratory lung volume (FRC). If this was the reason for loops failing to close, the situation would have to be transient and would correct itself in a very brief period of time. Failure of the tidal loops to self-correct would again raise the suspicion of a leak or pneumotach alinearity.

Resolution: There should be less than or equal to five percent difference between V_I and V_E. If the loops are moving back and forth, this difference would be considered normal variation of the end-expiratory lung volume. If the loops are walking in one direction only, a leak or pneumotach alinearity should be suspected, even if the error falls within the +/- 5% variance.

Compliance/Resistance

Equipment-related problems

1. Calibration drift

   a. **zero (baseline) drift**
      plugged pressure transducer line
      water in the lines
      failure of O-ring seal (leak and obstruction)

   b. **gain drift**
      pneumotach incorrectly assembled
      failure of O-ring seal (leak)

2. Water in pneumotach port or line

   a. **drift (see above)**
   b. **noise**
   c. **increased response time**
   d. **prevention - note position of pneumotach port relative to gravity and drainage of condensate.**

It was noted that measurements made with patients on the ventilator require additional care in that condensate accumulates more rapidly when testing these patients.

3. Sticky valve

   a. **use the Calibration/Verification software routine to apply continuous pressure to the valve to unstick**

   b. **check air supply (requires 50-60 psi)**

   c. **clean and lubricate the valve using Dow-Corning Molykote^{R} 33) (see attached data sheet from Hans-Rudolph on the care and cleaning of Hans-Rudolph products and Sections 14.2 and 14.3 of the Sensormedic manual on the cleaning of maintenance of the Occlusion and FRC valve)**

   d. **as a last resort, use the calibration syringe wrench to free up a "frozen" valve**

Compliance/Resistance
Equipment-related problems (cont.)

4. 100 LPM pneumotach yields "bumpy" curves, difficult to evaluate

The "bumpy" C/R curves when using the 100LPM pneumotach was noted but the cause is not known. It was suggested that if you switched to a 30 LPM pneumotach and the appearance of the C/R curves obtained remained consistent with what was seen using the 100 LPM pneumotach, then the cause of the problem was most likely to be the child. If the appearance changed, then the problem was most likely to be the pneumotach.

5. Incorrect pneumotach size

Selection of the correct pneumotach is based on the expected normal V_E for the child being tested. It was suggested that the 30 LPM pneumotach be used for most Tidal Loop and Compliance/ Resistance studies and the 100 LPM pneumotach be used only for bigger kids (> 10 kg.) with higher pressures. The 30 LPM pneumotach is suitable for all studies on infants < 10 kg. For babies on ventilators (not commonly performed for P2C2 study) the 30 LPM pneumotach is suitable for Compliance/Resistance and Tidal Loops and the 100 LPM pneumotach should be used for Hug studies. If it is desired to change pneumotachs after measurements have been made, it is necessary to exit the testing software and inform the 2600 (via software) what pneumotach size will be used. In general, a flat top appearance to the expiratory curve indicates flow is exceeding the pneumotach rating.

6. Poor mask fit
see Discussion under Tidal Flow-Volume Loops

7. Mask dead space - **refer to table, below**

Description	Sensormedic Part #	Dead Space
Neonate	462534	7 cc
Infant	462533	22 cc
Toddler	462532	35 cc
Child	462531	65 cc

If using a mask not supplied by Sensormedic, use a syringe and sterile saline or water to fill the sealed mask/connector apparatus to obtain an estimate. Slight variations can occur during actual use because of mask distortion under pressure while making an airtight seal.

Compliance/Resistance
Patient-related problems

1. High respiratory rate; difficult or unable to properly adjust flow threshold

This was noted to be a difficult area to standardize a procedure. The inspiratory flow threshold adjustment can be used to start the movement of the shutter earlier and more appropriately time the closing of the occlusion valve. This valve takes 150 milliseconds (ms) to close fully. For measurements made with the patient on the ventilator, two valves must move and the time increases to 325 ms. Adjustment of the inspiratory flow threshold is empirical and no other guidelines were suggested.

