MASON RESEARCH INSTITUTE

THE APPLICATION OF THE FLUORESCENT-ANTIBODY TECHNIQUE TO BLOOD GROUP DIFFERENTIATION OF DRIED BLOODSTAINS

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THE APPLICATION OF THE FLUORESCENT-ANTIBODY TECHNIQUE TO BLOOD GROUP DIFFERENTIATION OF DRIED BLOODSTAINS

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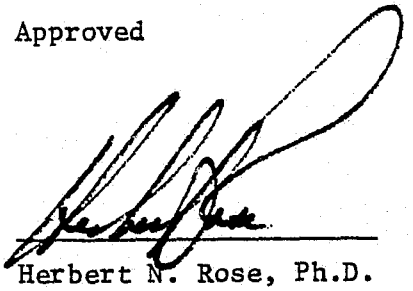
Approved



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Project Director

2. ABSTRACT

The fluorescent antibody technique was studied to determine applicability to blood group differentiation of dried two-week old bloodstains. Initial studies with an Aminco-Bowman Spectrophotofluorometer indicated possible feasibility; that differences could be detected between group A and B antigens reacted with homologous antibodies. Further experimentation was directed towards developing methodology, reagents and instrumentation for a "Low Angle, Direct Fluorescence Tagging Method". This method incorporated the analysis of bloodstained fabrics and dried blood particulates. A prototype instrument was developed, and its problem areas defined.

Methodology examined included: direct fluorescent staining, by tagging human plasma and fractions that contained high titers of anti-blood group antibodies with fluorescein isothiocyanate, indirect fluorescent staining, utilizing conjugated-goat anti-human IgG and/or IgM, subsequent to reaction with unlabeled plasma with high titers of blood group antibodies. Antibody purification was attempted by heat-elution of tagged γ-globulins from intact erythrocytes. The eluate was not adequate as a blood typing reagent with the immunofluorescent method. The major immunologic problem was non-specific binding of fluorescent-protein.

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5. ACKNOWLEDGEMENTS

Herewith, we wish to acknowledge that -

. The concept of the "Low Angle, Solid Phase, Immunofluorescent Approach to the identification of blood group antigens in bloodstains, was the creative result of discussions between Arthur E. Bogden, Ph.D., and Henry J. Esber, Ph.D. of the Mason Research Institute and Frederic Siegelman, Ph.D. of the ORTEC Company.

. The immunoserological studies and equipment modifications leading to the Feasibility Study were designed and directed by Dr. Bogden and Dr. Esber with the very capable assistance of James H. Gray, M.S. and Miasnig Hagopian, Ph.D., also of the Mason Research Institute.

. The design and construction of the LABS-1, a "breadboard" fluorometer with the configuration unique to the requirements of "Low Angle, Solid Phase, Immunofluorescence Analysis", in time to be used in the Feasibility Study, is credited to Dr. Siegelman and the ORTEC Company.

Grateful acknowledgement and thanks to the several official and unofficial consultants to this project whose knowledge was tapped and incorporated into the design of these studies.

6. SUMMARY

Introduction

One of the most common physical clues found at the scene of a violent crime are human blood stains. Such evidence is available in approximately 40% of the major criminal cases, such as murder, rape and assault, yet the application and usage of such immunoserological evidence by U.S. criminalists except for ABO typing has been minimal. This is primarily due to the lack of simplified, rapid and inexpensive analytical procedures. Even major criminalistic laboratories are limited in providing such services, due to case loads involving drug and alcohol analysis, lack of funds, space, personnel, time, etc.

In order to raise the quality and availability of these foresnic services, the Law Enforcement Assistance Administration has sponsored The Aerospace Corporation's Bloodstain Analysis System program. The specific objectives of this program are the development of forensic analysis and instrumentation leading to increased blood characterization and establishment of a blood data bank to increase knowledge of the frequency of the genetic variants throughout the U.S. population. The ultimate goal of this program is the increased utilization of bloodstain analysis results as evidence in the nations' courts of law.

Background

The physical characteristics of every person are determined by their genetic make-up. This includes a number of constituents

found in human blood. Blood is composed of a liquid portion and a solid portion. The liquid portion, plasma, contains a great number of proteins and enzymes. The solid segment contains red blood cells, white blood cells and platelets, and are composed of and contain numerous proteins. A large number of the ingredients found in blood involve inherited variants, and each person possesses certain variants, depending on their genetic make-up. The identification and classification of a sufficient number of these variants makes possible the individualization of a blood sample, similar to a fingerprint.

The application of the less commonly used genetic systems, by U.S. criminalists has been very limited. Only three genetic marker systems, (ABO, MN, and Rh) all residing on the red blood cell, are extensively utilized by U.S. crime laboratories. The existing forensic methodologies were developed by adaptation of clinical serological procedures.

The absorption-inhibition method was used initially in forensic laboratories. Samples of bloodstained fabrics were added to a test solution containing a known quantity of an antibody. After removal of the stain, the antibody remaining was determined by the addition of the appropirate indicator cells. Comparison of the titers before and after absorption with the bloodstain hopefully gave the scientist sufficient information to determine the antigenic make-up of the stain in question. This method, because of inherent difficulties with determining antibody titer endpoints, non-specific absorption, antigen availability, and operator subjectivity was eventually replaced by the absorption-elution method.

In testing bloodstains by this method, the stained fabric was treated with the appropriate antibody solution. This time, the residual antibody solution was discarded, the stain washed, and the absorbed antibody removed from the stained fabric by heat. The presence of eluted antibody was determined by the addition of the appropriate indicator cells. A positive reaction was indicated by the agglutination of the indicator cells. The absorption-elution method remains the current method for typing bloodstains, however, the results are often subjective and the interpretation requires considerable experience.

The described absorption-elution method is an immunological procedure, involving antigens on the surface of the red cells (from the bloodstain) and antibodies either naturally occurring or raised by immunization. The purpose of indicator cells is to visualize the antibody-antigen reaction. Other means of observing antigen-antibody are available, notably radioactive and fluorescent labeling. These labeling techniques involve attaching a tag, a small molecule, to the antibody which provides a convenient and sensitive detection method. Mason Research Institute preferred not to use the radioactive labels because the Statement of Work prohibited the use of hazardous materials or reagents. The most frequently used, currently available, fluorescent compounds are fluorescein isothiocyanate and rhodamine.

Bloodstain Analysis System Goals

This contract called for the development of a Bloodstain Analysis System. It was proposed to develop an immunofluorescent detection method, instrumentation based on the detection method, and immunological techniques to utilize the fluorescent procedures and instrumentation

developed would be suitable for utilization by criminalistic laboratories at the county government level. It was the goal of the Bloodstain Analysis System to devise the means for overcoming the difficulties associated with available methodology and reagents. At a minimum, the program was to improve current immunological methodology and equipment for the qualitative analysis of blood constituents, for those antigens where methods of detection were currently available, and to bring about a significant improvement in blood individualization. The development of new methods of analysis, to include blood groupings not currently being analyzed, such as the Kell, Kidd and Duffy antigen systems, and new equipment designed to increase the speed, reduce the cost, and remove subjectivity of interpreting the results, were the objectives.

This report concerns itself with one aspect of the Bloodstain Analysis System, the qualitative analysis of the A, B, and Rh erythrocyte antigens on dried bloodstains. This goal-orientated research effort has been guided by the cost of reagents and equipment limitations specified as goals for the Bloodstain Analysis System by The Aerospace Corporation. Within the limitations specified by the Statement of Work and funding constraints, Mason Research Institute's approach to the problem was designed to:

1. Increase the sensitivity of the currently used forensic bloodstain analysis techniques.

2. Increase discrimination capabilities by permitting multiple tests on the same bloodstain sample.

3. Decrease test time.

4. Simplify testing procedures.

5. Minimize operator interaction by simplifying and automating those areas involving subjective influences on decision making.

6. Elimination of the need for fresh erythrocytes as indicator systems.

In order to develop a Bloodstain Analysis System, the expertise of two subsidiaries of the Applied Technology Group of EG&G, Incorporated were utilized: The Mason Research Institute, a life-science research and development organization with long and varied experience in immunobiology and immunogenetics and ORTEC, Incorporated, engaged in the design, development, manufacture and marketing of sophisticated electronic and electrophoretic equipment.

Fluorescent Immunological Methodologies

Initially, the Mason Research Institute proposed a novel methodology for the detection of erythrocyte antigens, the "Low Angle, Solid Phase, Direct Fluorescence Tagging Method". This method was based upon the insolubility of stromal antigens in dried bloodstains. The need for fresh red blood cells as an indicator of an antigen-antibody reaction was eliminated by using antibodies labeled with a fluorescent dye. Following the reaction the reacted components were to be thoroughly washed and examined by low-angle ultraviolet excitation and the degree of surface fluorescence quantitated with a fluorometer designed by ORTEC. The sensitivity of such measurements were to be greatly increased by employing digital photon analysis. Read-out of the data was digital, with the differentiation of positive and negative

reactions to be completely objective.

The Aerospace Corporation requested a four-month feasibility study, to be followed by a Feasibility Demonstration of the Low Angle, Solid Phase, Direct Fluorescence Tagging Method. Aerospace also requested that the Mason Research Institute suggest two alternative procedures if the primary method proved infeasible.

Studies were initiated in December, 1975 on all three approaches (a) the primary method, the Low Angle, Solid Phase, Direct Fluorescence Tagging Method; and the alternate methods, (b) designated as the "Direct Fluorescence Elution Method" and (c) the "Solid Phase Indirect Immunoassay Method".

Detection and differentiation of two erythrocyte antigen systems was to be developed, blood groups A and B of ABO system and groups C, D and e of the Rh system. The intial work-effort was concentrated on defining the various parameters, non-specific fluorescence in cloth, differentiation of positive and negative reactions, reagent concentrations and ratios, reaction times and temperatures, excitation and emission wavelengths, and sample presentation angles. Fluorescence was measured in an Aminco-Bowman Spectrophotofluorometer that was modified to measure fluorescence from a solid surface. Three sample holders were designed for this purpose, allowing pieces of bloodstained cloth or single threads to be inserted in the instrument at any desired angle. Using an indirect fluorescent staining technique, it was possible to define a set of reaction conditions that would differentiate type A and B bloodstains, but the conditions had not been optimized for differentiation of types AB and O.

Work was begun on all three proposed methods simultaneously and experiments performed to determine the effect of fluorescein isothiocyanate conjugation on antibody agglutination activity, the effect of temperature on antibody binding capacity, and the effect of detergents on antibody binding capacity. Difficulty with reproducibility was encountered when fresh bloodstains were tested with these methods. Investigation of the problem revealed that the wash procedures removed not only hemoglobin and serum proteins, but variable amounts of erythrocyte stroma. The effects of biological fixatives were assessed, and fixation techniques were devised that retained erythrocyte antigens. A great deal of effort was concentrated on studies dealing with solid surface fluorescence scanning.

After two months into the feasibility study, an assessment was made of the rate of progress achieved by the three methods. A decision was made to discontinue the research efforts on the two alternative methods and concentrate on the primary approach. In the remaining time, an all-out effort was made:

1. To identify and eliminate variables pertinent to bloodstain sample preparation.

2. To design and construct an instrument with a configuration suited to the Low Angle, Solid Phase, Direct Fluorescence Tagging Method.

3. To design improved bloodstain sample holders for the Aminco-Bowman Spectrophotofluorometer.

Effort was concentrated on preparation of pieces of fabric, single fibers, and removal of stain particulates from a surface and impinging on a filter surface. The reaction and washing times for

sample preparation were standardized, as excessive washes were found to be detrimental. Although the conditions for serological reactions had not been optimized, the results with the primary method indicated that the degree of fluorescence was proportional to the sample angle in the Aminco-Bowman. In most instances, the Rh and A and B blood groups were distinguishable (see Table 6.1). The degree of wetness of the samples was found to effect the observed fluorescence and apparently was a factor in the observed inconsistencies.

Dr. F. Siegleman of ORTEC, Incorporated, must be commended for his efforts. He hurridly constructed a prototype instrument out of readily available and some specially fabricated parts. The instrument, named LABS-1 was based on the methodology requirements and was intended only for a short term of usage before being replaced by a more appropriate configuration. LABS-1 was equipped with a 150 watt slide projector halogen lamp for sample excitation, at a fixed angle of 28 degrees. The LABS-1 fluorometry reduced a number of instrumentation variables in surface fluorometry analysis that were inherent in the configuration of the currently available Aminco-Bowman unit. The ORTEC instrument had permitted a clearer definition of the problems related to the variability encountered in the "Low Angle, Solid Phase, Fluorescence Tagging Method". As a result we reached the stage in methodology and equipment development that permitted a direct examination of the influence of reagent specificity and purity on the sensitivity and reproducibility of the primary method. Unfortunately, the LABS-1 was only available for testing one week prior to the Feasibility Demonstration.

For the Feasibility Demonstration Mason Research Institute was presented with ten, two-week old bloodstains that included blood groups A, B, AB, O, C, D and e. Samples were numbered and the code was revealed on 2 April 1975, on the second day of the Feasibility Demonstration, after Mason Research Institute's results had been tabulated and discussed. The results of the Feasibility Demonstration are summarized in Table 6.1, the actual blood types, determined by The Aerospace Corporation and verified by its consultants, and Mason Research Institute's test results are compared.

The assessment of the test results are:

The Aerospace Corporation - Correct identification for A and B antigens was made in 70% of the specimens, 10% were incorrect and 20% were questionable (Blood groups O and AB are included in these percentages). For the Rh system, the values were 57%, 30% and 13% for correct, incorrect and questionable identifications, respectively.

The Mason Research Institute - Correct identification of A and B bloodstains was made in 100% of the specimens, AB was a questionable positive and of blood group O two were questionable and 3 out of 5 were incorrect. For the Rh system 70% of group C, 60% of group D and 80% of group E were correctly identified. Mason Research Institute considers a ± (trace) reaction as a positive test that ideally should have confirmation testing. It is a standard procedure both in clinical and forensic serology to consider ± (trace) reactions as weak positives and to retest for confirmation. Due to the length of time alloted for the Feasibility Demonstration, confirmation testing was not possible.

TABLE 6.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
A	11-3	anti-A	13,468	A
		anti-B	9,877	
		anti-AB	12,936	
		Control	8,708	
A	11-6	anti-A	24,341	A
		anti-B	16,609	
		anti-AB	22,492	
		Control	12,664	
B	11-5	anti-A	8,666	B
		anti-B	11,033	
		anti-AB	10,036	
		Control	7,343	
B	11-10	anti-A	11,994	B
		anti-B	15,159	
		anti-AB	14,617	
		Control	10,798	
AB	11-4	anti-A	12,235	AB
		anti-B	13,503	
		anti-AB	15,067	
		Control	11,144	

* Coded bloodstains provided by The Aerospace Corporation.

TABLE 6.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
O	11-1	anti-A	12,788	not A
		anti-B	12,466	not B
		anti-AB	14,380	(AB)✓
		Control	12,064	
O	11-1	anti-A	9,085	A ✓
		anti-B	8,020	
		anti-AB	8,175	
		Control	7,020	
O	11-7	anti-A	31,141	not A
		anti-B	27,196	not B
		anti-AB	32,744	
		Control	30,643	
O	11-8	anti-A	19,832	A ✓
		anti-B	16,702	
		anti-AB	19,484	
		Control	16,372	
O	11-9	anti-A	11,940	not A
		anti-B	11,684	not B
		anti-AB	12,357	(O)
		Control	11,197	

* Coded bloodstains provided by The Aerospace Corporation.

( ) Parentheses indicate possible blood type.

✓ Indicates an error.

TABLE 6.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
C^+	11-2	anti-C	4,603	C^+
		Control	4,233	
C^+	11-4	anti-C	5,569	C^+
		Control	5,255	
C^+	11-5	anti-C	5,147	C^+
		Control	4,686	
C^+	11-6	anti-C	13,673	C^- ✓
		Control	13,808	
C^+	11-7	anti-C	23,654	C^+
		Control	22,294	
C^+	11-9	anti-C	16,982	C^+
		Control	14,343	
C^+	11-10	anti-C	11,473	C^+
		Control	11,008	
C^-	11-1	anti-C	9,828	C^-
		Control	10,481	
C^-	11-3	anti-C	4,930	C^+ ✓
		Control	4,600	
C^-	11-8	anti-C	19,622	C^+ ✓
		Control	17,758	

* Coded bloodstains provided by The Aerospace Corporation.

✓ Indicates an error.

TABLE 6.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
D^+	11-2	anti-D Control	5,041 4,773	D^+
D^+	11-3	anti-D Control	4,872 4,844	±
D^+	11-4	anti-D Control	5,474 3,743	D^+
D^+	11-5	anti-D Control	3,964 4,702	D^- ✓
D^+	11-6	anti-D Control	11,716 12,904	D^- ✓
D^+	11-7	anti-D Control	21,848 21,158	D^+
D^+	11-9	anti-D Control	13,311 13,150	±
D^+	11-10	anti-D Control	15,417 15,139	±
D^-	11-1	anti-D Control	10,627 10,355	D^+ ✓
D^-	11-8	anti-D Control	17,222 16,387	D^+ ✓

* Coded bloodstains provided by The Aerospace Corporation.

± Indicates a trace reaction, test should be repeated.

✓ Indicates an error.

TABLE 6.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
e^+	11-1	anti-e	8,268	e^+
		Control	7,306	
e^+	11-2	anti-e	4,984	e^- ✓
		Control	5,000	
e^+	11-4	anti-e	5,206	e^+
		Control	4,475	
e^+	11-5	anti-e	5,559	e^+
		Control	5,323	
e^+	11-6	anti-e	15,324	e^+
		Control	12,611	
e^+	11-7	anti-e	19,931	e^- ✓
		Control	22,974	
e^+	11-8	anti-e	18,122	e^+
		Control	13,544	
e^+	11-9	anti-e	13,039	±
		Control	12,941	
e^+	11-10	anti-e	21,967	e^+
		Control	13,822	
e^-	11-3	anti-e	5,486	e^-
		Control	5,637	

* Coded bloodstains provided by The Aerospace Corporation.

± Indicates a trace reaction, test should be repeated.

✓ Indicates an error.

TABLE 6.1

INTERPRETATION OF FLUOROMETER COUNTS

(Continued)

Blood Group	Rationale
A	Positive when anti-A count is greater than anti-B, and control count is equal to or less than anti-B.
B	Positive when anti-B count is greater than anti-A, and control count is equal to or less than anti-A.
AB	Positive when anti-AB count is greater than either anti-A or anti-B, and control count is less than either anti-A or anti-B.
C	Positive when control count is less than anti-C.
D	Positive when control count is less than anti-D.
e	Positive when control count is less than anti-e.

The Aerospace Corporation judged that feasibility had not been demonstrated, but agreed to an additional month of study in order to improve the analysis techniques. At that time, a second feasibility test would take place. During the two weeks that followed, it had been made clear to us that for a "Feasibility Demonstration" the timing and methods used need not be optimized nor have any direct relationship to those eventually to be developed. However, we pointed out that regardless of whether we address ourselves to demonstrating feasibility of the "Low Angle, Solid Phase, Direct Tagging Method" or oriented to the final, practical method, that to meet the requirements of 95% to 98% accuracy as required for the Feasibility Demonstration, reagents must be tested and developed, and reagent concentrations and reaction times must be optimized to a very high degree. In the remaining two weeks, it was not possible to obtain sufficient quantities of commercial raw antiserum for blood groups A, B, C, D and e, carry out the necessary purification steps, and test the reagents with phenotypically defined erythrocytes. A meeting with The Aerospace Corporation resulted in Aerospace's conclusion that the probability of success by this approach was not indicated at that time. However, the evidence as interpreted by The Aerospace Corporation, did indicate that "an Indirect Fluorescence Staining Method coupled with the LABS-1 Detection System would not work, but a potential for feasibility using the Direct Method was not ruled out". This technique differed from the primary method in that the human typing reagent was labeled directly with a fluorescent compound. The primary demonstrated method employed a fluoresceinized

goat antibody directed against human gamma-globulins, an indirect fluorescence staining technique.

In July 1975, a new Study Plan for the continuation of the feasibility verification effort was approved by The Aerospace Corporation. This Study Plan detailed specifically the guidelines and limitations for continued studies on the LABS-1 fluorometer and for the preparation of the fluoresceinized reagents required for typing blood groups A, B, AB and O. The guidelines and limitations stipulated in the Study Plan were to be strictly adhered to, and any modifications to procedures were to be first approved by The Aerospace Corporation.

Instrumentation Modification

The data accumulated prior to the Feasibility Demonstration suggested that the optical filter used for the emission detector transmitted some of the excitation light, resulting in spurious signals. This, and other problems would be examined and corrected by:

1. Obtaining a diffuse white reflector reference sample.
2. Generating a stable fluorescent reference sample.
3. Obtaining optical filters with more optimum characteristics in regard to peak and bandwidth.
4. Evaluating broad bandpass and cut-off filters.
5. Utilizing a constant voltage transformer.
6. Performing a proposed signal normalization.
7. Determining the consequences of variation in excitation intensity.

The instrument was inspected, light leaks repaired, and rewired to increase the effectiveness of the constant voltage transformer. New excitation and emission filters were selected based on data collected with a white reflectance reference standard and a fluorescein standard generated by incorporating fluorescein isothiocyanate into the reflectance standard. The object was to find a filter combination that would give the greatest fluorescence intensity and the largest ratio of fluorescence standard reading over the white reflectance standard reading.

Additional data was collected on instrument operation and a second modification was planned. It was found necessary to reevaluate the optical filters, using actual bloodstains and bloodstains saturated with fluorescein-conjugated antibodies. Physical alterations were made to the instrument in order to reduce heating of the sample chamber and photo-multiplier housing and to mask filters in order to eliminate light leakage. Excitation and emission filters were selected on the basis of a compromise between the maximum signal increase noted with fluoresceinized bloodstains and the minimum signal ratio achieved with the white reflectance standard and the dark count.

Reagent Purification

The direct staining technique decided upon by The Aerospace Corporation required a fluoresceinized antibody reagent for each antigen to be determined. In the ABO system, two different fluorescein-conjugates had to be developed, an anti-A conjugate and an anti-B conjugate.

The plan of work called for:

1. Development of procedures that enabled fluorescein isothiocyanate to be conjugated to hyperimmune human plasma proteins so that minimal specific antibody activity was lost.

2. Purification of the labeled-antibodies by absorption onto intact red blood cells and elution by heating.

Immunochemical examination of the heat-eluted proteins yielded unexpected results. Gamma-globins were detected, as well as large quantities of serum albumin. The albumin was fluoresceinized, rendering the reagent useless. To circumvent the problem of the contamination of the antibody reagent with tagged albumin, permission was granted by The Aerospace Corporation to alter the purification methodology. It was mutually agreed that albumin was to be removed from the bleed prior to fluorescein conjugation, the fluorescein/gamma-globulin ratio was to be increased to assure that all antibody was tagged, and the scheduled Feasibility Verification Test date was postponed one week to 30-31 October, 1975. At the request of The Aerospace Croporation, this date was subsequently changed to 6-7 November, 1975. The removal of albumin from plasma was accomplished by the repeated salting-out of the gamma-globulins with sodium sulfate. It was also possible to substantially increase the fluorescein/protein ratio without significantly altering the biological activity of the antibody.

Attempts to distinguish blood types A, B, AB and O on the modified LABS-1 with the albumin free tagged-gamma-globulin preparations were unsuccessful. It was difficult to determine whether the problem was

immunochemical, serological or instrumental. Absorption-elution experiments were performed on dried, aged bloodstains using the same tagged antibody preparations. When the proper indicator red blood cells were added, it was possible to correctly type A, B, AB and O bloodstains. In view of the serological reactions obtained with the tagged gamma-globulin preparations, it would appear that the problem was with the instrument.

The approved Study Plan outlined a Criteria for Success study to be demonstrated before proceeding to the feasibility verification test. This Criteria for Success study was to determine sample reproducibility, the increase in value for positive over negative tests, and the contribution to fluorescence readings due to non-specific absorption of the anti-serum. Specifically, in order to successfully complete this study, the following had to demonstrated:

1. After subtraction of the dark current and normalization using the reference reading, the scatter in the fluorescence value shall be small for all samples treated similarly from the same specimen at the identical stage of analysis.

2. After correction, the increase in fluorescence reading for positive test shall be large relative to the possible increase for a negative test.

3. After correction, the difference in readings between positive and negative test shall be significantly greater (a minimum of 10 X and a goal of 20 X) than the scatter obtained in each individual reading.

The "Criteria for Success" study was performed prior to the

scheduled Feasibility Verification Test, with types A, B and O stains and a fluoresceinized anti-A gamma-globulin antibody preparation. The fluorescence values from the LABS-1 were normalized according to the protocol. In order to demonstrate success, it was necessary to have a valid increase for the positive control over the negative tests. Comparison of these normalized values by the Student's t-test indicated no significant increase in fluorescence for the positive control when compared with either negative control.

Changes in procedure which were instituted resulted in greatly increased processing time of the blood reagents. The time remaining prior to the Feasibility Verification Demonstration was insufficient to allow preparation of the reagents and the optimization of reaction conditions. Consequently, the Feasibility Verification Demonstration scheduled for 6-7 November, 1975 was cancelled.

At the onset of this study it was recognized that this research was of a high risk nature but with the potential of a large payoff if successful. The study plan adopted called for simultaneous research on the primary method of fluorescent tagging and on two alternative methods. Research was to be conducted on all three of the techniques; if any of the methods proved to be infeasible they would be dropped. However, exploratory efforts directed to the simultaneous determination of feasibility of all three approaches within a four month period were discontinued in order to concentrate on development of the primary approach. Although the results supported the methodology proposed for the primary approach, they also showed that the state of the art was not sufficiently advanced to permit development,

within the allocated time frame, of the accuracy necessary or desirable for application of the method to the forensic laboratory. It is felt that if sufficient resources and man-power were available to conduct the required basic research, one of these approaches could be developed into a technique of value to the forensic serologist.

7. REPORT

7.1 OBJECTIVES

The functional capacity of law enforcement agencies is greatly enhanced by the ability to positively relate bloodstains obtained at the scene of a crime to either a suspect, or a victim. At this time, only a limited capability toward this objective exists in the United States. British forensic scientists have expanded their bloodstain analysis capability considerably by the adaptation of procedures used in genetic research for detecting polymorphic variants of blood proteins and enzymes to bloodstains. By working in terms of genetically controlled polymorphisms that occur in the blood, and by calculating from population data the frequency of occurrence of the particular combination of groups obtained, one can attempt to individualize blood in the same way one can fingerprint.

The National Institute of Law Enforcement and Criminal Justice of the Law Enforcement Assistance Administration of the U.S. Department of Justice has undertaken a program having as its mandate ". . . to encourage research and development to improve and strengthen law enforcement". In furtherance of this, the Equipment System Improvement Program has been established within the National Institute of Law Enforcement and Criminal Justice to contribute to the solution of law and criminal justice problems by developing, demonstrating and evaluating new or improved equipment systems and procedures. The Aerospace Corporation is under

contract to manage the development, demonstration, and evaluation of the new equipment systems. In support of this goal, the Bloodstain Analysis System, initiated by the Law Enforcement Development Group of The Aerospace Corporation has, as its general objectives: a) the improvement of current immunological and electrophoretic methodology for qualitative analysis of blood constituents, b) the pursuance of other approaches having potential for increased bloodstain individualization.

The program calls for techniques which will be effective for dried bloodstain individualization and be acceptable for use by criminalistic laboratories, and as evidence in courts of law.

7.2 BACKGROUND

The classic method for the detection of red cell antigens is the simple agglutination test. The red cells in bloodstains are obviously incapable of agglutination, so other immunological procedures have been developed for the detection of antigens in stains. Two methodologies have been employed: a) the absorption-inhibition method and b) absorption-elution.

7.2.1 Absorption-Inhibition Method

The inhibition method is based on the ability of the antigens in the stain to specifically absorb antibody as measured by titration of the antiserum before and after the addition of stain material. This technique was described by Wiener for the ABO and MN antigen systems (1). The Rh antigens (c, C, D, e) were also successfully detected by this method (2). Other red cell antigen systems (Gm, Kell) have been shown to be active in stains by the inhibition method (3-6). The technique is sensitive and can be carried out on small quantities of material, often only one or two bloodstained fibers. However, there are limitations. The method requires experienced personnel to be reliable. Also sufficient viable antigen must remain in the stain to be capable of absorbing more than half of the antibody content of the antiserum before significant results are obtained. Determining the proper proportions of reagents necessary to yield significant data often could not be done with the amount of stained material available. Detergent residues were often associated with bloodstains on cloth.

Large amounts of detergents resulted in hemolysis of the indicator red blood cells (7). Based on available information, the absorption-inhibition methodologies required a total of 1-50 mg of dried blood to yield reliable results (9, 10).

7.2.2 Absorption-Elution Method

In the absorption-elution technique, specific absorption of an antibody takes place by immersion of the antigen in the antiserum. Afterwards, the antiserum was discarded and the test material was washed until all uncombined antibody was removed. Known typed red blood cells were added, and heated together with the absorbed antigen in a moist chamber. The specific antibody that was combined with the antigen was dissociated by the heat, and reacted with the indicator red blood cells producing agglutination.

The earliest attempt to apply this principal was made in 1923 (11). The technique was not used again until 1960, when Kind published the method for ABO grouping of dried blood smears, without the knowledge of the work done 37 years ago (12, 13). Nickolls and Pereira adapted the method and extended it for the M and N antigens (14, 15). Similar techniques quickly followed for the detection of the Rh antigens (16-18). This technique did not suffer from many of the drawbacks of the absorption-inhibition method. The sensitivity has been demonstrated to be 10 µg of dried stain for A and B substances (9).

The absorption-elution method has now found general usage

and acceptance. Numerous modifications of Kind's first method have been devised. Cutteridge developed the first technique for the routine forensic laboratory (19). Microtechniques were refined by Nickolls and Pereira (20), and Cutteridge (21). A complete battery of assays based on the absorption-elution method are now available, allowing the routine testing of the antigens A, B, H, M, N, S, C, C^W, D, E, e and c in the forensic serological laboratory (22).

7.2.3 Fluorescent Antibody Technique

Agglutination and precipitation are classical examples of gross effects produced by antigen-antibody interactions. Other methods are available that allow an observer to "see" antigen-antibody reactions that do not produce gross effects in vitro. One such device is to label one of the reactants, usually the antibody, and use it as a tracer. There are two types of labels in common use, one involving radioisotopes, the other fluorochromes, each having its place in immunological studies.

Fluorochromes are dyes that absorb light, of a particular wavelength, become excited, and emit light of a different wavelength. This emission phenomenon ceases almost immediately after withdrawal of the excitation radiation. These fluorochrome dyes have been converted to derivatives that can be coupled to proteins. In this way, antibodies can be covalently labeled with a tracer substance that is readily detected. With proper technique,

the labeled proteins retain their specific immunological reactivity so that an antigen-antibody reaction can be visualized.

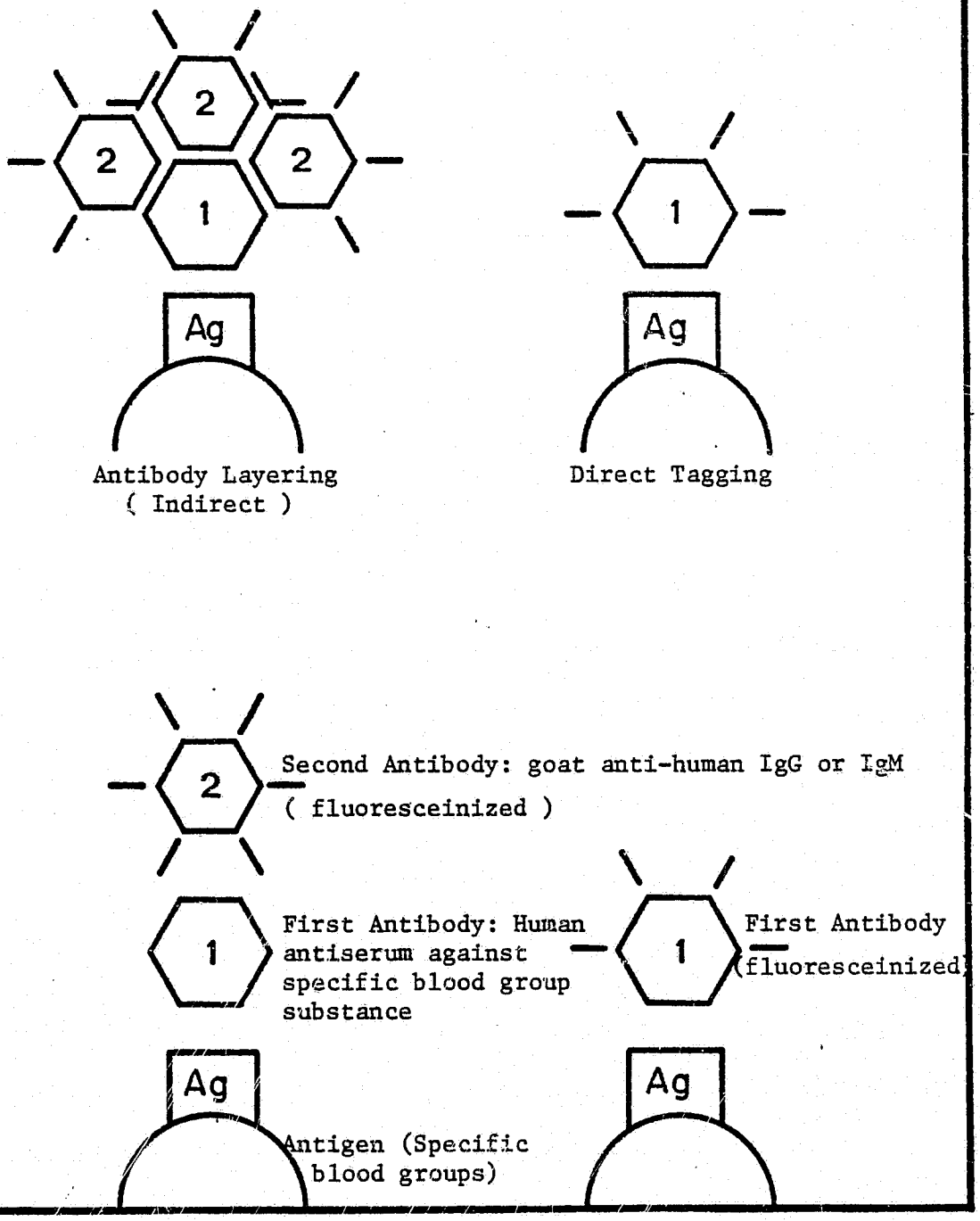
This technique is due to the work of Dr. A.H. Coons, who published it in 1941 (23). This type of procedure may be used either with conjugated antibody of known specificity in order to detect antigen, or with antigen in order to test an unknown solution for the presence of corresponding antibody.

Two basic immunofluorescent methods have developed, the direct staining and the indirect staining procedures. In the direct staining method, the antibody against the antigen to be detected is labeled with fluorochrome. The labeled antibody is allowed to react with the antigen and the excess fluorescent reagent is removed. Remaining fluorescence is the result of specific antigen-antibody reactivities. Most commonly used is the indirect method, in which preparations are treated with an appropriate antiserum, thoroughly washed, and then tested for any antibody remaining specifically combined with antigen by using a conjugated antiglobulin preparation of the proper specificity. A comparison between the direct and indirect immunofluorescent methods is illustrated in Figure 7.2.1

A practical difficulty which accompanies these immunofluorescent methods is the occurrence of "non-specific" fluorescence, e.g. fluorescence that is not directly attributable to the specific antigen-antibody reaction under study. Autofluorescence of the material under examination, due to non-immunological

FIGURE 7.2.1

FLUORESCENCE TAGGING OF ANTIGENS



adherence of the fluorochrome-labeled protein to the specimen, or the presence of irrelevant antibodies in the conjugate that specifically react are some of the sources of unwanted fluorescence.

There are various reports on blood grouping by immunofluorescent techniques (24, 27). Hasebe described blood grouping of dried stains on glass slides and other smooth surfaces such as metal and stone. Crusts of dried blood were powdered and stained specifically with fluorescent antibody in a three-step indirect method (28). The crust was reacted with human anti-A or anti-B serum. The resulting specific complex was reacted with rabbit anti-human globulin, then with fluorescent-labeled sheep anti-rabbit globulin. However, Hasebe was unable to type the blood group antigens from stains on cloth, paper or wood, due to non-specific fluorescent interference. Attempts to use immunofluorescent techniques for the detection of blood-group antigens continued to meet with only partial success. Kind and Cleevely, after an intensive three-year study developed procedures that could type blood group antigen under certain conditions (29). In their hands, the fluorescent antibody technique was successful only when it was not necessary to extract the antigens from the stains. Kind was utilizing a fluorescence microscope for detection. Once the organized structure of the red blood cells are destroyed by extraction, he lost the non-fluorescing background used for visual comparison, making it very difficult to detect any low level of fluorescence.

7.3 SPECIFIC APPROACHES

The Statement of Work prepared by The Aerospace Corporation, dated 5 February 1974, defined the basic goals and requirements for a blood and bloodstain analysis procedure and associated equipment which are summarized in Section 8.10.

The general objective of the program to which the Mason Research Institute responded were defined by The Aerospace Corporation, as follows (Statement of Work, Annex B, par. 2):

> At a minimum to improve current immunological and electrophoretic methodology (the "Culliford" method) and equipment for qualitative analysis of blood constituents.
>
> As a desired goal, to bring about a significant improvement by pursuing other approaches having potential for increased blood individualization, such as new methods of analysis, blood grouping not currently being analyzed, and new equipments to markedly increase speed and reduce complexity of the analysis procedure.

This report, however, concerns itself only with that aspect of the Bloodstain Analysis Program that Mason Research Institute (MRI) was permitted to address itself, i.e., the qualitative analysis of the erythrocyte antigens on dried bloodstains. This goal oriented, research effort by MRI has been guided by cost of reagents and equipment limitations specified as goals for a bloodstain analysis system by The Aerospace Corporation, and by the consideration that whatever techniques were developed would be amenable to the interests and capabilities of the general criminalistic laboratory at the county government or lower government level.

Therefore, within the limitations specified by the Statement

of Work and the constraints of available funding, MRI's approach to the problem was designed to:

- Increase sensitivity of currently used forensic blood analysis techniques.
- Increase discrimination capabilities by permitting multiple tests on the same bloodstain sample.
- Decrease test time.
- Simplify testing procedures.
- Minimize operator interaction by simplifying and automating those areas involving subjective influences on decision making, e.g., automatic readout of results.
- Eliminate the need for fresh erythrocytes as indicator systems.
- Use commercially available reagents.
- Satisfy the cost restraint for reagents and equipment as specified in the Statement of Work.

It was recognized early from the scope of the problem that a meaningful research and development effort would require an integration of capabilities in fundamental immunology as well as in equipment development. Therefore, the relevant capabilities and expertise of two subsidiaries of the Applied Technology Group of EG&G, Inc., were combined; the Mason Research Institute, a life sciences research and development organization with long and varied experience in immunoassay and immunogenetics, and ORTEC, Inc., engaged in the design, development, manufacture and marketing of highly sophisticated electronic and electrophoresis systems.