It was recommended that the pressure plateau time could be reduced in patients with a high respiratory rate. The default value is 100 ms and reduction is empirical. The general guideline would be to reduce the pressure plateau time by as little as possible necessary to obtain good compliance/resistance curves. It was noted that always using a reduced pressure plateau time increased the likelihood of measuring "noise".

One Center suggested that if all else fails, try removing the mask for a few minutes and retry. They report some success with this, suggesting that perhaps mask dead space exceeds tidal volume and the presence of the mask induces a hypercapnic responce test.

2. Position of head/neck

No recommendations.

3. Upper airway obstruction-"snorers"

No recommendations.

4. Improper sedation

No recommendations. It was noted that babies in the south are more "laid back" and sleep more soundly.

5. Age of patients (if > 9 months is Hering-Breuer reflex present?)

<u>Resolution:</u>

<u>Always</u> use the 2600 software to insure the presence of the Hering-Breuer reflex before making Compliance/Resistance measurements. If % prolongation is less than 100%, do <u>not</u> proceed with compliance/resistance measurements. Doing this will prevent wasting time attempting to make compliance measurements on kids without a H-B reflex present.

Compliance/Resistance
Patient-related problems (cont.)

6. Exhalation not relaxed

These should be discarded. By definition, the static compliance measurement must be made while the child is relaxing against the closed orifice (see #5, above).

The sequence of testing was discussed. An earlier resolution from a conference call involved the sequence of processing and detailed the need to process only hug data in the NIH software. All other studies should be processed using the 2600 software. The actual sequence of testing at each Clinical Center was requested and is reported below:

Baylor	Boston Children	Boston City	Mt. Sinai	Columbia	UCLA
Tidal	Tidal	Tidal	Tidal	Tidal	Tidal
Hug	HB	Hug	Comp/Res	Comp/Res	Comp/Res
HB	Comp/Res	Comp/Res	FRC	FRC	FRC
Comp/Res	Hug	FRC	HB	Hug	Hug
FRC	FRC		Hug		

Prior to 1/93, Boston City was performing tests in the following sequence: Tidal, C/R, FRC followed by Hugs.

It was suggested that the CCC analyze the FRC (and Comp/Res?) data to determine if there is a significant difference between the three centers that perform the hug before FRC and those that perform it after.

Compliance/Resistance
Processing problems - see Processing Resolutions, below

1. Curves without substantial straight-line portions - **discard if not straight within the 65-90% range**

2. Two "good" curves with different slopes - **process only the 65-90% portion of the curve; if this portion contains more than one slope, discard. If both curves show good 65-90% segments, they may have been collected at different lung volumes or may have been influenced by inspiratory or expiratory efforts, inspect the volume and pressure tracings at the top of each screen. Process according to your best judgement.**

3. Curves that appear to be flat - **inspect volume and pressure tracings in upper portion of screen for evidence of "true passivity" - discard if it appears there could be an inspiratory effort just preceeding the opening of the shutter.**

4. Curves that appear too steep - **inspect volume and pressure tracings in upper portion of screen for evidence of "true passivity" - discard if it appears there could be an expiratory effort just preceeding the opening of the shutter.**

5. "Peaks" in negative slope - **This was taken to mean "noise" or flutter. Discard if in the 65-90% segment.**

6. Difficulty in getting full passive exhalation - **discard if not passive. During collection, try reducing the pressure plateau time.**

7. Curve that appears to have two different time constants - **process only the 65-90% segment of the curve. If this section shows more than one time constant - discard it.**

Compliance/Resistance Processing Resolutions:

1. Inspect the pressure-time and volume-time tracings at the top of the screen to verify relaxation. Discard if any evidence that the patient was not relaxed just prior or just after valve opened.

2. Process only the segment that the software chooses as a default. This is set from 65% to 90% of V_E. Do not adjust the processing boundaries. Note: the software may choose values just slightly lower or higher than 65% and 90%. This is acceptable.

3. Inspect the 65-90% segment. Discard if there is evidence of:

   more than one slope

   excessive noise

   inspiration

   active or forced expiration

4. Collect a sufficient number of curves to allow a valid estimation of mean and coefficient of variation after processing. The minimum acceptable number of valid curves for the session is eight. After processing, press the letter "R" to view statistics. The coefficient of variation should not exceed 15%.