A review of the serological methodology being applied currently in various criminalistic laboratories, as well as a review of the literature pertinent to forensic serology including "The Examination and Typing of Bloodstains in the Crime Laboratory" by

B.J. Culliford, revealed that the procedures basic to dried bloodstain analysis and to erythrocyte grouping as a whole, have been essentially unchanged for over thirty years. Some developments which have been made have not been oriented to the capabilities of the average criminalistic laboratory, or have not met the stringent criteria required to hold up as evidence in courts of law. It must be acknowledged, however, that the more sophisticated criminalistic laboratories, working under heavy case loads, have not had the opportunity to muster the necessary professional expertise and instrumentation capabilities required for new methodology development.

In the studies to be described, consultants expert in blood banking procedures were employed rather than forensic serologists, because all studies were primarily of an exploratory and developmental nature designed to conduct a feasibility demonstration of the fluorescent immunoassay methods proposed by MRI. It was recognized that input from the forensic scientist in modifying methodologies for practical application in field testing would be essential once feasibility of the method had been demonstrated.

The initial Program Plan prepared by MRI which was based upon the requirements set forth in the Statement of Work prepared by The Aerospace Corporation, indicated that the primary method to be demonstrated was the "Low Angle, Direct Fluorescence Tagging Method" described in MRI's original proposal. This method incorporated the analysis of bloodstained fabrics, single fibers and dried blood particulates. Concurrently, a demonstration was to be

provided of two alternative methods also proposed by MRI, the "Direct Fluorescence Elution" and the "Indirect Solid Phase Immunoassay" methods. The latter were to be demonstrated only if the primary method proved infeasible.

The application of any immunofluorescent method involves four separate procedure areas: (1) the preparation of antigen (bloodstain) into a form suitable for use; (2) preparation and characterization of the fluorescent reagent; (3) staining procedures, and (4) a suitable detection method. Each area contains numerous variables, and they must be systematically evaluated, individually, and in relation to the other areas, in order to develop an immunofluorescent assay.

All three methodologies proposed by MRI are based on proven immunofluorescent principles and techniques. The three proposed methods and the four procedure areas have been tabulated in a matrix format and presented as Table 7.3.1. Figures 7.3.1 and 7.3.2 compare current forensic techniques and the proposed methods discussed below.

7.3.1 Low Angle, Solid Phase and Direct Fluorescence Method

The primary method, the Low Angle, Solid Phase, Direct Fluorescence Method allowed the antigen (bloodstain) to be prepared in numerous ways. The bloodstain could be fixed to cloth or threads, or crusts could be removed from objects and the antigenic material deposited on membranes. The fluorochrome selected was fluorescein, which is commercially available as the isothio-

TABLE 7.3.1.

MATRIX TABLE

PROPOSED BLOODSTAIN METHODOLOGIES VS. PROCEDURE AREAS

	Antigen (bloodstain) Preparation (1)	Fluorescent Reagent (2)	Stain Procedure (3)	Detection Methodology (4)
I. Low Angle Fluorescence	fixed — fabric, threads, crusts	FITC-γ-globulins* FITC IgM* FITC IgG*	Indirect	Solution
	unfixed -impinged on membranes	FITC - anti A† FITC - anti B†	Direct	Surface
II. Direct Fluorescence Elution	fixed — fabric, crusts on filters	FITC IgM* FITC IgG*	Indirect	Solution
III. Solid Phase Indirect Assay	fixed fabric	FITC IgM* FITC IgG*	Indirect, on polystyrene beads	Solution

* Goat antihuman antibodies, Cappel Laboratories

† Not commercially available, prepared by MRI

(1), (2), (3), (4) explanation, please see text section 7.3

FIGURE 7.3.1

KEY TO SYMBOLS

BLOODGROUP ANTIGENS

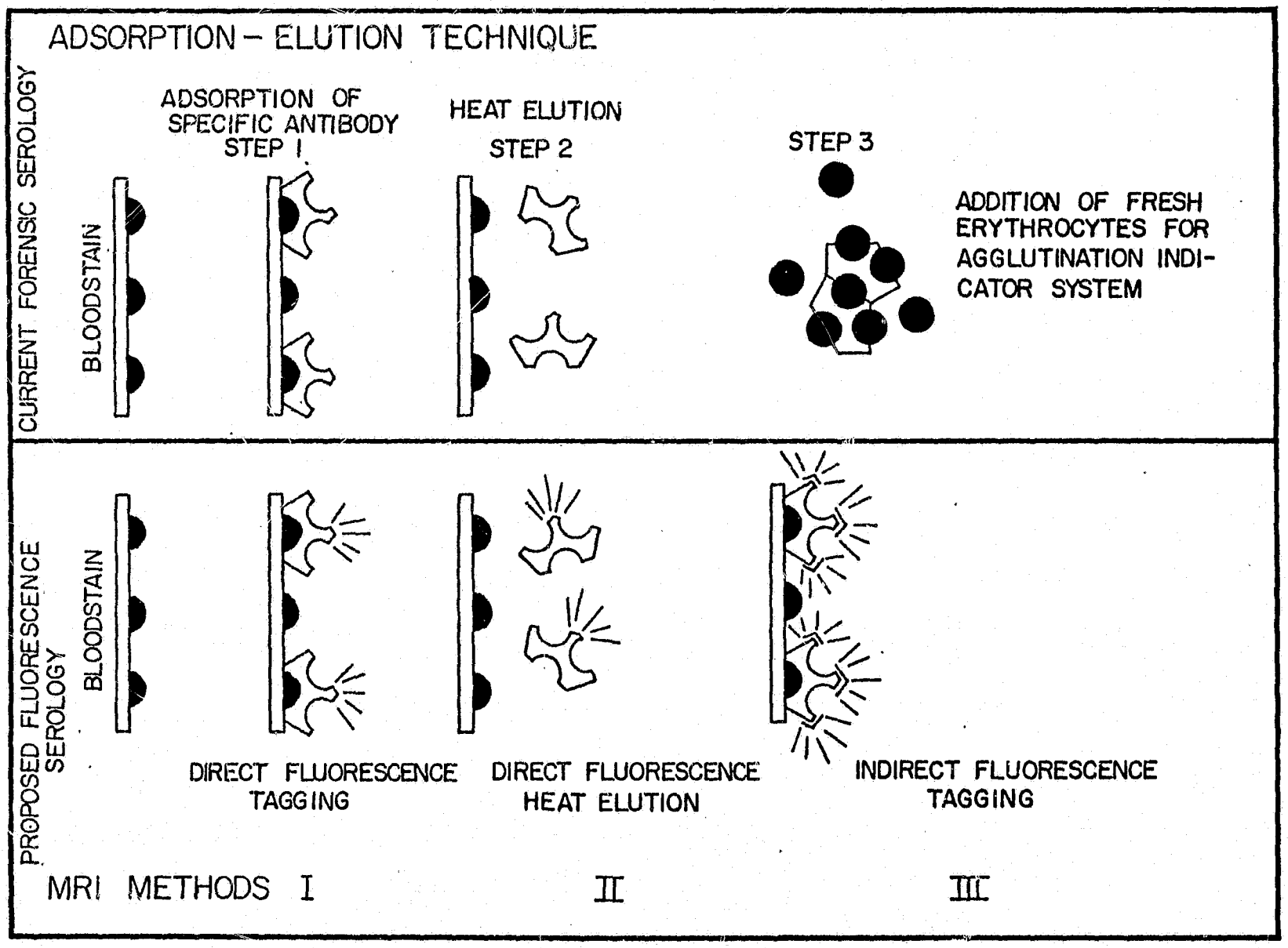
ANTIBODY SPECIFIC FOR ANTIGENS (human r-globulin)

FLUORESCEIN TAGGED ANTIBODY SPECIFIC FOR ANTIGENS

FLUORESCEIN TAGGED GOAT ANTIBODY (anti-human r-globulin)

FLUORESCEIN TAGGED GOAT ANTI-HUMAN r-GLOBULIN REACTED WITH HUMAN r-GLOBULIN (antibody)

FIGURE 7.3.2


ADSORPTION – ELUTION TECHNIQUE
CURRENT FORENSIC SEROLOGY
BLOODSTAIN
ADSORPTION OF SPECIFIC ANTIBODY STEP 1
HEAT ELUTION STEP 2
STEP 3
ADDITION OF FRESH ERYTHROCYTES FOR AGGLUTINATION INDICATOR SYSTEM
PROPOSED FLUORESCENCE SEROLOGY
BLOODSTAIN
DIRECT FLUORESCENCE TAGGING
DIRECT FLUORESCENCE HEAT ELUTION
INDIRECT FLUORESCENCE TAGGING
MRI METHODS I
II
III

cyanate derivative. This derivative allows direct coupling to proteins. Antiserum with the proper specificity and affinity, or isolated gamma globulins or a single class of gamma globulins (IgG, IgM) can be successfully conjugated with fluorescein isothiocyanate (FITC) so that the material is fluorescent and immunoreactive. Both generalized staining procedures (direct and indirect) are compatible with this method. In the direct staining procedure, the primary antibodies directed against the blood group substances (A, B, C, D, e, etc.) are conjugated with FITC. The indirect staining procedure involves the use of unconjugated primary antiserum. After reaction with the bloodstain antigens and removal of unreacted proteins, a secondary fluorescent antibody that is specific for the primary antibody is added to the reaction mixture. The novelty of this first methodology was the detection technique. In the research laboratory, fluorescence was quantitated by means of a spectrophotofluorometer, a versatile, sophisticated instrument designed to measure fluorescence in solution. This proposed procedure would utilize an instrument designed to quantitate fluorescence reflected from a solid surface. The design of such an instrument was part of the Bloodstain Analysis System contract.

Two alternative methods were to be developed concurrently, to be used if the primary method proved infeasible.

7.3.2 Direct Fluorescence Elution Method

The Direct Fluorescence Elution Method was also designed

for testing of either bloodstains fixed on fabric or removed and impinged on filters, and utilized the same fluoresceinized antibodies as the primary method. The staining method of choice was the indirect method, as this technique, when optimized, could decrease the contribution of non-specific fluorescence, and increase the number of available binding sites. Staining was accomplished by reacting the prepared bloodstain with the appropriate unconjugated antibody, followed by FITC-antihuman antibodies. Fluorescence was quantitated both before and after reaction, a positive reaction resulting in a decrease in fluorescence in the secondary antibody solution. The specifically absorbed conjugated antibody was then heat-eluted from the bloodstain, isolated and quantitated. Fluorescence was quantitated in solution utilizing conventional fluorometry equipment. A positive reaction was indicated by loss of fluorescence in the supernatant and/or increased fluorescence in the heat eluate.

7.3.3 Solid-Phase Indirect Immunoassay Method

The Solid-Phase Indirect Immunoassay was initially designed to accept fixed bloodstains as the antigen source. However, if the method was promising, alternate techniques of bloodstain preparation could be developed. The fluorescent reagent was commercially available secondary antibody, as used in the previous methods. This method differs from the others in the application of the stain procedure. The fixed bloodstain was reacted with optimized quantities of non-fluoresceinized anti-blood group antibodies.

Following heat elution, the antibody globulin was absorbed onto polystyrene particles and subsequent treatment of the coated particles with fluorescein-conjugated goat anti-human γ-globulin removed a proportionate amount of fluorescent antibody. Detection of initial and final fluorescence values was by standard fluorometry in solution.

7.3.4 Feasibility Demonstration

Before a firm development approach was selected and submitted for approval to The Aerospace Corporation, MRI was to conduct feasibility demonstrations and research or analyses designed to assist in the formulation of the approach. Specifically, MRI agreed to conduct a feasibility demonstration covering the fluorescent immunoassay methods described. The primary method to be demonstrated was the "Low Angle, Direct Fluorescence Tagging Method". Concurrently, a demonstration was to be provided of the alternate methods, the "Direct Fluorescence Elution" and the "Indirect Solid Phase Immunoassay" methods, the latter to be demonstrated only if the primary method proved infeasible. Initially, work-effort was to be concentrated on developing all three methods during the four month feasibility period. As soon as sufficient evidence was available concerning the infeasibility of any of the two alternate methods, work on that method was to be terminated. Conversely, as soon as sufficient evidence was available that the primary method was feasible for demonstration, work on the alternate method demonstrations were to be terminated.

7.4 DEVELOPMENT OF METHODOLOGY (6 December 1974 - 31 May 1975)

Antisera to blood group types A, B, C, D and e in bulk volumes, were obtained from Dade Company, Miami, Florida. The antisera were individually fractionated by ammonium sulfate precipitation and following dialysis the globulin fractions were conjugated to fluorescein isothiocyanate according to the method of Rindernecht (36). Separation of the unconjugated fluorescein was accomplished by passing the tagged antibody through a column of Sephadex G-25.

7.4.1 Determination of Antibody Titer Pre and Post Fluorescein Conjugation:

In order to determine the effect of fluorescein conjugation on antibody titers, both conjugated and non-conjugated antisera of the same lot were tested. Serial dilutions of the antisera were made in saline or albumin solution using 0.1 ml aliquots. An equal volume of the proper indicator cell system was added to all dilutions, mixed well, and centrifuged at 3,000 rpm. Agglutination was scored macroscopically. The results presented in Table 7.4.1 indicate that there was a 1-2 fold decrease in antibody titer as a result of the fluorescein conjugation.

7.4.2 Effect of Temperature on Antibody Binding Capacity:

Because fluoresceinized antiserum was to be used in the Direct Fluorescence Elution Method, an attempt was made to determine

TABLE 7.4.1

EFFECT OF FLUORESCEIN CONJUGATION ON

SERUM ANTIBODY TITER

	Antibody Titer*	
Antisera	Unconjugated	Conjugated
Anti-A	1:512	1:128
Anti-B	1:1024	1:256
Anti-D	1:256	1:128

* Titer is expressed as the highest serum dilution showing 1+ or more agglutination reaction.

the effect of temperature on the fluoresceinized reagents. For this purpose aliquots of fluoresceinized anti-A were incubated at 40, 42, 45, 48 and 50° C for 5, 10, 20 and 30 minutes. Following incubation, anti-A was serially diluted in phosphate buffered saline (PBS) pH 7.3 from 1:20 - 1:320. Thrice washed human A cells were then added to each dilution, mixed well and centrifuged at 3,000 rpm. The results shown in Table 7.4.2 indicate that temperatures of 40-50° C do not significantly affect the antibody titer of fluoresceinized anti-A. From the raw data presented in Table 8.1.2, one can note that temperature of 45, 48 and 50° C eliminated only the trace (±) reactivity which was observed in the control and lower temperatures at 1:20 dilution. Time of incubation was not a factor in the reduction of antibody titer.

7.4.3 Effect of Commercial Detergents Coupled with Temperature on Antibody Binding Capacity:

The effect of commonly used commercial detergents on antibody activity was evaluated by incubating an aliquot of fluoresceinized and non-fluoresceinized anti-A with 1 cm^2 pieces of prewashed fabric at various temperatures for varying periods of time and then reacted with human type A cells as shown in Table 7.4.2. The results presented in Table 7.4.3 though not consistent, indicate that there was a slight reduction in antibody titer as a result of this treatment.

TABLE 7.4.2

EFFECT OF TEMPERATURE ON ANTIBODY BINDING CAPACITY

	Agglutination Titer*			
	Time of Incubation in minutes			
Temperature	5	10	20	30
40°C	1:160	1:160	1:160	1:160
42°C	1:160	1:160	1:160	1:160
45°C	1:160	1:160	1:160	1:160
48°C	1:160	1:160	1:160	1:160
50°C	1:160	1:160	1:160	1:160
Control	1:160	1:160	1:160	1:160

* Titer is the reciprocal of the highest antibody dilution showing 1+ or more agglutination reaction.

TABLE 7.4.3

EFFECT OF DETERGENTS ON ANTIBODY ACTIVITY

Temperature (C°)	Agglutination Titer* Time of Incubation in minutes 5	10	20	30
		(CHEER DETERGENT)		
40	1:80	1:80	1:40	1:40
42	1:80	1:40	1:40	1:20
45	1:40	1:40	1:40	1:40
48	1:40	1:40	1:20	1:20
59	1:20	1:20	1:40	1:20
Control	1:80	1:80	1:80	1:80
		(ALL DETERGENT)		
40	1:80	1:80	1:80	1:80
42	1:80	1:80	1:80	1:80
45	1:40	1:40	1:40	1:40
48	1:40	1:40	1:40	1:40
50	1:40	1:40	1:40	1:40
Control	1:80	1:80	1:80	1:80

* Titer is the reciprocal of the highest antibody dilution showing 1+ or more agglutination reaction.

7.4.4 Direct Fluorescence Elution Method:

Preliminary studies similar to those reported by Myhre (37) were carried out as follows: Serial dilution of fluoresceinized anti-A ranging from 1:2 - 1:64 were made into 5 sets of tubes. Ten (10), 50 and 100 µl of 50% washed human A cells were added to the first 3 sets of dilution whereas B and O cells were added to the other 2 sets. All reactants were thoroughly mixed and allowed to incubate for 10 minutes at room temperature in the dark. After determining the agglutination titer, the cells were washed (3x) with large volume of PBS, resuspended in 0.5 ml in the same buffer and incubated for 15 minutes at 50° C with intermittent gentle mixing. Following centrifugation at 2,000 rpm for 5 minutes, the supernatant of each dilution was read on the Aminco-Bowman Spectrophotofluorometer. As can be seen from Table 7.4.4, satisfactory results were obtained in the first study when using erythrocyte suspensions prepared from heparinized whole blood. This experiment was confirmed in a second study and the results are also presented in Table 7.4.4. Consistently, eluates of A cells gave higher fluorescence values than either the B or O cells eluates.

7.4.5 Direct Fluorescence Elution Using Fresh Stains:

Fresh blood stains (A, B and O) were prepared on different pieces of cloth (1 cm^2) and allowed to dry for 30 minutes at room temperature. The stained cloths were washed (3x) with distilled

TABLE 7.4.4

DIRECT FLUORESCENCE ELUTION

Fluoresceinized Anti-A Dilutions	Fluorescence Units of Eluates* Volume and Type of Cells				
	10 µl A	50 µl A	100 µl A	100 µl B	100 µl O
			(First Study)		
1:2	15	21	21	9	12
1:4	12	11	11	6	9
1:8	9	11	11	6	9
1:16	8	9	9	6	6
1:32	6	8	9	3	6
1:64	8	6	8	5	5
			(Second Study)		
1:2			29	15	15
1:4			21	16	19
1:8			21	18	19
1:16			25	14	18
1:32			24	11	19
1:64			21	15	12

* Aminco-Bowman settings:

Excitation: 486 nm

Emission: 520 nm

Slits: 1,2,1,1,2,2 mm

water and then while moist, they were allowed to react with 1:2 dilution of fluoresceinized anti-A for 90 minutes at room temperature in the dark. Following incubation the unreacted, tagged anti-A was removed and the cloths were washed (3x) in PBS. The elution procedure, which is described in Section 8.4, was the same as listed in paragraph 7.4.4. The results are presented in Table 7.4.5. When the fluoresceinized antiserum was applied to blood stained fabrics, unlike whole blood, it produced variable results and reproducibility was a major problem.

An investigation of this problem revealed that the initial washing of blood stained fabrics to remove the plasma proteins for isoenzyme electrophoresis, not only removed the hemoglobin and proteins, but washed off varying amounts of erythrocytes (ghosts and cell membranes) as well. According to a conversation with officials at Ciba-Geigy, Cranston, R.I., we were informed that many synthetic fibers are being treated to be "stain resistant" resulting in a less adhesive bloodstain.