Compliance/Resistance

Side Study - The effect of thoracic squeezes on respiratory compliance and resistance measurements

It was noted that one Clinical Center performs the hug on their subjects prior to collecting compliance/resistance. Since it is believed that the thoracic squeeze maneuver may cause transient shifts in end-expiratory lung volumes and that shifts in lung volume can affect compliance and resistance measurements, it was resolved that each Clinical Center would collect compliance/resistance measurements before and after hugs on children that appear to be very well sedated.

The Clinical Coordinating Center will forward data collection forms for this study. It is suggested that each Center submit the data to the Clinical Coordinating Center by mail as the data is collected. When several records have been collected, the CCC will pool the data and perform a power analysis to refine the estimate of appropriate minimum sample size necessary to test the null hypothesis.

Functional Residual Capacity

Equpiment-related problems

1. Nitrogen analyzer

   a. calibration drift (must reboot to recalibrate) - **will forward Sensormedic's response to this complaint. The manual recommends that the vacuum pump must be turned on 20 minutes prior to performing the N2 washout.**

   b. needle valve peaking - **Resolution: peak the nitrogen needle valve before testing each patient - see "Nitrogen Needle Valve Calibration", section 14.5 of the Sensormedic manual (attached).**

   c. spikes in nitrogen tracing - **related to improperly "peaked" N2 needle valve - see "Nitrogen Needle Valve Calibration", section 14.5 of the Sensormedic manual (attached).**

   d. vacuum pump oil viscosity - **Sensormedic 2600 manual recommends the vacuum pump oil be changed monthly regardless of usage. Use only Sensormedic's vacuum pump oil. The oil level should cover about 2/3 of the glass on the oil level gauge when the pump is running. The pump holds approximately 6 oz. of oil.**

   e. leaks around mask/tubing - **see Tidal Flow-Volume Curves, discussion**

   See attached N2 troubleshooting chart.

2. Sticky switch-in valve

   see Compliance/Resistance, equipment-related problems, section 3: sticky valve

3. Low O2 flow, (PTIF exceeds regulator capacity of 20 LPM) - **will forward Sensormedic's response**

4. Drift in O2 flow (when using E cylinders) - **not discussed.**

5. Occasional "Invalid" message - **appeared when the nitrogen analyzer did not fall to zero (but < 0.8%) in the presence of oxygen during the FRC measurement, and is probably appropriate. Mike Weisner will inquire to Sensormedics.**

Functional Residual Capacity
Patient-related problems

1. Arousal (last test in sequence) - **noted to be a problem with no easy solution.**

2. Switch-in error at high respiratory rates - **as above, recognizing that the valve switch-in speed is 150-200 ms, this problem may have no easy solution**

3. "Inadequate interval between repeat tests lowers FRC" - **since oxygen resident in the lung from a previous measurement lowers the amount of nitrogen in the lung at FRC, it will lower the measured FRC.**

   Resolution: The minimum interval between repeat FRC tests (N2 wash-in time) will be three times the "washout" time from the previous test.

4. "Analyzer interprets yawns as FRC volume" - **Yawns during the course of an FRC measurement should appropriately be considered as part of the FRC volume. If the inspiratory flow during the yawn exceeds the regulator capacity of 20 LPM,** ???room air dilution or flow restriction, Sensormedic.

Standardization

1. Definition of "good" or acceptable N2 washout

   a. progressive decline of expired N2

   b. "smooth" washout curve

   c. no spikes in expired N2 tracing

   d. multiple efforts show similar washout time and curve shape

   Note: Sighs may produce an expired N2 reading slightly higher than the previous breath if the larger tidal breath opens a poorly ventilating or previously non-ventilating lung compartment. There should then be a progressive decline with each subsequent breath. If increases in N2 cannot be associated with larger breaths, suspect and look for a leak.

2. Definition of "unacceptable" N2 washouts

   a. "spikes" in N2 curve

   b. non-reproducible FRCs (implies minimum of 3 measurements)

Functional Residual Capacity

3. Methodology

Use the predicted FRC for a low syringe calibration value calibration value and 150% of the predicted value for a high calibration value.

Resolutions:

Peak the nitrogen needle valve before each patient.

Minimum number of FRC tests: two, if within 5%; three if within 10%.