A similar experiment was carried out using A, B and O blood crusts previously fixed in acetone instead of bloodstained fabrics. The crusts were reacted with various dilutions of fluoresceinized anti-A reagent. After removal of the crusts, the supernatent tagged anti-A solution was placed in the Aminco-Bowman spectrophotofluorometer to determine tha amount of unabsorbed antibody as a function of antibody dilution. Since this was a measurement of antibody absorption, a decrease in the fluorescence of the supernatent labeled anti-A indicated a positive reaction.

TABLE 7.4.5

DIRECT FLUORESCENCE ELUTION USING FRESH BLOOD STAINS

Blood Stain Type	Fluorescence Units of Eluates *	
A	30	
A	12	Average = 19
A	14	
B	8	
B	15	Average = 11
O	25	
O	15	Average = 17
O	12	
Plain Cloth	7.5	Average = 7.5

* Aminco-Bowman Setting:

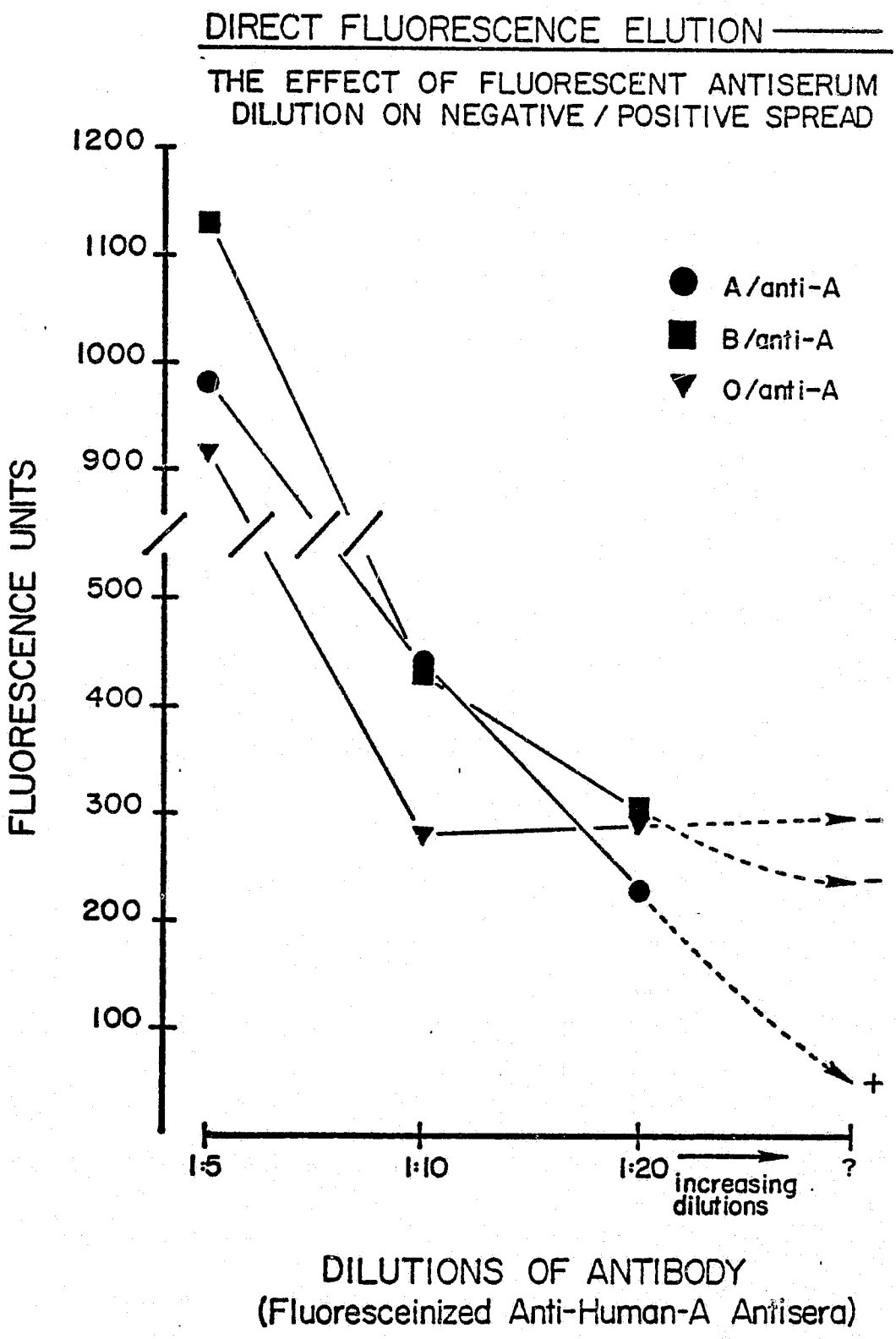
Excitation: 486 nm
Emission: 520 nm
Slits: 1, 2, 1, 1, 2, 2 mm

The results illustrated in Figure 7.4.1 clearly demonstrated the importance of optimizing the concentration of the reagents for use in the absorption step of the Direct Fluorescence Elution Method. When conjugated anti-A was used at dilutions of 1:5 or 1:10, no difference in intensity of fluorescence (spread) could be detected between bloodstains of type A (positive test) and bloodstains of type B or O (negative controls). However, by increasing the dilution of the conjugated antiserum (1:20 dilution or greater), a definite differentiation between the positive and negative bloodstains was observed.

7.4.6 Low Angle, Solid Phase, Direct Fluorescence Tagging Method:

Anticipating problems in the "Low Angle, Solid Phase, Direct Fluorescence Tagging Method" resulting from loss of erythrocyte antigens from bloodstained fabrics during washing steps, several biological fixatives (acetone, alcohol and ether) used in the fixation of tissues for fluorescent microscopy, were tested against bloodstains. Fixation of bloodstains was accomplished by incubating bloodstained fabrics in acetone or 95% ethanol for 10 minutes at room temperature, or by incubating in an ether: ethanol (50:50) mixture for 10 minutes at room temperature followed by 20 minutes in 95% ethanol at 37° C or at room temperature. The latter proved to be the best method for fixation based upon microscopic examination of the eluate collected and stained on Millipore membrane filters. When fixed in this manner, washing with buffered saline did not elute the blood.

FIGURE 7.4.1



NOTE: Positive reaction is indicated by a decrease in fluorescence.

A great amount of effort was concentrated on studies dealing with solid surface fluorescence scanning. Blood crusts or direct bloodstained fabric samples (type A, B, O, C, D and e) were initially fixed in a 50:50 ether-ethanol mixture (v/v) for 10 minutes at room temperature followed by 20 minutes in 95% ethanol at 37° C. The appropriate unlabelled antiserum was then allowed to react with the antigenic sites on the bloodstained fabric. After incubation, the samples were thoroughly washed in buffered saline and incubated in the presence of 22% albumin for 20 minutes. Following aspiration of the excess albumin, the samples were reacted with a monospecific labelled anti-human IgG (1:10 dilution), washed throughly to remove excess labelled anti-IgG and mounted on stainless steel holders for determining the fluorescence in the Aminco-Bowman spectrophotofluorometer (See detailed procedures in Section 8.5).

Employing the indirect fluorescent antibody procedure, the following were investigated:

a. Comparison of fixed angle and angle optimization for maximal fluorescence in the A/anti-A, B/anti-B, D/anti-D, C/anti-C and e/anti-e blood type systems (See Tables 7.4.6 thru 7.4.10).

b. Comparison of the effect of relative wetness of bloodstain preparation of fluorescence (Table 7.4.10).

c. Comparison of the fluorescence of blood crusts and fresh erythrocyte suspensions (Tables 7.4.12 and 7.4.13).

TABLE 7.4.6

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING

METHOD: COMPARING FIXED ANGLE AND VARIABLE ANGLE FOR MAXIMUM FLUORESCENCE*

Bloodstain Type/Antiserum (Cloth Samples)	Fluorescence Units: Angle Fixed at 45°	Fluorescence Units: Maximum Fluorescence
A/anti-A	4,100	33,000
A/anti-A (Average)	5,200 (4650)	72,000 (52500)
B/anti-A	1,900	2,100
O/anti-A (Average)	3,000 (2450)	12,000 (7050)

Fluorescein tagged antibody was goat anti-human IgG

Cloth samples mounted directly on stainless steel holders

* Maximum fluorescence determined by rotation of the sample within the excitation beam.

Reactions	Increase in Fluorescence Units Resulting From Angle Optimization
Positive (A/anti-A)	11.3 times
Negative Controls	2.9 times

NOTE: Limitations of the instrumentation did not permit accurate measurement of the angle.

TABLE 7.4.7

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING METHOD: COMPARING FIXED ANGLE AND VARIABLE ANGLE FOR MAXIMUM FLUORESCENCE*

Bloodstain Type/Antiserum (Cloth Samples)	Fluorescence Units		
	Angle Fixed at 45°	Maximum Fluorescence	Angle
B/anti-B	5,500	86,000	55°
B/anti-B	4,400	95,000	50°
B/anti-B	9,900	>100,000	52°
(Average)	(6,600)	(93,666)	
A/anti-B	3,700	25,000	50°
O/anti-B	3,500	52,000	50°
(Average)	(3,600)	(38,500)	
Oxoid Membrane	500	500	45°

Fluorescein tagged antibody was goat anti-human IgG
Cloth samples were mounted on stainless steel holders against an oxide membrane background.

Reactions	Increase in Fluorescence Units Resulting From Angle Optimization
Positive (B/anti-B)	14.2 times
Negative Controls	10.7 times

* Maximum fluorescence determined by rotation of the sample within the excitation beam.

TABLE 7.4.8

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING

METHOD: COMPARISON OF FIXED ANGLE AND EXPERIMENTALLY DETERMINED ANGLE FOR MAXIMUM FLUORESCENCE*

Bloodstain Type/Antiserum (Cloth Samples)	Fluorescence Units	
	Angle Fixed at 45°	Angle Fixed at 50°
C+/anti-C	9,000	13,800
C+/anti-C	7,500	12,000
(Average)	(8,250)	(12,900)
C-/anti-C	2,500	7,100
C-/anti-C	5,000	8,500
(Average)	(3,750)	(7,800)
Oxoid Membrane	260	7,000

Fluorescein tagged antibody was goat anti-human IgG.

Cloth samples were mounted on stainless steel holders against an oxoid membrane background.

* Maximum fluorescence determined by rotation of sample within the excitation beam.

TABLE 7.4.9

LOW ANGLE, SOLID PHASE DIRECT FLUORESCENCE TAGGING METHOD: COMPARING FIXED ANGLE AND VARIABLE ANGLE FOR MAXIMUM FLUORESCENCE*

Bloodstain Type/Antiserum (Cloth Samples)	Fluorescence Units		
	Angle Fixed at 45°	Maximum Fluorescence	Angle
D+/anti-D	>100,000	>100,000	45°
D+/anti-D	35,000	>100,000	50°
D+/anti-D	93,000	93,000	45°
(Average)	(76,000)	(>97,666)	
D-/anti-D	2,200	7,000	50°
Oxoid membrand/anti-D	9,600	TF	TF
Oxoid membrane/PBS+	1,080	2,400	55°

Fluorescein tagged antibody was goat anti-human IgG.
Cloth samples were mounted on stainless steel holders against an oxoid membrane background.

+PBS = phosphate buffered saline.

* Maximum fluorescence determined by rotation of sample within the excitation beam.

TABLE 7.4.10

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING

METHOD: COMPARING THE EFFECT OF RELATIVE WETNESS OF BLOODSTAIN PREPARATION ON FLUORESCENCE*

Bloodstain Type/Antiserum (Cloth Samples)	Fluorescence Units	
	With Coverslip	Without Coverslip
e+/anti-e	TF	480
e+/anti-e	15,000	710
(Average)	(15,000)	(595)
e-/anti-e	12,000	390
e-/anti-e	6,900	300
(Average)	(9,450)	(345)

* Fluorescein tagged antibody was goat anti-human IgG.
Angle of excitation beam was fixed at 45°.
Preparations were moistened with phosphate buffered saline, and samples covered with coverslips were relatively more moist.
Cloth samples were mounted on stainless steel holders against an oxoid membrane background.
TF = technical failure

TABLE 7.4.11

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING

METHOD: SPECIFIC FLUORESCENCE OF BLOODSTAIN CRUSTS ON GLASS*

Blood Type/Antiserum (Bloodstain Crusts)	Fluorescence Units
B/anti-B	3,300
A/anti-B	96
A/anti-B	110
O/anti-B	54

* Crusts were prepared on glass-slides (1.5 x 4 cm), covered with a glass coverslip, sealed with paraffin and mounted on stainless steel holders. Angle of excitation beam was fixed at 45°. Fluorescein tagged antibody was goat anti-human IgG.

TABLE 7.4.12

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING

METHOD: COMPARISON OF BLOODSTAIN CRUSTS AND FRESH ERYTHROCYTE SUSPENSIONS

Blood Type/Antiserum	Fluorescence Units	
	Fresh Erythrocyte Suspensions	Bloodstain Crusts
A/anti-A	2,430*	12,000
A/anti-A	14,400*	15,600
B/anti-A	160	290
O/anti-A	10	60
Unreacted A bloodstain crusts		10
Phosphate buffered saline		1

Crusts or erythrocyte suspensions were prepared on glass slides (1.5 x 4 cm) covered with a glass coverslip, sealed with paraffin and mounted on stainless steel holders.

Angle of excitation beam was fixed at 45°.
Fluorescein tagged antibody was human anti-A.

* Variability may be due to a) individual differences of various donors and b) different amount of cells used.

TABLE 7.4.13

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING METHOD: PERCENT INCREASE IN FLUORESCENCE OF POSITIVE REACTIONS OVER THAT OF NEGATIVE CONTROLS *

Bloodstain Type/Antiserum	Fluorescence Units	
	Angle Fixed at 45°	Maximum Fluorescence
Bloodstain Fabric		
A/anti-A	190%	750%
B/anti-B	180%	>240%
C^+/anti-C	220%	170%
D^+/anti-D	345%	>140%
e^+/anti-e	160%	NT
Bloodstain Crusts		
A/anti-A	7890%	NT
B/anti-B	3810%	NT
Fresh Erythrocytes		
A/anti-A	9900%	NT

NT = not tested

Percent Increase = Fluorescence Units (Test)/Fluorescence Units (Control) X 100

* Maximum fluorescence determined by rotation of the sample within the excitation beam.

d. Determination of the percent increase in fluorescence of positive reactions over that of negative controls (Table 7.4.13).

The initial data presented in Tables 7.4.6 - 7.4.13 indicate that the fluorescence signal from the known positive samples was higher than the corresponding negative control samples. This difference in the fluorescence readings was maintained under various conditions of sample preparation and presentation in the spectrophotofluorometer. These experimental variables include angle optimization (Tables 7.4.6 - 7.4.10) and wetness of test samples (Table 7.4.10).

As a result of an assessment made of the progress achieved with the three methods proposed for bloodstain analysis, the problems peculiar to each method still to be solved, and the time remaining before the date of Feasibility Demonstration, a decision by MRI personnel was made to discontinue research effort on the alternative methods and to concentrate all work effort on the primary "Low Angle, Solid Phase, Direct Fluorescence Tagging Method".

Therefore, research effort was concentrated on -

a. Identifying and eliminating serological variables pertinent to bloodstain preparation for fluorescence analysis;

b. Designing and constructing instrumentation with a configuration of excitation and emission monochromators

more ideally suited for the Low Angle, Solid Phase, Direct Fluorescence Method; and

c. Designing better bloodstain sample holders for the Aminco-Bowman Spectrophotofluorometer to be used in the period interim to the design and construction of idealized instrumentation.

. Serological Variables: Work effort in this area was concentrated on three forms of bloodstain preparations for fluorescence analysis:

1. Pieces of fabrics
2. Single threads or fibers
3. Membrane filters with bloodstain particulates removed by ultrasonic vibration and impinged upon the filter surface.

In order to assure adequate time for equilibration reactions and washes and minimize any interfering factors, standardized procedures were established (See Sections 8.7-8.9). You will note, for example, care was taken to identify fabric surfaces to be compared (negative controls were taken directly from adjoining test sections), both antiserum reactions and washes were accomplished on automatic rotators or rocker mechanisms, and timing of washes strictly defined. The reason for the latter modification is that excessive washes can be detrimental and that processed bloodstain samples cannot be kept for prolonged periods in saline solutions at room temperature before being read for specific

fluorescence.

7.4.7 Low Angle, Solid Phase, Direct Fluorescence Tagging Method: Pieces of Fabrics

In an effort to determine the optimal dilution of anti-A or anti-B to be used in the indirect fluorescent antibody technique, a study was undertaken using various dilutions of antisera ranging from undiluted to a dilution of 1:160. All other steps in the procedure were kept the same. The results presented in Table 7.4.14 indicate that the undiluted antisera provided a consistent difference between a positive and negative reaction and, therefore, should be used in order to differentiate between type A or B bloodstain. Because of this finding it was determined that undiluted antisera will be used for typing in all our future experiments.

Different pieces of cloth were tested as described in Section 8.6. Measurement of fluorescence were made in the Aminco-Bowman Spectrophotofluorometer at angles of 10, 20 and 30°. The results for the ABO system and the Rh system are presented in Tables 7.4.15 and 7.4.16 respectively. Although conditions for serological reactions had not been optimized, the results indicate that the degree of fluorescence was proportional to the angle used. In most instances, blood types A and B were easily distinguished.

The fluorescence obtained with AB serum (See Table 7.4.15) was unexpectedly high. This might be attributed to the adherence

TABLE 7.4.14

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD: USING PIECES OF FABRIC

	Fluorescence Units					
	Angle			Angle		
Dilution	10	20	30	10	20	30
	(A/Anti-A)			(A/Anti-B)		
	9100	17000	24000	6500	10100	17000
1:5	3600	6300	9400	3700	5200	8300
1:10	1900	3400	4800	1800	4300	6300
1:20	2700	4100	5500	1700	3200	5000
1:40	3000	4700	6600	1750	3850	5700
1:80	2700	3850	5650	2500	3700	5700
1:160	1500	4000	6100	2350	3500	5400

Excitation: 460

Emission: 520

Slits: 4, 2, 2, 4, 0, 2

TABLE 7.4.15

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD USING PIECES OF FABRIC

Bloodstain Type	Angle 10°	20°	30°	10°	Angle 20°	30°	10°	Angle 20°	30°
		(Anti A)			(Anti B)			(AB Serum)	
				Study #1					
A	2300	3800	5500	1200	2050	3150	900	2300	3500
A	2300	3650	4800	2200	3800	5450	1700	3600	4600
B	2500	3150	4300	2600	3300	3800	1900	3350	4500
O	1900	3050	4200	2100	3700	5300	1900	2900	3800
				Study #2					
A	1500	2400	2800	1100	3300	5100	1800	2800	3700
A	1600	2600	3550	2300	3500	4700	950	1400	2100
B	1150	1800	2400	2500	3200	4800	1050	2400	3600
O	1900	3900	5400	1950	3300	4400	1500	2600	3200

Excitation: 460 nm

Emission: 520 nm

Slits: 4, 2, 2, 4, 0,2

525 nm cut on filter in place

AB Serum: Serum was obtained from an AB blood type individual

See section 7.4.7

CONTINUED

1 OF 4

TABLE 7.4.16

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD USING PIECES OF FABRIC

Test System	Angle 10	20	30	Test System	Angle 10	20	30
			Study #1				
C^-/anti-C	1500	2300	3300	C^+/anti-C	1250	1800	2850
C^-/AB Control	1600	2700	3700	C^+/AB Control	1600	2700	3700
D^-/anti-D	2150	3700	5400	D^+/anti-D	2300	4000	5700
D^-/AB Control	3200	5500	7400	D^+/AB Control	2850	4900	6900
			Study #2				
C^-/anti-C	1300	3100	4550	C^+/anti-C	2300	3700	5200
C^-/AB Control	1550	3700	5750	C^+/AB Control	2000	3500	5700
D^-/anti-D	900	1500	2250	D^+/anti-D	1100	1700	2300
D^-/AB Control	1100	2300	3050	D^+/AB Control	1500	3200	4300

Excitation: 460 nm

Emission: 520 nm

Slits: 4, 2, 2, 4, 0, 2

525 nm cut on filter in place

AB Serum: Serum was obtained from an AB blood type individual

See section 7.4.7

of extraneous serum proteins in the AB serum onto the red cells, which upon reaction with the fluoresceinized anti-human γ-globulin, may have resulted in non-specific fluorescence.