Provide a minimum interval between repeat measurements equivalent to or exceeding three times the washout time.

Hug PEFV

Systematic application of incremental hug pressures

1. Starting pressure - **40 cmH2O**

2. Pressure increment - **20 cmH2O may be OK, but 10 cmH2O may be better in sicker kids.**

3. Number of hugs at each pressure while incrementing - **1 or 2 at each increment.**

3. Determination that "ideal" pressure has been exceeded - **appearance of concavity where none existed at lower pressures. Appearance of noise (flutter) where none existed at lower pressure?**

4. Determination of "ideal" pressure - **compare flow at FRC. When no change in flow at FRC in spite of increase in hug pressure, flow limitation has been demonstrated.**

Equipment-related problems

1. Flow exceeds pneumotach rating - **Sensormedics, pneumotach flow limits. If a pneumotach is changed after several efforts have been collected, you must exit to the point in the software where you can specify the size of pneumotach you are using. Do not process loops collected with different size pneumotachs together. -**

Choice of pneumotachs should be guided by the child's predicted V_E.

The following table shows each pneumotach's volume ranges:

Pneumotach name	Volume Range
10 LPM	0 - 127 ml
30 LPM	0 - 255 ml
100 LPM	0 - 2048 ml

2. Flow/volume lines drift

   a. **Zero drift?**
      temperature
      water
      O-ring failure

Equipment-related problems (cont)

Drifting flow/volume lines

b. Change in apparent end-tidal point

O-ring failure
water
inspiratory / expiratory asymmetry (calls for linearization)

Note: true changes in end-tidal point (end-expiratory lung volume) are self-limiting i.e. a new end-tidal equilibrium will be reached and end-expiratory drift will cease. Back-and-forth end-expiratory changes can occur and may simply indicate end-expiratory instability. Drifts of the end-expiratory point that consistently move in one direction only, without stabilization usually indicate a pneumotach that needs to be linearized.

3. Squeeze late

a. low pressure in reservoir

b. low air supply pressure

c. loose jacket (large jacket volume)

d. O-ring failure in V1 or V2 lines

e. Leak in hugger jacket circuit

jacket or hose failure
balloon valve related leak
tank (mouth) pressure line leak (O-ring)

4. Balloon in hugger valve leaks

a. mandrel (balloon support) failure

b. balloon leaks

c. balloon is "sucked in"

5. T-connector pops off at high pressures - **noted to be an occasional problem at higher pressures. No solution.**

Hug PEFV
Patient-related problems

1. Airway secretions

2. Inadequate sedation

3. Upper airway closure - **was noted to occasionally be related to head and neck positioning.**

4. Head/neck positioning - **As positioning may change slightly during measurement, readjustment may be necessary.**

5. Stage of sleep

6. Tachypnea

Hug PEFV
Processing

Number of curves:

Minimum goal is to obtain at least three acceptable curves, one of which was obtained using a different hug pressure so as to demonstrate true flow limitation. The ideal goal is to obtain eight acceptable curves, one of which was obtained using a different hug pressure.

Choose "best" curve:

Pick the curve that has the largest V_E and the smoothest trailing portion (latter portion of the expiratory curve).

Align other curves for processing of composite:

Line up the flow-limited segment of subsequent breaths without regard for FRC (start of tidal breath preceeding hug), start of expiratory curve, or end of expiratory curve.

Isolate the flow-limited segment:

Adjust the volume scale to approximate a 45 degree slope of the apparent flow-limited segement. Do not consider whether or not the flow-limited segment you are choosing includes FRC. Inspect the more distal (higher numbers on V_E scale) portion of the curve. The flow-limited segment should constitute at least 20% of the curve. If the curve appears two have two or more slopes, process the most distal slope (unless the curve has a convex or "rock of Gibraltar" shape; see processing convex curves, below) such that $RV_{EXTRAPOLATED}$ defined by the slope always exceeds V_E.

Processing convex curves:

If the curve has a convex appearance ("rock of Gibraltar" shape) inspect the proximal (lower numbers on V_E scale) portion of the expiratory curve. The low overlay segment (OS_{low}) should be no further left than 20% of the V_E. {Example - if the V_E is 200ml, the OS_{low} should be placed no further left than 40 ml}. Placement of the OS_{high} should be guided by the appearance of the composite. The sharp dropoff of the typical convex curve probably represents inspiratory effort and should not be included in the flow-limited segment, even if a good overlap is noted.

Hug PEFV

Miscellaneous

It was noted that there could be two separate and distinct methods and purposes for defining the flow-limited segment. It was resolved that the method described above would be used for the purpose of assigning a value for $RV_{extrapolated}$ that would always equal or exceed V_E. A second method would extend the overlay segments to include as much of the curves that overlapped sufficiently to be called a flow-limited segment. Isolation of this segment could be used to tag flow rates as being derived from the flow-limited segment of the curve. It was suggested that flows that were derived from the portion of the curve defined by this "largest best-fit" method be called *Hug Decile Flows or* HDF_{40}, HDF_{50}, *etc.*

Note: The NIH software does not currently allow the isolation of this second, "largest, best-fit" section of the curves. This may be included in future software revisions. For the present, process hugs according to the guidelines given on the previous page.

How should hugs that demonstrate an $RV_{extrapolated}$ that vastly exceeds V_E (i.e. near-horizontal slope) be handled? This was not discussed before the end of the meeting, unknown.

Is a drop in heart rate (around 10%) with increasing hug pressure a cause for concern? Unknown, further information needed.

APPENDIX 17

SECTION 6. MAINTENANCE AND CALIBRATION

MAINTENANCE

Cleaning

The only maintenance routinely required is that the Monitor and accessories are kept clean and are handled and used according to the instructions provided here and in the Service Manual (776-368).

The exterior of the Monitor may be wiped clean with a cloth slightly dampened with mild detergents.

- Do *not* immerse unit.
- Do *not* clean with isopropyl alcohol or other solvents.
- Cuffs and hoses should be cleaned with a cloth slightly dampened with mild detergent.
- Do *not* immerse hoses.
- Do *not* immerse cuffs without prior application of cuff hose caps.

Storage

If it becomes necessary to store the Monitor for an extended period of time, attach the original packing inserts and place the unit into the original shipping carton. Refer to Section 3, Physical Description, Environmental Specifications for storage temperature information.

CALIBRATION

Calibration of the Monitor should be checked at least once a month or when there is doubt about the validity of the pressure readings.

CAUTION

Calibration equipment should always be kept dry and free of particulate matter. Moisture or foreign substances introduced into the pneumatic system can cause damage to the unit.

To perform a calibration check, follow these procedural steps:

1. Obtain the calibration kit supplied with the unit.

CALIBRATION
Continued

2. Connect a mercury manometer with sufficient range or a digital pressure gauge to the Monitor using the parts supplied with the calibration kit as shown in Figure 6-1.

NOTE
In calibration procedure, use an adult cuff and 12-foot hose.

3. Plug the Monitor into the specified line power outlet.

4. Press and hold the SET switch while pressing the POWER ON switch. Flashing 88s in CYCLE MINUTES display confirms that calibration mode has been entered. The unit will turn on, will light the CUFF indicator, and will display cuff pressure in the MAP display.

5. Using the inflation bulb, manually pump up the pressure to 200 mmHg, ±1 mmHg, as indicated by the mercury manometer and close pneumatic release valve on manometer bulb.

6. Verify that the pressure indicated by the mercury manometer does not change more than 12 mmHg in 60 seconds for all cuff and hose combinations.

NOTE
If the leakdown is greater than 12 mmHg in 60 seconds, check cuff and hose junctions and rubber "O" rings for cracks, tears, or breaks. If no cuff or hose leaks are found, refer to qualified service personnel.

7. Verify that the MAP display indicates the correct pressure at the following pressure levels. Refer to Table 6-I, Calibration Check Pressure Levels.

TABLE 6-1. CALIBRATION CHECK PRESSURE LEVELS

MANOMETER INDICATED PRESSURE LEVEL	MAP DISPLAY
200 mmHg, ±1 mmHg	200 mmHg, ±5 mmHg
150 mmHg, ±1 mmHg	150 mmHg, ±4 mmHg
100 mmHg, ±1 mmHg	100 mmHg, ±4 mmHg
50 mmHg, ±1 mmHg	50 mmHg, ±4 mmHg
0 mmHg	0 mmHg, +1 mmHg −0 mmHg

CALIBRATION
Continued

8. If the indicated pressures are not within tolerance, the Monitor must be calibrated. Refer to qualified service personnel.