Interpretation of the O results was difficult. The feasibility demonstration was to be on the identification of the presence or absence of the antigens A, B, C, D and e, efforts to solve these problems were delayed in order to concentrate on what was required for feasibility.

The results of testing the Rh system (Table 7.4.16), unoptimized conditions, were similar to that obtained with the ABO, the greater the angle the greater the intensity of fluorescence. This indicated that the condition of the samples at time of presentation were a factor in the inconsistencies observed. Thus, the effects of wetness and dryness of test samples were also investigated. Both studies demonstrate that when the samples are presented dry, the fluorescence units were, in the majority of cases, slightly higher (See Table 7.4.17). The statistical significance of the reported fluorescence was not known.

7.4.8 Low Angle, Solid Phase, Direct Fluorescence Tagging Method: Single Threads or Fibers

Samples were prepared as described in Section 8.8. The results of this study are summarized in Table 7.4.18. It can be noted that when the thread was rotated (120^{o}) so as to expose three surfaces, 5 out of 6 tests (83%) were correct, indicating that this approach was feasible for determining blood types on

TABLE 7.4.17

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD: EFFECT OF

WETTNESS

Donor	Test System	Condition at Time of Presentation to Aminco-Bowman: Wet	Dry
		(Study #1)	
Divna	A/anti-A	3700	3450
	A/anti-B	2300	3000
Patty	A/anti-A	2200	2500
	A/anti-B	2200	2300
Jane	B/anti-A	2900	3100
	B/anti-B	2400 (x)	2500 (x)
Kathy*	O/anti-A	3850	4000
	O/anti-B	3200	3300
		(Study #2)	
Divna	A/anti-A	3600	3600
	A/anti-B	3000	3000
Jane	B/anti-A	2300	2300
	B/anti-B	2800	2900
Kathy*	O/anti-A	4500	4700
	O/anti-B	3800	4450

Excitation: 460 nm — 525 nm cut on filter in place

Emission: 520 nm

Slits: 4, 2, 2, 4, 0, 2

* This anomaly was not explainable at this point in time.

TABLE 7.4.18

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD: USING SINGLE THREADS

Test System	Fluorescence Units Angle of Rotation 60°	90°	120°
C^+/anti-C	570	400	330
C^+/AB Control	420	460	520
D^+/anti-D	310	410	540
D^+/AB Control	270	300	400
e^+/anti-e	550	570	570
e^+/AB Control	220	190	180
C^-/anti-C	210	200	230
C^-/AB Control	380	550	490
D^+/anti-D	330	300	370
D^+/AB Control	570	500	350
e^-/anti-e	350	330	350
e^-/AB Control	770	710	660

Excitation: 460 nm

Emission: 520 nm

Slits: 4, 2, 0.5, 2, 0, 2

NOTE: test is negative when test fluorescence is < AB control reaction.

single threads of fibers when samples are small.

7.4.9 Instrumentation - LABS 1:

LABS is an acronym for LEAA Aerospace Bloodstain System, and was the name given to the prototype instrument developed during this study. The apparatus was built by the Life Sciences Division of ORTEC, Inc., under a subcontract agreement to the Mason Research Institute.

The inconsistencies of the results obtained and the sample centering difficulties encountered with the Aminco-Bowman apparatus, were felt to be largely due to the fixed 90° configuration of the excitation and emission monochromators. Dr. Siegelman and the ORTEC Company with some input from MRI personnel designed an instrument to more realistically meet the needs of the Low Angle, Solid Phase, Direct Fluorescence Tagging Method. The instrument that had been designed and constructed was much simpler and more practical for low angle fluorescence excitation having the following general specifications:

a. Angle of the excitation beam to sample is 28° using a quartz halogen lamp as light source.

b. Intensity can be varied from 0-150 watts with excitation set at 470 nm using a 3 cavity interference filter having a band width of 7nm.

c. Emission is set at 530 nm using a cavity interference filter having a band width of 8.2 nm and an IP21

photomultiplier tube.

d. The basic instrument is connected to a photon counting electronics system with a digital readout capability.

e. The LABS-1 instrument is adapted to accept a variety of samples, e.g., fabrics, threads, membrane filters and microscope slides. Both excitation and emission can be varied by the use of specialized filters.

f. A sample holder had been designed that would present identical surface areas of test and control samples for excitation.

A major amount of effort at this time was concentrated on employing the LABS-1 instrument in the Low Angle, Solid Phase, Direct Fluorescence Tagging Approach Method for pieces of fabrics and for bloodstained particulates impinged on Millipore or Oxoid membrane filters.

7.4.10 Low Angle, Solid Phase, Direct Fluorescence Tagging Method with LABS-1 Using Piece of Fabric:

The procedures employed were as described in detail in Sections 8.7 and 8.8. The results of the AB system are presented in Table 7.4.19 and 7.4.20. Based on the reactivities with anti-A and anti-B, bloodstains of groups A and B were correctly identified in 75% of the cases (See Table 7.4.19) according to the procedure detailed in Section 8.6. However, slight modification of this procedure in relation to time of incubation with the first

TABLE 7.4.19

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD: LABS-1 USING PIECES OF FABRIC*

(A, B System)

Test System	Photon Counts Set #1		Set #2	
B/anti-A	1778		1782	
B/anti-B	2110		1918	
A/anti-A	2225		2189	
A/anti-B	1882		1807	
B/anti-B	1881	(X)	1914	(X)
B/anti-A	2055		2260	
A/anti-A	1957		2041	
A/anti-B	1667		1745	

* Fluoresceinated goat anti-IgM was substituted for goat anti-IgG.

and second antibody, resulted in 100% accurate identification of group A and B bloodstains (See Table 7.4.20). Procedures for the identification of group O and AB bloodstains were not attempted.

Table 7.4.21 summarizes the results of the Rh system when pieces of fabric were used. Note that test is negative when AB control reading is equal to or greater than test reading, and (X) indicates incorrect reading. It is quite clear from the results that time of incubation plays a major role in the identification of Rh types C, D and e. Only 50% were correctly identified when the specific first antibody (anti-C, anti-D and anti-e) was allowed to incubate for only 15 minutes with the bloodstained fabrics (See Set #1 in Table 7.4.21). Varying the incubation time with the first antibody resulted in 75-84% correct identification of blood Rh groups, (C, D and e), as can be seen in Set #2-Set #5 in Table 7.4.21.

In order to clarify whether the fluorescence observed was due to the reactivity of fluorescent goat anti-human globulin or anti-IgM reacting with the globulin portion of the bloodstain itself, a precoating step with non-fluoresceinized goat anti-IgM (same lot as used for fluorescent tagging) was inserted in the procedure prior to incubation with the first antibody. Theoretically, this was to combine with the globulin sites and block them from reacting with the fluoresceinized antiserum. From the results presented in Table 7.4.22 (Study #1), precoating with non-fluoresceinized goat anti-human IgM did not increase the spread

TABLE 7.4.20

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE
TAGGING METHOD: LABS-1 USING PIECES OF FABRIC

(A, B System)

Photon Counts			
Set No. 1*		Set No. 2**	
A/anti-A	1788	A/anti-A	7443
A/anti-B	1547	A/anti-B	6292
		AB Control	5894
A/anti-A	1937	B/anti-A	6451
A/anti-B	1755	B/anti-B	6832
		AB Control	5900
B/anti-A	1459	B/anti-A	5632
B/anti-B	1669	B/anti-B	6150
		AB Control	5165
B/anti-A	1542		
B/anti-B	1870		
A/anti-A	2094		
A/anti-B	1662		

* Set No. 1 was treated with first and second antibody for 90 minutes each at room temperature.

** Set No. 2 was incubated for 60 minutes at 37°C and an additional 60 minutes at room temperature with first antibody. Incubation with second antibody was for 90 minutes.

NOTE: AB Control is normal serum for Type AB donor

TABLE 7.4.21

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD WITH LABS-1 USING PIECES

OF FABRICS

(Rh System)

	Photon Counts											
Set #1 *			Set #2 **			Set #3 ***			Set #4 †			Set #5 ††		
C^-/anti-C	3493		D^+/anti-D	4368		C^+/anti-C	4004		C^-/anti-C	3042	(X)	C^+/anti-C	4719	
AB Control	4012		D^+/22%/Albumin	3908		AB Control	3829		AB Control	2340		AB Control	4375	
			AB Control	3905										
D^+/anti-D	3560	(X)	D^+/anti-D	4247		D^+/anti-D	3988	(X)	D^-/anti-D	2802		C_2^+/anti-C	4469	
AB Control	3796		D^+/22%/Albumin	3658		AB Control	4325		AB Control	2897		AB Control	4251	
			AB Control	3750										
e^+/anti-e	3762		e^+/anti-e	3142		e^+/anti-e	3895		e^+/anti-e	2633		$D_{\frac{1}{2}}^+$/anti-D	4059	(X)
AB Control	3706		e^+/22%/Albumin	2346		AB Control	2551		AB Control	2608		AB Control	4247	
			AB Control	2954										
C^+/anti-C	3194	(X)	e^+/anti-e	2587		C^-/anti-C	3317		C^+/anti-C	2885		D_2^+/anti-D	4363	
AB Control	4620		e^+/22%/Albumin	2722	(X)	AB Control	3689		AB Control	2682		AB Control	4028	
			AB Control	3021										
D^+/anti-D	3689	(X)				D^+/anti-D	3287		D^+/anti-D	2761		e_1^+/anti-e	4895	
AB Control	3870					AB Control	3262		AB Control	2635		AB Control	3891	
e^+/anti-e	4592					e^+/anti-e	3714		e^+anti-e	2608		e_2^+/anti-e	4219	
AB Control	3877					AB Control	3555		AB Control	2404		AB Control	3238	

* Set #1 was incubated at 37°C for 15 minutes with first antibody. Second antibody incubation was for 90 minutes at room temperature.

** Set #2 was incubated at 37°C for 60 minutes. Second antibody incubation was for 90 minutes at room temperature.

*** Set #3 was incubated for 60 minutes at 37°C and an additional 60 minutes at 4°C with first antibody. Second antibody incubation was for 90 minutes at room temperature.

† Set #4 was incubated at 37°C for 2 hours with first antibody. Second antibody incubation was for 90 minutes at room temperature.

†† Set #5 was incubated at 37°C for 60 minutes and then overnight at 4°C with first antibody. Second antibody incubation was for 90 minutes at room temperature.

TABLE 7.4.22

LOW ANGLES, SOLID PHASE, DIRECT FLUORESCENCE TAGGING METHOD WITH LABS-1: EFFECT OF PRECOATING WITH NON-FLUORESCEINIZED GOAT ANTI-IgM

(A, B System)

	Photon Counts		
	1st reading	2nd reading	Final Difference
	Study No. 1*		
A/anti-A	9544	25546	16,002
A/anti-B	9894	21451	11,557
A/AB Control	8739	13163	4,424
B/anti-A	9670	14835	5,165
B/anti-B	8353	16640	8,287
B/AB Control	8859	12431	3,752
	Study No. 2**		
A/anti-A	10759	28475	17,716
A/anti-B	10425	23826	13,401
AB Control	10955	25391	14,436
B/anti-A	8440	18093	9,653
B/anti-B	8978	19006	10,028
AB Control	8659	17285	8,627
AB/anti-A	8734	13248	4,890
AB/anti-B	8736	13568	4,831
AB Control	8879	13624	4,745
O/anti-A	8667	14659	5,992
O/anti-B	8806	14279	5,473
AB Control	8705	15026	6,321

* Precoating with nonfluoresceinized anti-IgM for 60 minutes and fluoresceinized anti-IgM for 60 minutes. First antibody incubation was for 2 hours at 37°C. Second antibody incubation was 90 minutes.

** Steps were the same as Study No. 1 but the precoating with fluoresceinized anti-IgM was eliminated.

NOTE: Test is negative when AB Control count is highest in grouping.

in photon counts between A/anti-A and A/anti-B are truly due to the antigen antibody reactivity and not to non-specific fluorescence resulting from reactivity of globulins present in the bloodstain with the second antibody which was fluoresceinized anti-human immunoglobulins.

7.4.11 Low Angle, Solid Phase, Direct Fluorescence Tagging Method With LABS-1 Using Sonicated Bloodstain Particulates Impinged on Membrane Filters:

The procedure for sonication and impingement of bloodstain particulates on membrane filters is presented in Section 8.9. Various modifications of the original procedure were attempted as can be seen in Set #1-4 in Table 7.4.23. The results expressed as % correct, for Set #1, 2, 3 and 4 were 100, 100, 100 and 50% respectively. Collectively, the results were 88% correct and suggest that it is quite feasible to apply this method for identifying blood groups A and B in bloodstain particulates.

The effect of sonication time was also studied by varying the time of disrupting the bloodstain particulates from 2-10 minutes. The results obtained indicated that within the 10 minute time frame there were no measurable differences.

The results of the Rh system as studied by the membrane filter-impingement system are presented in Table 7.4.24. Eighty-four percent (84%) of the C, D and e bloodstains when tested against an experimental AB serum/albumin control were correct, indicating again that this approach was feasible for bloodstain analysis.

TABLE 7.4.23

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD: LABS-1 USING

SONICATED BLOODSTAIN PARTICULATES IMPINGED

ON MEMBRANE FILTERS

(AB System)

Photon Counts							
Set No. 1*		Set No. 2**		Set No. 3†		Set No. 4††	
A/anti-A	10,641	A/anti-A	8294	A/anti-A	5994	A/anti-A	6071
A/anti-B	8,872	A/anti-B	7251	A/anti-B	5919	A/anti-B	6211
		A/AB control	8945				
B/anti-A	9,050	B/anti-A	7729	A/anti-A	6169	A/anti-A	6616
B/anti-B	9,208	B/anti-B	8727	A/anti-B	5910	A/anti-B	6755
		B/AB control	7683				
				B/anti-A	5717	B/anti-A	6356
				B/anti-B	6226	B/anti-B	7226
				B/anti-A	5897	B/anti-A	6127
				B/anti-B	6505	B/anti-B	6956

* Set No. 1 was incubated for 2 hours at room temperature with first antibody which was obtained fresh from donors in our laboratory. Second antibody incubation was for 60 minutes at room temperature using fluoresceinized goat anti-IgM.

** Set No. 2 was incubated for 2 hours at 37°C with first antibody which was obtained fresh from donors in our laboratory. Second antibody incubation was for 90 minutes at room temperature using fluoresceinized goat anti-IgM.

† Set No. 3 was incubated for 2 hours at 37°C with first antibody using commercial reagents (Dade). Second antibody incubation was for 90 minutes using fluoresceinized goat anti-IgM, IgG and IgA diluted (1:30).

†† Set No. 4 was treated in a similar fashion to Set No. 3. The results represent the average of three bloodstain determinations.

TABLE 7.4.24

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE

TAGGING METHOD: WITH LABS-1 USING SONICATED

BLOODSTAIN PARTICULATES IMPINGED ON

MEMBRANE FILTERS

(Rh System)

Photon Counts					
Set #1 *		Set #2 **		Set #3 **	
C^+/anti-C	6277	C^+/anti-C	25404	C^+/anti-C	19293
D^+/anti-D	6097	C^+/AB Control	20053	C^+/AB Control	12155
e^+/anti-e	6207				
AB/albumin control	5177				
C^-/anti-C	6398 (X)	D^+/anti-D	26196	D^+/anti-D	14404
D^-/anti-D	4570	D^+/AB Control	24598	D^+/AB Control	10360
e^+/anti-e	6139				
AB/albumin control	5512				
		e^+/anti-e	22930	e^+/anti-e	12977
		e^+/AB Control	20313	e^+/AB Control	11234
		C^-/anti-C	21490	C^-/anti-C	13923
		C^-/AB Control	22504	C^-/AB Control	9282 (X)
		D^-/anti-D	30421 (X)	D^-/anti-D	10267
		D^-/AB Control	20930	D^-/AB Control	12477
		e^+/anti-e	25911	e^+/anti-e	14274
		e^+/AB Control	23068	e^+/AB Control	11137

* Set #1 was incubated for 4 hours at 37°C and overnight at 4°C with first antibody. Second antibody incubation was for 2 hours at room temperature using fluoresceinized goat anti-IgG diluted (1:10).

** Set #2 and Set #3 were treated in a similar fashion to Set #1.

AB/albumin control is a serum obtained from an AB individual and diluted 1:1 with 22% albumin.

7.4.12 Low Angle, Solid Phase, Direct Fluorescence Tagging Method Using LABS-1 - Effect of Light Intensity:

The effect of excitation light intensity was also investigated. While there appears to be a greater number of photon counts with the use of high intensity light (See Table 7.4.25), both low and high intensity light were equally predictive of the bloodstain type.

7.4.13 Statistical Significance of Fluorescence Measurements

7.4.13.1 Aminco-Bowman Spectrophotofluorometer:

Statistical data were not collected on the studies involving the Aminco-Bowman Spectrophotofluorometer. This instrument was used only in the initial stages of the study in order to collect sufficient information to design a new fluorescence detection apparatus specifically for bloodstain analysis. The fluorescence measurements made were assumed to be valid, based on the number of experiments performed and the trend of the results. The trend indicated that it was possible to consistently differentiate type A and B bloodstains. No effort was made to distinguish types AB and O bloodstains.

7.4.13.2 LABS-1 Fluorescence System:

In order to determine the precision of individual measurements and the validity of observed differences between samples, the following experiment was performed: Dried, aged bloodstains (type

TABLE 7.4.25

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING METHOD USING LABS-1: EFFECT OF LIGHT INTENSITY

(AB System)

Test System	Intensity	
	Low	High
	(Set No. 1)	
A/anti-A	8451	363,347
A/anti-B	6735	187,578
A/AB Control	7226	234,184
B/anti-A	6731	193,640
B/anti-B	6800	202,056
B/AB Control	6309	188,872
	(Set No. 2)	
A/anti-A	7243	371,341
A/anti-B	6446	310,887
A/AB Control	6425	259078
B/anti-A	6626	242,712
B/anti-B	6769	263,860
B/AB Control	6689	360,892

* Samples were incubated for 2 hours at 37°C and 1 hour at 4°C with first antibody. Second antibody incubation was for 90 minutes using fluoresceinized goat anti-IgM.

A and B) were fixed on cloth and reacted with either anti-A or anti-B, in duplicate. All the specimens were then reacted with fluoresceinized goat anti-human IgM as described in Section 8.6, with one modification; incubations with albumin and subsequent washes were omitted. The entire experiment was repeated the next day.

Five successive 10 second photon counts were recorded for each sample. The precision of the fluorescent measurement, expressed as the standard deviation, varied from ± 1.41% to ± 3.72%, with an average variation of ± 2.48% (Table 8.3.2). The validity of the observed differences between matched samples (A/anti-A vs A/anti-B, and B/anti-A vs B/anti-B) was tested by applying Student's t-test. In every case, the differences between the positive and corresponding negative were highly significant ($p < 0.001$).