9. *Slowly* pump up the manometer at a rate of approximately 10 mmHg per second (avoiding pressure spikes) using the manometer bulb and, as pressure increases between 305 mmHg and 320 mmHg, verify that the Monitor briefly blanks the displays, opens the deflate valves, and then issues an 800 alarm.

10. If the overpressure point is not within tolerance (310 mmHg +10/-5 mmHg), the overpressure switch must be adjusted. Refer to qualified service personnel.

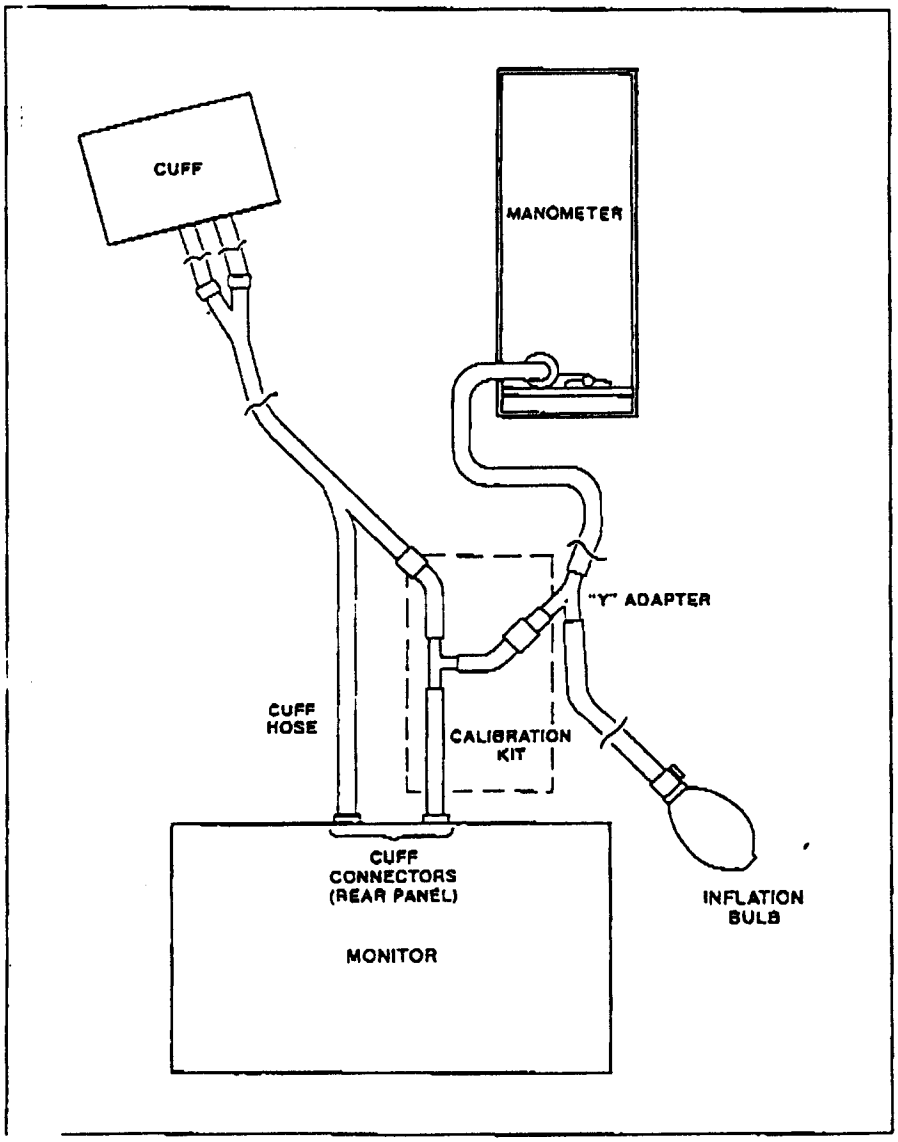

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Figure 6-1. Calibration Check Setup