7.5 FEASIBILITY DEMONSTRATION

7.5.1 Summary of Milestones Completed Prior To Feasibility Demonstration

Initially, research emphasis and work effort was concentrated on exploring the methodology, optimizing reaction conditions and defining the problems relevant to the primary approach identified as the "Low Angle, Solid Phase, Direct Fluorescence Tagging Method", as well as the alternative approaches identified as the "Direct Fluorescence Elution Method" and the "Solid Phase Indirect Immunoassay Method". The primary objective of all three approaches was to eliminate the need for fresh erythrocytes as a detecting system, and to eliminate subjectivity in determining positive and negative serological reactions. Problems investigated included the effects of background fluorescence and scatter, the quenching of both specific and undesired fluorescence by blood and blood proteins and the effect of laundry detergents, bleaches and whiteners on antibody absorption to the bloodstain surfaces. It quickly became apparent, however, that to meet the Feasibility Demonstration deadline, a time limitation of four months, all effort would have to be concentrated on one approach rather than three.

An analysis was made of the problems that were unique to each approach and an estimate of the time that would be required to resolve them. From the data available, data resulting from our own studies as well as that in the scientific literature, it was decided that the primary method proposed had the greatest potential for meeting

feasibility requirements within the designated time frame.

The decision to discontinue research effort on the alternative methods and to concentrate all work effort on the primary "Low Angle, Solid Phase, Direct Fluorescence Tagging Approach" was made on March 1, 1975. Therefore, during the two months prior to the Feasibility Demonstration work effort was concentrated on the following:

a) Identifying and eliminating serological variables pertinent to bloodstain preparation for fluorescence analysis of bloodstain surfaces.

b) Comparison of fixed angle and angle optimization for maximizing desired specific fluorescence.

c) Designing better bloodstian sample holders for the Amin-co-Bowman spectrophotofluorometer to be used in the period interim to the construction of an ORTEC fluorometer more closely approximating the requirements for analysis of fluorescent surfaces.

d) Designing and constructing instrumentation (ORTEC) with a configuration of excitation and emission monochromotors more ideally suited for the Low Angle, Solid Phase, Direct Fluorescence Tagging Method; and

e) Designing a sample holder for the ORTEC instrument that would present identical bloodstain surface areas to the excitation and emission monochromotors.

Bloodstain sample preparations included three forms for fluorescence analysis:

a) pieces of fabric, 4 x 10 mm in size

b) single threads or fibers, and

c) membrane filters with bloodstain particulates removed from fabric by simple ultrasonic vibration and impinged upon the filter surface for analysis.

The first ORTEC fluorometer was hastily constructed from available off-the-shelf items in time to meet the Feasibility Demonstration. It was designed to meet the requirements of the Low Angle, Solid Phase, Direct Fluorescence Tagging Method within the cost constraints stipulated in the Statement of Work. The angle of the excitation beam to sample was empirically determined, and represented the position where maximal fluorescence was achieved. This angle was found to be 28^{o}. A quartz halogen lamp was used as a light source. Intensity was variable form 0-150 Watts with excitation set at 470 nm using a 3 cavity interference filter having a band width of 7 nm. Emission was set at 520 nm using a 3 cavity interference filter having a band width of 8.2 nm and a IP21 photomultiplier. This simple instrument was connected to a photon counting electronics system having a digital readout.

Thus, differentiation of a positive and negative reaction was a matter of a difference in the number of fluorescence units, the fluorescence signal from a positive sample being higher than from a corresponding negative control sample. This instrument was made available to the Immunoserologists at the Mason Research Institute for testing on 19 March 1975, twelve days prior to the date of the Feasibility Demonstration. Laboratory personnel were trained in its use

and serological procedures were firmed-up. The procedure used during the Feasibility Demonstration are given both in detail and in summary outline in Sections 8.6 and 8.7.

Although Section 8.6 contains procedures for typing group AB bloodstains, research effort during the four months prior to the Feasibility Demonstration had included relatively few AB or O blood type specimens. It had been understood by MRI that the five blood type specimens selected for Feasibility Demonstration were A and B of the ABO system, and C, D and e of the Rh system. Preliminary probes using AB and O stains had indicated that these two blood groups had unique problems for identification that hinged on the development of adequate control sera. Serologically, the presence of antigens A and B indicate a type AB blood group, and the absence of antigens A and B indicate a type O blood group. However, the fluorometric readings were not as easily interpretated because of the different inherent problems of the fluorescent-antibody technique. One major problem might be the non-specific binding of fluorescent serum globulins onto other bloodstain components.

7.5.2 Test Results

For the Feasibility Demonstration MRI was presented with ten, two-week old bloodstains that included blood groups A, B, AB, O, C, D and e. Samples were numbered and the code was revealed on 2 April 1975, on the second day of the Feasibility Demonstration, after MRI's results had been tabulated and discussed. The results of the Feasibility Demonstration are summarized in Table 7.5.1, actual blood

TABLE 7.5.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
A	11-3	anti-A	13,468	A
		anti-B	9,877	
		anti-AB	12,936	
		Control	8,708	
A	11-6	anti-A	24,341	A
		anti-B	16,609	
		anti-AB	22,492	
		Control	12,664	
B	11-5	anti-A	8,666	B
		anti-B	11,033	
		anti-AB	10,036	
		Control	7,343	
B	11-10	anti-A	11,994	B
		anti-B	15,159	
		anti-AB	14,617	
		Control	10,798	
AB	11-4	anti-A	12,235	AB
		anti-B	13,503	
		anti-AB	15,067	
		Control	11,144	

* Coded bloodstains provided by The Aerospace Corporation.

TABLE 7.5.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
O	11-1	anti-A	12,788	not A
		anti-B	12,466	not B
		anti-AB	14,380	(AB)✓
		Control	12,064	
O	11-1	anti-A	9,085	A ✓
		anti-B	8,020	
		anti-AB	8,175	
		Control	7,020	
O	11-7	anti-A	31,141	not A
		anti-B	27,196	not B
		anti-AB	32,744	
		Control	30,643	
O	11-8	anti-A	19,832	A ✓
		anti-B	16,702	
		anti-AB	19,484	
		Control	16,372	
O	11-9	anti-A	11,940	not A
		anti-B	11,684	not B
		anti-AB	12,357	(O)
		Control	11,197	

* Coded bloodstains provided by The Aerospace Corporation.

( ) Parentheses indicate possible blood type.

✓ Indicates an error.

TABLE 7.5.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
C^+	11-2	anti-C	4,603	C^+
		Control	4,233	
C^+	11-4	anti-C	5,569	C^+
		Control	5,255	
C^+	11-5	anti-C	5,147	C^+
		Control	4,686	
C^+	11-6	anti-C	13,673	C^- ✓
		Control	13,808	
C^+	11-7	anti-C	23,654	C^+
		Control	22,294	
C^+	11-9	anti-C	16,982	C^+
		Control	14,343	
C^+	11-10	anti-C	11,473	C^+
		Control	11,008	
C^-	11-1	anti-C	9,828	C^-
		Control	10,481	
C^-	11-3	anti-C	4,930	C^+ ✓
		Control	4,600	
C^-	11-8	anti-C	19,622	C^+ ✓
		Control	17,758	

* Coded bloodstains provided by The Aerospace Corporation.

✓ Indicates an error.

TABLE 7.5.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
D^+	11-2	anti-D	5,041	D^+
		Control	4,773	
D^+	11-3	anti-D	4,872	±
		Control	4,844	
D^+	11-4	anti-D	5,474	D^+
		Control	3,743	
D^+	11-5	anti-D	3,964	D^- ✓
		Control	4,702	
D^+	11-6	anti-D	11,716	D^- ✓
		Control	12,904	
D^+	11-7	anti-D	21,848	D^+
		Control	21,158	
D^+	11-9	anti-D	13,311	±
		Control	13,150	
D^+	11-10	anti-D	15,417	±
		Control	15,139	
D^-	11-1	anti-D	10,627	D^+ ✓
		Control	10,355	
D^-	11-8	anti-D	17,222	D^+ ✓
		Control	16,387	

* Coded bloodstains provided by The Aerospace Corporation.

± Indicates a trace reaction, test should be repeated.

✓ Indicates an error.

TABLE 7.5.1

RESULTS OF FEASIBILITY DEMONSTRATION BY BLOOD GROUP

(Continued)

Bloodstain Blood Group*	Aerospace Code No.	Blood Typing Reagent	Fluorometer Counts/30 sec.	MRI Interpretation
e^+	11-1	anti-e	8,268	e^+
		Control	7,306	
e^+	11-2	anti-e	4,984	e^- ✓
		Control	5,000	
e^+	11-4	anti-e	5,206	e^+
		Control	4,475	
e^+	11-5	anti-e	5,559	e^+
		Control	5,323	
e^+	11-6	anti-e	15,324	e^+
		Control	12,611	
e^+	11-7	anti-e	19,931	e^- ✓
		Control	22,974	
e^+	11-8	anti-e	18,122	e^+
		Control	13,544	
e^+	11-9	anti-e	13,039	±
		Control	12,941	
e^+	11-10	anti-e	21,967	e^+
		Control	13,822	
e^-	11-3	anti-e	5,486	e^-
		Control	5,637	

* Coded bloodstains provided by The Aerospace Corporation.

± Indicates a trace reaction, test should be repeated.

✓ Indicates an error.

TABLE 7.5.1

INTERPRETATION OF FLUOROMETER COUNTS

(Continued)

Blood Group	Rationale
A	Positive when anti-A count is greater than anti-B, and control count is equal to or less than anti-B.
B	Positive when anti-B count is greater than anti-A, and control count is equal to or less than anti-A.
AB	Positive when anti-AB count is greater than either anti-A or anti-B, and control count is less than either anti-A or anti-B.
C	Positive when control count is less than anti-C.
D	Positive when control count is less than anti-D.
e	Positive when control count is less than anti-e.

types (determined by The Aerospace Corporation and verified by its consultants) and MRI's test results are compared. The interpretation of the fluorescence values as positive or negative results was made solely by the Mason Research Institute.

The assessment of the test results are:

The Aerospace Corporation - Correct identification for A and B antigens was made in 70% of the specimens, 10% were incorrect and 20% were questionable (blood groups O and AB are included in these percentages). For the Rh system, the values were 57%, 30% and 13% for correct, incorrect and questionable identifications, respectively.

The Mason Research Institute - Correct identification of A and B bloodstains was made in 100% of the specimens, AB was a questionable positive and of blood group O two were questionable and 3 out of 5 were incorrect. For the Rh system 70% of group C, 60% of group D and 80% of group e were correctly identified. MRI considers a ± (trace) reaction as a positive test that ideally should have confirmation testing. It is a standard procedure both in clinical and forensic serology to consider ± (trace) reactions as weak positives and to retest for confirmation. Due to the length of time alloted for the Feasibility Demonstration, confirmation testing was not possible.

7.6 REAGENT PURIFICATION (12 AUGUST - 31 OCTOBER 1975)

This study was a follow-on contract to establish the feasibility of the concept to identify the red cell antigens in bloodstains. The work effort and its duration were detailed in a Study Plan modification revised and submitted to The Aerospace Corporation on 30 July 1975. Incorporated in the Study Plan were those changes to the methodology requested by The Aerospace Corporation via telephone conferences and those changes resulting from conferences with the Mason Research Institute's consultants and sub-contractors.

It was explicitly stated that: "The guidelines and limitations set forth by The Aerospace Corporation will be strictly adhered to. Further, it is understood that the purification of the fluoresceinized antisera shall be performed only after the detailed procedures are critically reviewed and approved . . ."

7.6.1 Initial Screening of Antiserum

The criteria for selection of suitable plasma to be used as sources of anti-A and anti-B were established by The Aerospace Corporation in the "Amendments to the Statement of Work for a Bloodstain Analysis System". A "bleed" consisted of 500-700 ml of plasma from a single donor of known blood group phenotype. The minimum acceptable criteria were:

Bleed checked for anti-Rh and anti-MN activity. If such activity is present, the antiserum was considered unsuitable and another bleed obtained. Anti-Rh activity included any of the following antibodies: anti-C, anti-D, anti-E, anti-c, anti-e.

Bleed checked for the cold agglutinins, anti-H, anti-I, anti-Lewis and anti-P. If present, they were considered unsuitable and another bleed obtained.

Bleed checked for specific blood group substance antibody (anti-A or anti-B) by hemagglutination titration, a minimum titer of 256 was acceptable.

In accordance with the protocol, MRI's immunochemist and senior serological technician visited the laboratory of Dr. A. Konugres of the Boston Lying-In Hospital. In her role as consultant, Dr. Konugres performed the initial screening tests on aliquots of antisera, so that we could verify techniques and endpoint definitions. Based on the data chosen as initial starting materials for reagent purification. Additional bleeds were purchased as required. Table 7.6.1 summarizes the data. The anti-B bleeds were accepted with a titer of 128 on Dr. Konugres' advice, as anti-B titers were often lower.

7.6.2 Cold Agglutination Absorption

All "bleeds" were to be absorbed with O^+P^+ red blood cells, regardless of whether such agglutinins were detectable or not. A volume of packed, washed red blood cells equal to the volume of plasma being absorbed was satisfactory. Cells for absorption of cold agglutinins (O^+P^+) were obtained from the American Red Cross blood bank. They were prepared for absorption by washing with cold isotonic phosphate-buffered saline (PBS) pH 7.2 four times. Sedimentation of the erythrocytes was accomplished by centrifugation in an IEC PR-2 centrifuge for 30 minutes at 1500 rpm.

TABLE 7.6.1

DETERMINATION OF ANTIBODY TITER

Sample	Antibody	Titer
S-397	Anti-A	512
* S-398	Anti-A	256
S-399	Anti-A	512
AL-377-14759	Anti-A	512
23-50600	Anti-A	512
23-46887	Anti-A	>512
S-400	Anti-B	128
* S-401	Anti-B	64
* S-402	Anti-B	64
BL-116-13112	Anti-B	256
* 23-72165	Anti-B	32
40-49117	Anti-B	512

* Rejected

Hemagglutination titer , Table 8.2.1

One volume of washed O^+P^+ RBC's was added to the bleed, incubated for 30 minutes at 4° C, then removed by centrifugation at 2500 rpm for 30 minutes. A fresh aliquot of O^+P^+ cells was then added to the bleed and incubated for 2 hours at 4° C, followed by removal by centrifugation. Blood group antibody determination following absorption with O^+P^+ washed red cells indicated a decrease of antibody titer (Table 7.6.2).

Antibody loss at this point was unexpected, and the absorption procedure was changed as follows:

1. Centrifugation speed used for O^+P^+ red cell washing was increased to 2500 rpm, in an effort to avoid a dilution effect.

2. Antiserum absorption changed to one absorption at 37° C for 30 minutes then at 4° C for 2 hours instead ot two aliquots at 4° C. This change was implemented on the advice of Dr. Konugres.

The absorption experiment was repeated incorporating these changes as well as varying the proportion of O^+P^+ cells. Under these conditions, there was no loss of antibody titer upon absorption with O^+P^+ red cells at a red cell/bleed ratio of 1:1 (Table 8.2.3).

7.6.3 Fluorescein Conjugation

An aliquot of anti-A and anti-B that had been absorbed with O^+P^+ red cells was diluted with PBS, pH 7.4 to a final protein concentration of 10 mg/ml. Fluorescein isothiocyanate (FITC) was dissolved in PBS to a concentration of 1 mg/ml. The FITC solution was added to the diluted plasma dropwise with continuous stirring in the cold (4° C). A quantity of FITC was added that equalled 2%

TABLE 7.6.2

DETERMINATION OF ANTIBODY TITER AFTER

ABSORPTION FOR COLD AGGLUTININS

Sample	Antibody	Procedure	Titer
S-397	Anti-A	unabsorbed	384
		absorbed O^+P^+	96
S-399	Anti-A	unabsorbed	384
		absorbed O^+P^+	96
S-400	Anti-B	unabsorbed	96
		absorbed O^+P^+	96

Titration data - see Table 8.2.2

(by weight) of the protein in the sample. The mixture was incubated in an ice bath for 6 hours, then dialyzed against PBS for 48 hours. Antibody activity was determined by hemagglutination titration. The fluoresceinization procedure resulted in almost complete loss of anti-A and anti-B activity (Table 8.2.4).

A variety of modifications of the conjugation procedure was tried in an attempt to label the absorbed bleed and retain antibody activity. The modifications were as follows:

1. Protein concentration 10 mg/ml, pH 9.0, FITC (1 mg/ml), 2% final concentration.

2. Protein concentration 20 mg/ml, pH 9.0, FITC (crystalline), 2% final concentration.

3. Protein concentration 30 mg/ml, pH 9.0, FITC (crystalline), 2% final concentration.

4. Protein concentration 60 mg/ml, pH 9.0, FITC (crystalline), 2% final concentration.

After a search of the literature, pH 9.0 was chosen (38). Prior to conjugation, pH was adjusted to 9.0 ± 0.1 either by the addition of 15% sodium carbonate or by dialysis against 0.1 M carbonate/bicarbonate buffer. FITC was added as a crystalline solid to the final concentration noted. The reaction proceeded at 4° C for 6 hours. After conjugation, samples were dialyzed against PBS, pH 7.4 in order to remove unconjugated fluorescein. The procedure for determining the presence of unconjugated fluorescein was as follows:

An Amicon Centriflow CF-50 was prepared as per the manufacturers directions, and centrifuged at 1000 rpm for 10 minutes to

remove excess moisture. Approximately 7 ml of the material to be tested was added to the cone and centrifuged until a sufficient volume of filtrate (2-3 ml) has been obtained. The fluorescein-bound protein remains in the Centriflow cone, and free fluorescein passes through the membrane. Dialysis was continued until the Centriflow filtrate was fluorescein-free. In the above experiments, 48-72 hours were sufficient.

Titration of the FITC-conjugated bleed for anti-A activity demonstrated that the biological antibody activity could best be retained when the final protein concentration was high and the reaction carried out a pH 9.0 (modification 4, Table 7.6.3).

7.6.4 Absorption-Elution Procedures

The initial procedure for the purification of fluorescein conjugated antiserum reflects the guidelines established by The Aerospace Corporation and its consultant, Dr. P. Sturgeon, in the "Amendments to the Statement of Work for a Bloodstain Analysis System" and in conference with Dr. A. Konugres.

Fresh erythrocytes (group A_2 and B) were washed four times in cold PBS, pH 7.4 and then packed tightly by centrifugation. The optimum ratio of packed erythrocytes:antiserum for antibody absorption must be determined for each bleed. Initially, one ratio was tested, 1:2. The reactants (FITC-anti-A and A_2 cells, FITC-anti-B and B cells) were mixed every 5 minutes by gentle agitation for 20 minutes at 4° C. The cells were then separated by low speed centrifugation

TABLE 7.6.3

FITC-CONJUGATION OF ANTI-A BLEED

Sample	Procedure	Protein Conc. (mg/ml)	FITC*	Titer
Anti-A	Absorbed O^+P^+	-		512
Anti-A	FITC-Modif 1	10	A	64
Anti-A	FITC-Modif 2	20	S	128
Anti-A	FITC-Modif 3	30	S	256
Anti-A	FITC-Modif 4	60	S	256

* FITC additions; A refers to aqueous (1 mg/ml), S refers to solid

All fluoresceinizations done at pH 9.0, reaction time of 6 hours at $4^{\circ}C$.

For titration data, see Table 8.2.5

for 5 minutes. After the red cells were thoroughly washed, they were added to a volume of PBS equivalent to 1/2 the original absorbed FITC plasma volume for elution. The PBS was preheated to 56° C and the suspension was then immediately centrifuged while maintaining a constant temperature of 56° C. The supernatant PBS containing the eluted fluorescent antibody was removed as quickly as possible from the pelleted red blood cells, and stored at 4° C. The antibody activity of the resulting eluates were very low. The eluates were then concentrated ten-fold in an ultrafiltration apparatus (Minicon B-15, Amicon Corp., Lexington, Mass.). The experiment was repeated, with similar result, on a different anti-A preparation (Table 7.6.4). The results of these experiments indicated that the available antibody binding sites on the A_2 and B cells were not saturated. Red cells to be used for elution must be charged with a plasma that contains an excess of antibody. This was clearly not the case, as the plasma conjugate lost all antibody activity after absorption. In order to increase the titer of the eluate, the volume of PBS used for elution must be decreased. To increase the stability of the eluate, elution into PBS containing human albumin (1-5%) was examined. The absorption-elution procedures were, therefore, modified as follows:

1. A suitable conjugate:erythrocyte ratio was to be determined. After absorption and prior to elution, the supernatant conjugate was titered for antibody content. If the hemagglutination assay indicated a low titer, the antibody-containing RBC's were mixed with a fresh aliquot of FITC-plasma. This process continued until the supernatant after absorption had a high antibody titer, within

TABLE 7.6.4

ABSORPTION - ELUTION

Procedure	Titer		
	Anti-A S-397	Anti-B S-400	Anti-A S- 399
absorbed O^+P^+	96	96	96
FITC-conjugated	12	6	48
after A_2 or B absorption	0	0	0
eluate	6	0	12
eluate, conc. 10x	6	0	6
eluate, conc. 25x	-	6	-

Data reference, Table 8.2.6

two dilutions of the initial titer, indicating the presence of excess antibody, implying that the red cells were saturated.

2. Elution was carried out with a volume of PBS (or PBS-albumin) equal to the volume of the packed red blood cells to be eluted.

The absorption-elution procedure was repeated incorporating the above changes with FITC-anti-A S-399. After the antibody was absorbed from four aliquots of FITC-anti-A, the cells were still not saturated, as indicated by the titer of the supernatant. This exhausted the entire supply of FITC-anti-A S-399. The absorbed cells were heat-eluted three times, with 1 ml of PBS for each eluate. The three eluates were maintained separately. Under these conditions, eluates with satisfactory antibody titer were produced. This data is presented in Table 7.6.5. These eluates were fluorescent, as determined by reading 20 µl applied to an Oxoid filter in the LABS-1 apparatus. The fluorescence was qualitatively proportional to the antibody titer, e.g. the higher the hemagglutination titer, the greater the observed fluorescence. The major technical problem at this point was hemolysis. All three eluates contained free hemoglobin, as determined by visual observation. It was felt by the consultants that elution into PBS containing serum albumin might reduce this problem.

7.6.5 Determination of Presence of A and B Substance in Human Serum Albumin

Human serum albumin was purchased from Sigma Chemical Co. (A-9511) as a crystallized and lyophilized preparation. Human serum

TABLE 7.6.5

ABSORPTION - ELUTION

Sample	Procedure	Titer	Fluorescence* photons/10 seconds
Anti-A S-399	FITC-conjugated	64	
aliquot 1	after 1st absorption	8	
aliquot 2	after 2nd absorption	16	
aliquot 3	after 3rd absorption	16	
aliquot 4	after 4th absorption	8	
eluate 1 **		128	274487
eluate 2		256	310369
eluate 3		64	243357

* Fluorescence data reference, Table 8.2.8

Data reference, Table 8.2.7

** All eluates contained hemoglobin

LABS-1 configuration - first modification

albumin was dissolved in PBS to a final concentration of 10 mg/ml (1%). If A or B substance were present in the albumin preparation, incubating the albumin solution with the appropriate antibody preparation would result in a decrease in titer. An anti-A and an anti-B bleed were diluted 1:3 with PBS. To each diluted antiserum was added 1 volume of 1% serum albumin. After 20 minutes incubation at 25° C, each sample was titrated. The data indicated that the 1% and 5% albumin preparations did not contain detectable quantities of A and B substances (Table 8.2.9) and could be used for the production of eluates.

7.6.6 Effect of Temperature on Heat-Elution

Since anti-B bleeds of acceptable titer were difficult to get in sufficient quantity, these studies were carried out with anti-A only. A new anti-A plasma (AL 377-14759) was absorbed, fluoresceinized and dialyzed according to developed methods. Absorption with fresh A_2 red blood cells was accomplished at room temperature at 1:10 ratio. After washing, the cells were divided into 2 aliquots. Three eluates were produced from each aliquot of antibody-saturated A_2 red blood cells, using 1% human serum albumin in PBS. One aliquot was eluted at 56° C, the other at 45° C. The antibody titers were essentially identical between the 45° -aliquot and the 56° -aliquot. As a result of this experiment, 45° C was chosen as the elution temperature (Table 8.2.10).

7.6.7 Preparation of Anti-B Eluates

The procedures developed for producing high titer anti-A

eluates were applied to a fresh bleed of anti-B plasma. The initial attempt did not yield an eluate of satisfactory titer. The data indicated that the type B red cells used for absorption (1:10 ratio) were not saturated with antibody prior to elution. Attempts were made to saturate 1 ml of washed type B cells with high-titer fluorescent anti-B plasma. After each absorption, the supernatant plasma was assayed for residual antibody activity. Antibody was detectable in the post-absorption supernatant only after 60 ml of anti-B had been absorbed. Three 1 ml eluates were produced at 45° C, in 1% human serum albumin, their titers were 128, 64 and 64, respectively. Alteration of the elution temperature to 56° C produced eluates of lower titer and increased hemolysis. The data is presented on Table 8.2.11. Examination of the type B red cells after elution at 45° C with a fluorescent microscope clearly demonstrated fluorescent red cells, indicating incomplete elution.

It was apparent that anti-B had a greater affinity for B-cells than anti-A has for A_2 cells. By comparison, A_2 is a weaker antigen than B, and it has been demonstrated that, on the whole, the weaker the antigen the more potent the eluate (30). No further experimentation on the production of eluted anti-B was undertaken, due to the time limitations defined by The Aerospace Corporation.

7.6.8 Preparation of Bloodstain Specimens

Since uniform distribution of blood on the cloth substrate was essential for determining the variability of the fluorescence reading values resulting from both scatter and quenching, bloodstain

specimens using A, B, AB and O type blood were prepared on a plain white sheet, 50% cotton, 50% dacron, measuring about 12 x 20 inches. Blood was drawn from donors by venipuncture using a plastic 20 ml syringe with an 18 gauge 1 1/2 inch disposable needle. The cloth was placed on wax paper and an area of the cloth was saturated with blood. The cloth was folded over the stain, and a second piece of waxed paper was placed on the cloth. The blood was spread evenly by hand. This was repeated three times on each cloth. Approximately three cloths were produced per donor. Each cloth was labeled as to donor, date and blood type, and was allowed to air dry.

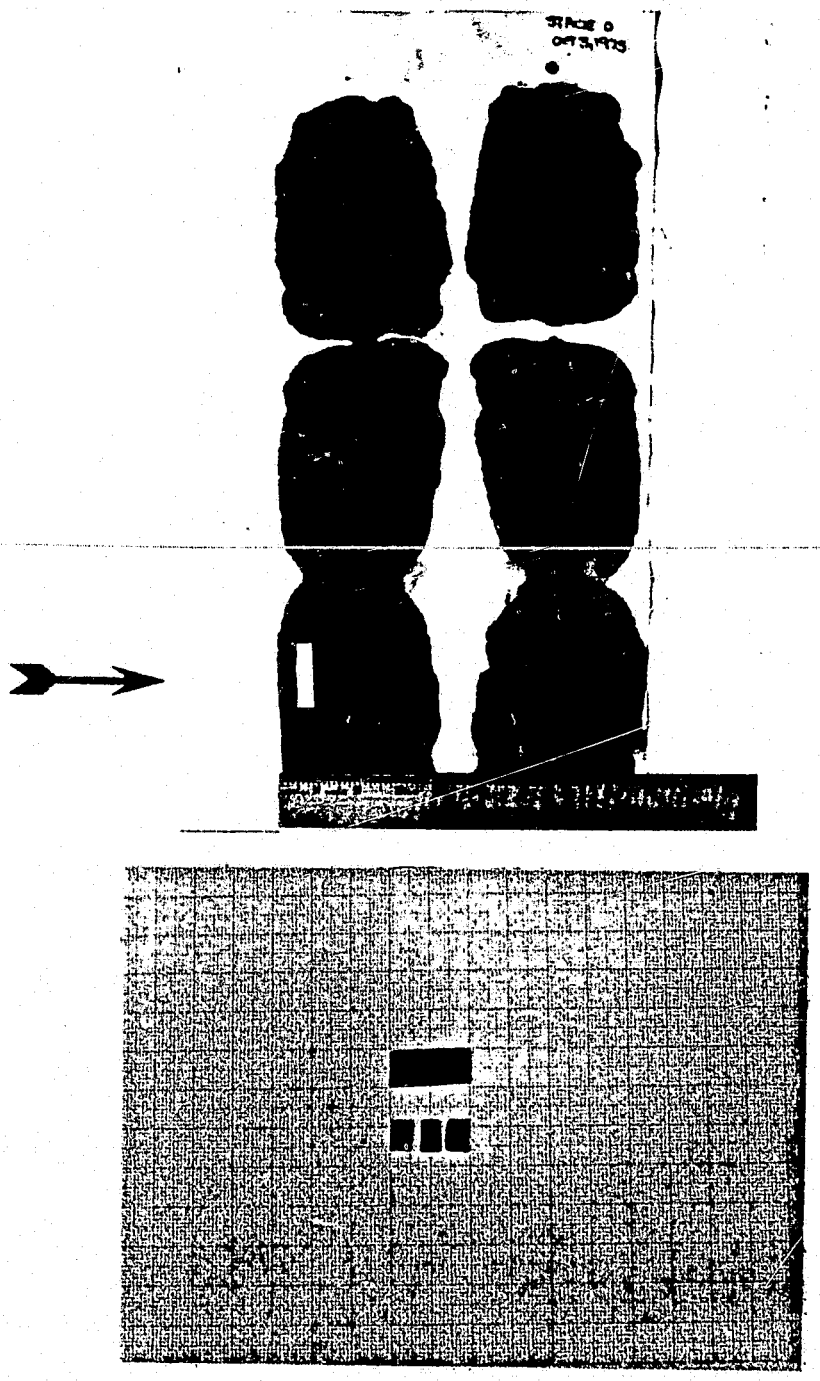
After aging two weeks, the stains were ready to be used for analysis. Zones of color could be seen on the stain. A section of stain approximately 1 x 4 cm was selected that appeared homogeneous and removed. The stain was marked with pencil so that all testing would be performed on adjacent pieces of stain and with identical orientation. Figure 7.6.1 illustrates the stained cloth, and cut pieces used for testing.

7.6.9 Fixation of Stains

Pieces of bloodstain were fixed by incubation of the cut stained cloth in 1 ml of 50:50 ethanol:ether for five minutes at room temperature. The supernatant fixative was removed by aspiration and the stain was then fixed in 1 ml of 95% ethanol for ten minutes. After aspiration, the stain was washed three times with 2 ml of PBS, ten minutes per wash. Fixation and washing was done on a rotary mixer that provided gentle and continuous agitation throughout the procedure.

FIGURE 7.6.1

PREPARATION OF BLOODSTAINS



Top: Aged bloodstains on piece of cloth. Note section of stain removed from lower left quadrant (arrow).

Bottom: Section of stain removed from large cloth and cut into individual segments for experimentation.

Scale, heavy lines = 1 cm.

7.6.10 Bloodstain Analysis with LABS-1

After fixation and washing, the pieces of bloodstained cloth were placed in the LABS-1 fluorometer and a ten-second prereading taken. The orientation of the cloth in the sample holder was noted, and always maintained. The sample was removed and reacted with 0.3 ml of the appropriate fluoresceinized antibody preparation. The samples were covered to minimize evaporation and incubated 45 minutes at room temperature, with mixing. The antibody solution was removed by aspiration, and the cloth was washed four times with 2 ml of PBS per wash for ten minutes. The samples were replaced in the sample holder in the same orientation as the preread, and read again for ten seconds. For comparative purposes, the difference between the preread and the final reading were compared. Bloodstain analysis was attempted with A, B, AB and O stains with FITC-anti-A plasma, FITC-anti-B plasma, as well as with eluates as they became available. These initial attempts were performed with antiserum and/or eluates that has less than optimum titers, and were intended as trials. This data is summarized in Table 7.6.6. These studies were continued as reagents of higher titer were available. More work was done with anti-A eluates than anti-B because of the difficulty of producing the necessary volumes of the latter (see section 7.6.7). Using the procedures outlined, it was obvious that it was not possible to discriminate consistently between types A, B, AB and O bloodstains (Tables 8.2.12-14).

This data also demonstrated:

1. Consistently high fluorescence values with type O

TABLE 7.6.6

PRELIMINARY BLOODSTAIN ANALYSIS

Bloodstain/Antibody	Photons/10 seconds (increase)
A/A (1)	823
B/A	1646
AB/A	1328
O/A	1607
A/B (2)	871
B/B	1776
AB/B	936
O/B	1242
A/A (3)	992
B/A	1200
AB/A	517
O/A	954
A/B (4)	899
B/B	1296
AB/B	639
O/B	1038

(1)	FITC - Anti A S-399	Titer = 64
(2)	FITC - Anti B BL 116-B112	Titer = 128
(3)	FITC - Anti A eluate	Titer = 128
(4)	FITC - Anti B eluate	Titer = 32

LABS-1 configuration - first modification

bloodstains.

2. A methodical decrease in preread values over time. For example, in Table 8.2.14, the preread count decays from 6241 to 5468. The effect appeared independent of the stain or antibody involved and was assumed to be instrumental or electronic in origin. The effect did not appear to influence the calculated differences between the pre and final readings.

3. Potent eluates with high immunological activity could be prepared.

Prior results indicated that serum albumin does have an affinity for intact erythrocytes. In order to test whether albumin could prevent some non-specific protein absorption to a fixed bloodstain, the following was tried: Two sets of duplicate stains were fixed in ethanol/ether in the prescribed manner. One set was incubated for 45 minutes with 5% human serum albumin. The excess albumin was removed with four consecutive ten minute washes with PBS. Both sets were then reacted with FITC-anti-A and FITC-anti-B, washed and read in LABS-1. Table 8.2.15 contains the averaged fluorescence values. Pretreatment with PBS containing albumin did not solve the problem.

The FITC-conjugated antibodies were regualrly reacted with the fixed bloodstain for 45 minutes at room temperature. To insure antibody saturation of the antigenic sites on the stains, two sets of stains were reacted with antibody, as usual. One set was then reinoculated with a fresh aliquot (0.3 ml/sample) of FITC-antibody, and incubated at 4^{o} C for 16 hours, then washed and fluorescence

determined on the LABS-1. The other set was processed immediately. The data is found in Table 8.2.16. Changing the antibody reaction conditions did not significantly alter the results. The system could distinguish type A stains from type B stains only in the absence of type O stains.

In this series, the ethanol/ether fixation and ethanol wash steps were omitted. The stains were washed with PBS, immediately blotted and prereading measurements taken. The moist stains were then reacted with FITC-antibody for 45 minutes at 25° C, washed with PBS and final reading made. The results (see Table 8.2.17) suggest that omission of the fixation reaction is not the correct approach to solve the problem. The noted increase fluorescence readings were, certainly in part, due to the fact that hemoglobin was washed out of the stains, resulting in a white reflective surface.

The problem of non-specificity remained after these experiments, and attention was turned to other aspects of the reagent purification problem, as time was becoming a most important parameter.

7.6.11 Characterization of Eluates

The antibody purification methodology described previously produced eluates from FITC-anti-A and FITC-anti-B plasma having good hemagglutination titers. These eluates were examined visually with ultraviolet light, as a qualitative indicator of fluorescence, by cellulose acetate electrophoresis, to identify types of protein

in the eluates and protein-bound fluorescence, and by Ouchterlony double-diffusion, to determine the presence of specific proteins and, if necessary to estimate quantities.

FITC-anti-A eluates were prepared according to developed procedures. Three 1 ml eluates were produced from a single absorption and examined under ultraviolet light. Visual observation clearly demonstrated that each of the eluates was fluorescent. Cellulose acetate electrophoresis of each eluate yielded no information, except that the protein concentration was too low for this type of analysis. The eluates were pooled and concentrated 25-fold in an Amicon concentrator. After electrophoresis, the cellulose acetate strips were examined under ultraviolet light and any fluorescent areas marked. Hemoglobin, being a colored protein, was visable at this time. The strips were then stained for protein with Ponceau S stain. With every electrophoretic run, the following standards were included: free fluorescein isothiocyanate, human plasma and human albumin. Electrophoresis of the concentrated anti-A eluate indicated; no free fluorescein, a narrow fluorescent band with the same mobility as human albumin, and a quantity of hemoglobin. The electrophoretic analysis was notable, in that it did not show any γ-globulins nor any fluorescence in the γ-globulin region. Ouchterlony double-diffusion in agar did indicate the presence of IgG, IgM and IgA in the concentrated anti-A eluates. It was not possible, however, to determine if the antibody was fluorescent, due to the quenching effect of the hemoglobin in the sample. The presence of fluorescein-conjugated albumin in the eluate rendered it useless as a reagent in the bloodstain analysis system.

No attempt, however, was made to remove the hemoglobin and thus determine if the eluted antibody was fluorescent. Based on mutual agreement, MRI was allowed to develop the appropriate methodology to produce an albumin-free reagent containing tagged antibody exhibiting high titer.

7.6.12 Protein Fractionation of Bleeds

Analysis of eluates from type A_2 red blood cells indicated that the major fluorescent eluted protein was albumin. In order to prevent non-specified binding of proteins to bloodstains, it was essential to remove all tagged non-γ-globulins from the bleeds. Two methods of plasma fractionation were considered, ammonium sulfate fractionation and sodium sulfate fractionation.

7.6.12.1 Ammonium Sulfate Fractionation

A saturated ammonium sulfate solution was prepared by the addition of solid analytical grade ammonium sulfate to distilled water at 4° C with continuous stirring. The solution was considered saturated only if some undissolved salt remained after 72 hours of mixing in the cold. Fractionation was accomplished by the slow addition of a volume of saturated ammonium sulfate to an equal volume of plasma, with constant stirring at 4° C. After the addition of the precipitating agent was completed, stirring was continued for 4 hours in the cold. After centrifugation at 5000 rpm for 45 minutes, the supernatant solution was removed by decantation, and the precipitate dissolved in an amount of PBS such that the final volume was one half

the original plasma volume. The γ-globulins were precipitated a second time by the addition of ammonium sulfate, as noted above. After the centrifugation step, the γ-globulin was dissolved in a minimal amount of carbonate/bicarbonate buffer, pH 9.0 then dialyzed against this buffer for 48 hours, to remove residual ammonium sulfate and to adjust the pH for fluorescein conjugation. Subsequent to dialysis, the volume of γ-globulin solution was adjusted to one half of the original plasma volume. The sample was now ready for biological testing and fluorescein conjugation.

7.6.12.2 Sodium Sulfate Fractionation

In this procedure the γ-globulins were separated from albumin by the addition of solid sodium sulfate to the bleed at room temperature to a final concentration of 1.0 Molar. The supernatant was separated from the precipitated globulins by decantation after centrifugation at room temperature (5000 rpm, 45 minutes). The globulins were dissolved in PBS to one half the original plasma volume. The γ-globulins were reprecipitated with sodium sulfate, and the precipitate was dissolved in a minimal quantity of 0.1 Molar carbonate/bicarbonate buffer, pH 9.0 and dialyzed against this same buffer for 48 hours.

7.6.13 Characterization of Fractionated Bleeds

The γ-globulin fractions of an anti-A bleed were isolated by the ammonium sulfate and the sodium sulfate methods as described. These preparations were labeled with fluorescein isothiocyanate,

and eluates prepared from fresh type A_2 red cells. Systematic analyses were performed: measuring hemagglutination titers, testing for bloodstain discrimination on the LABS-1, and immunochemical and electrophoretic profiles. The data indicated that:

1. Both ammonium sulfate and sodium sulfate fractionation methods yielded γ-globulin preparations with acceptable hemagglutination titers.

2. FITC-conjugation could successfully be accomplished with γ-globulin prepared by either method.

3. Eluates prepared from FITC-anti A-γ-globulins, regardless of methodology, had good hemagglutination titers. This segment of the data is detailed on Tables 8.2.18-8.2.21.

Examination of tagged anti-A γ-globulins and anti-B γ-globulins on the LABS-1 indicated inability to distinguish the four types of bloodstains (A, B, AB, O) on this instrument with these reagents (Table 8.2.22). Non-specific binding with type O stains continued to be the greatest problem.

Cellulose acetate electrophoresis of the γ-globulins prepared by ammonium sulfate fractionation consistenly demonstrated the presence of trace amounts of albumin, α_1-globulin and α_2-globulin. On the other hand, the γ-globulins prepared by sodium sulfate fractionation did not appear to contain albumin. Ouchterlony double-diffusion was positive for albumin in the sodium sulfate precipitated γ-globulins at a dilution of 1:8. This corresponded to approximately 2.0 µg of albumin per mg of γ-globulin. In order to insure complete fluoresceinization of the blood group antibodies

in the γ-globulin fraction, the FITC-conjugation procedure was examined. The conjugation time at 4° C was increased to 16 hours while the quantity of fluorescein isothiocyanate added was varied from 2-6%. The degree of fluorescein conjugation was calculated based on the extinction coefficient of protein-bound fluorescein at 495 nanometers and the protein concentration of γ-globulin mixture. The degree of fluorescein conjugation was calculated and reported as the F/P (fluorescein/protein) ratio as described by Mairn (31). This is a molar ratio, indicating the average number of molecules of fluorescein bound to a molecule of γ-globulin. The conjugates were evaluated by hemagglutination. The data indicated that increasing the F/P ratio did not drastically alter the biological activity of the antibody directed against the blood group substances (Table 8.2.23). The increase, however, did cause technical problems. The excess FITC was removed by dialysis against PBS. At 4% FITC, it was necessary to increase the dialysis time from 3-4 days to 9-10 days in order to remove the last traces of unbound fluorochrome.

Cellulose acetate electrophoresis indicated that the FITC-conjugated anti-A γ-globulin band was fluorescent. No albumin or fluorescence in the albumin region was discernable by cellulose acetate electrophoresis. Ouchterlony double-diffusion indicated the presence of IgM, IgG, IgA, and a trace quantity of albumin, when the conjugate was tested against specific antiserum.

7.6.14 Bloodstain Analysis by Absorption-Elution Method

In order to confirm the serological effectiveness of the prepared bloodstain reagents, classical absorption-elution experiments

on dried stains were performed according to the following procedure: two week old bloodstains on cloth (0.5 x 1 cm) were reacted with 0.3 ml of antisera preparations at 25° C for 90 minutes. After washing four times for 10 minutes each in PBS at 4° C the antibody was heat-eluted from the stains at 45° C for 20 minutes into 0.2 ml of PBS containing 1% human serum albumin.

The absorption-elution methodology was completely successful under the experimental conditions in properly determining all four blood types, A, B, AB and O (Table 8.2.24). Pretreatment of the unfixed bloodstains with 5% human serum albumin prior to reaction with specific antibody gave identical results with little difference in final titer (Table 8.2.25). Fixation with ethanol/ether (50:50) for 10 minutes did not alter the results (Table 8.2.26). Duplicate samples of each bloodstain type were fixed, read in LABS-1, then reacted with FITC-anti-A γ-globulin. After washing, the second reading was taken. The stains were then treated as described above, and the bound antibody heat eluted at 45° C into 1.0 ml of PBS containing 1% serum albumin. The eluates from the bloodstains were then characterized by hemagglutination and fluorescence in the Aminco-Bowman spectrophotofluorometer (Table 8.2.27). LABS-1 analysis did not discriminate successfully between the different bloodstains, however, hemagglutination performed on the eluted antibody clearly distinguished reactive from non-reactive antigens. The total fluorescence of the antibodies eluted from these stains were measured in the Aminco-Bowman spectrophotofluorometer. These measurements also did not correlate with the known blood types. Approximately 100 mg of washed, activated charcoal was added to each of the eluted antibody samples,

mixed continuously for 60 minutes, and centrifuged at 2000 rpm for 5 minutes. The supernatant was reread in the spectrofluorometer. If any of the fluorescein was unbound, it would have been removed by the charcoal, resulting in diminished second readings. The second readings were not diminished, indicating that all fluorescein was bound to the γ-globulins.

7.6.15 Criteria for Success

The criteria for success study was described in section 5.2.2.4 of the approved Study Plan. It was designed to determine:

1. Sample reproducibility.
2. The increase in value for positive over negative tests.
3. The contribution to fluorescence readings due to non-specific absorption of the antiserum.

The test was performed on 10 pieces of each type stain A, B and O using FITC-anti-A γ-globulin as the antibody reagent. The stains were fixed, and readings taken on the LABS-1. This prereading was designated "reading 1". The stains were reacted with the antibody, washed and "reading 2" recorded. Readings were also taken on a dark surface, a white reflectance standard and a fixed type A stain saturated with the fluorescent typing reagent. This final value was used as a fluorescent standard. The data was normalized after each reading according to the general formula:

$$S_N = \frac{M - D}{F - D}$$

Where S_N = the normalized signal, M = the measured reading (subscripts 1 and 2 were used to indicate reading 1 or reading 2),

D = the dark surface signal, and F = the signal from the bloodstain saturated with fluoresceinized anti-A γ-globulin typing reagent. Examination of S_{N1} and S_{N2} for each stain allowed evaluation of the sample reproducibility. The ratio of S_{N2}/S_{N1} was a measurement of the increase in fluorescence, and comparison of these values evaluated the increase for positive over negative tests. The raw data is found in Tables 8.2.28-8.2.30. In order to demonstrate success, it was necessary to have a valid increase in value for the positive control over the negative tests. The comparison of the S_{N2}/S_{N1} ratio for the positive control (A/anti-A) with the two negative tests (B/anti-A, O/anti-A) by Student's t-test indicated no statistically significant increase in value for the positive over either of the negative controls.

7.6.16 Termination of Work Effort

All work on this project terminated on 31 October 1975, even though complete evaluation of the fluoresceinized γ-globulin preparations and their eluates were not complete. The scheduled Feasibility Verification Demonstration was cancelled.

7.7 AMINCO-BOWMAN SPECTROPHOTOFLUOROMETER

Assuming that a technique could be developed to selectively attach fluorescein tagged antibody to specific erythrocyte antigens found in blood stains on solid matrices, analytical studies were undertaken for the direct detection of fluorescence from the solid phase. Initial studies utilized an Aminco-Bowman spectrophotofluorometer with modifications of the sample holder to expose small fabric surfaces to the light path.

From the outset it was clear that those factors which ultimately limit spectrofluorometric specificity and sensitivity when working with samples in relatively clear solutions would be greatly magnified in solid phase fluorometry. Much of our efforts were, therefore, directed towards reducing the effects of these interferring factors which include light scatter, non-specific (background) fluorescence and self-quenching. The instrumental variables which could help to increase selectivity and sensitivity of detection were optimized for reading surface fluorescence. The variables which were altered as sample spectra were recorded were the excitation and/or emission wavelengths, slit widths, angle of incidence of excitation light on sample surface, and installation of an optical filter (Ditric 525 nm cut-on-filter) in front of the photomultiplier tube.

7.7.1 Design of Sample Holders for Aminco-Bowman Spectrophotofluorometer

Three sample holders were designed for use in the Aminco-

Bowman Spectrophotofluorometer. With these holders, pieces of fabric and individual threads could be placed in the spectrophotofluorometer at a known and reproducible relationship to the excitation beam. These sample holders were detailed in Figure 7.7.1. The item marked Part 1 allowed a piece of cloth approximately 14 x 14 mm to be inserted in the spectrophotofluorometer and manually rotated to present the sample at any desired angle. Part 2 was designed for a single thread, and Part 5 for cloth pieces up to 13 x 16 mm. The latter had a fixed angle of 20°, and was designed after experimentation with Part 1.

7.7.2 Solid-Phase Spectrofluorometry

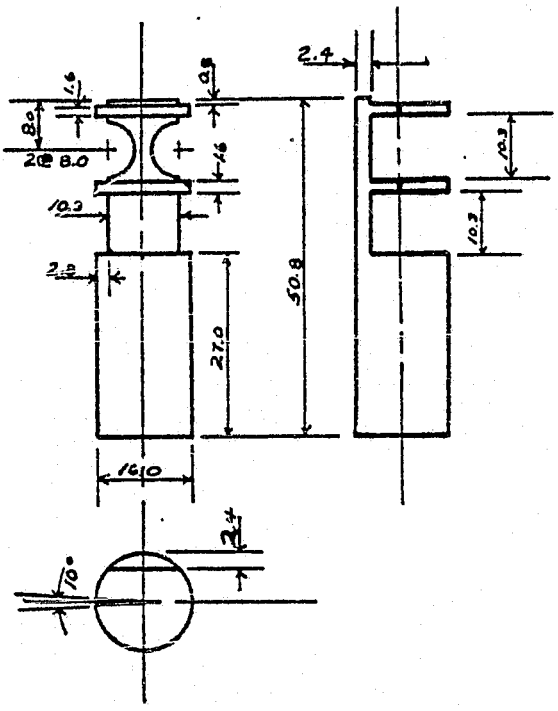
The following spectra of various experimental solid phase samples were recorded with a Moseley Model 2DR-2AM X-Y recorder connected to the Aminco-Bowman Spectrophotofluorometer. These illustrations are typical of the numerous spectrofluorometric readings that were obtained from various sample preparations during studies to establish those instrumental conditions that would give the highest signal to noise ratio. The reliable detection of a minimal signal arising from a fluorescent antibody reacted with a blood stained surface containing a specific erythrocyte antigen was the goal of these studies.

Instrument calibration with quinine sulfate (Figure 7.7.2)

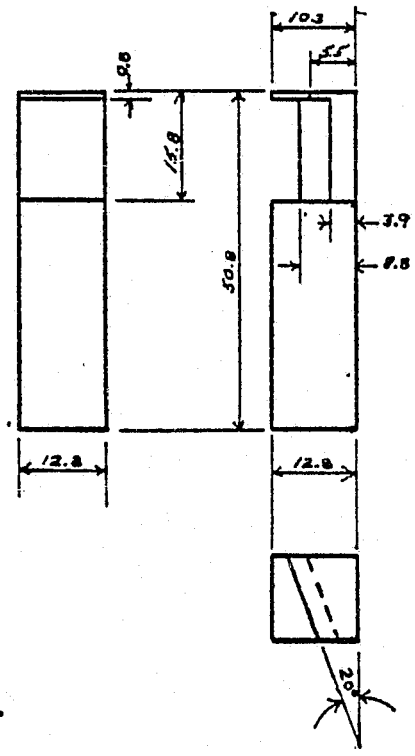
Quinine sulfate spectra was run frequently to check instrument sensitivity and monochrometer allignments. Quinine sulfate has two excitation peaks, at 250 and 350 nm, and one emission maxima.

FIGURE 7.7.1

SAMPLE HOLDERS FOR AMINCO-BOWMAN SPECTROPHOTOFLUOROMETER



Part 2



Part 5

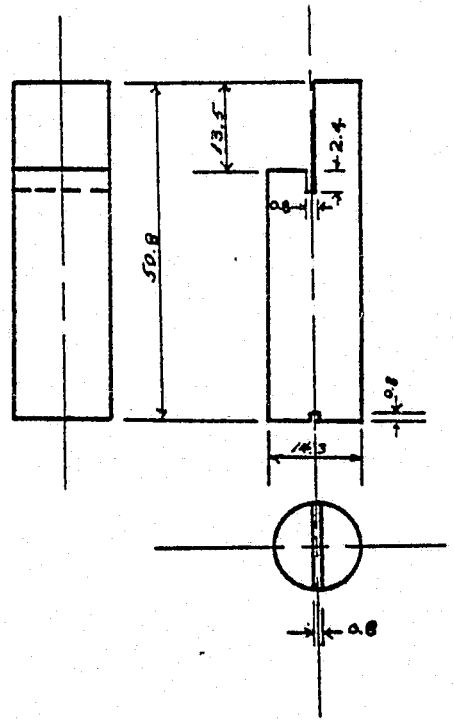
Note:

ALL dimensions in m.m..

Part 1: Holder for cloth - variable angle

Part 2: Holder for thread - variable angle

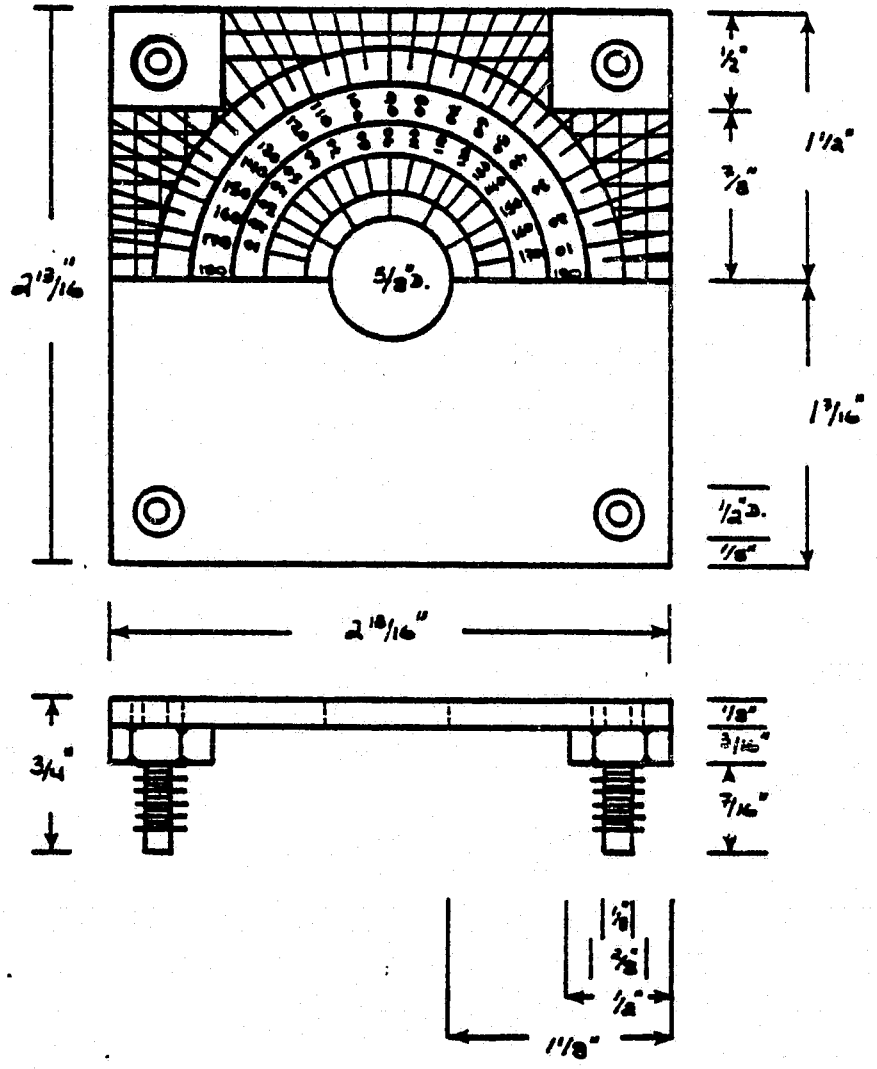
Part 5: Holder for cloth - fixed angle



Part 1

FIGURE 7.7.1 (continued)

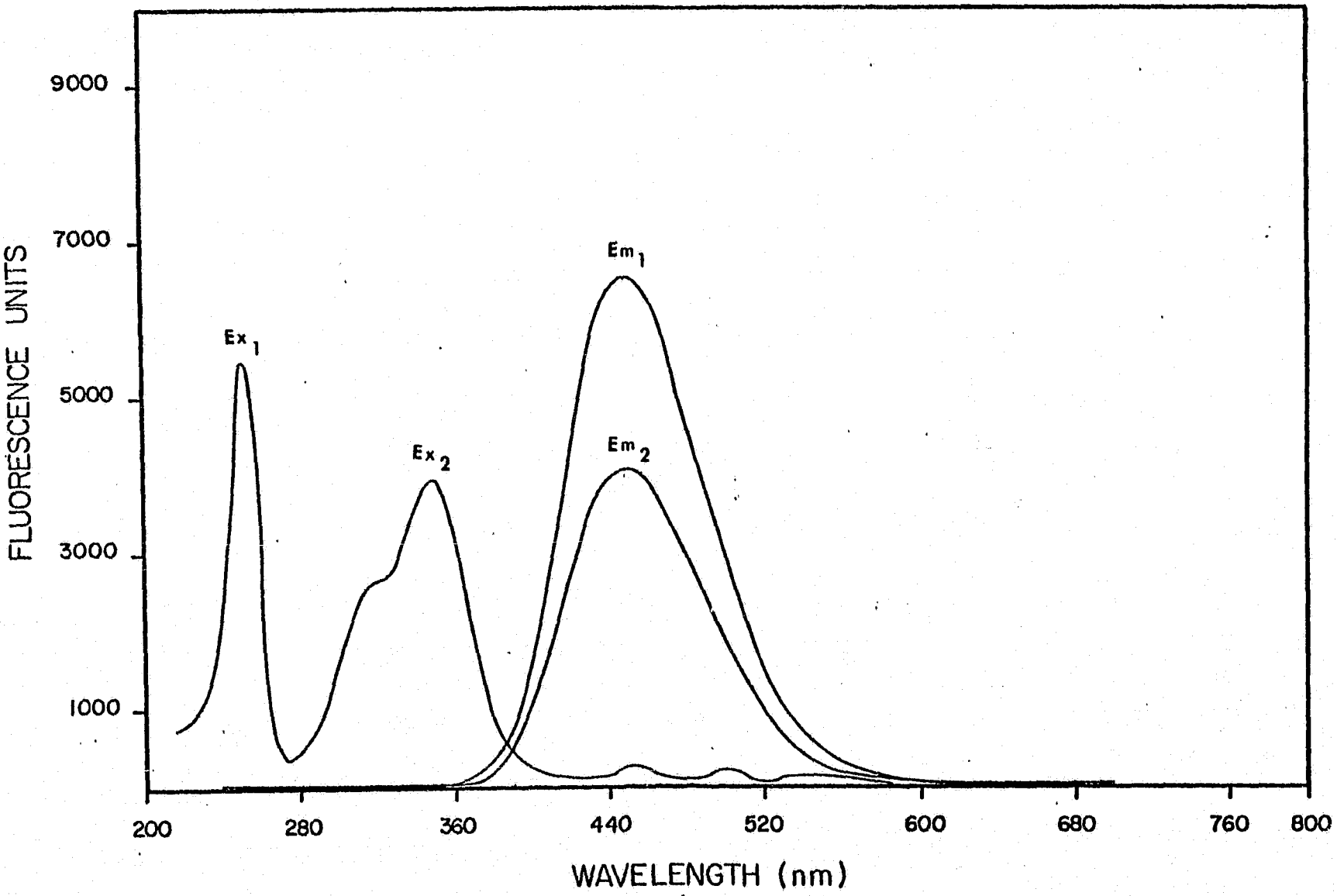
SAMPLE CHAMBER ADAPTER FOR AMINCO-BOWMAN SPECTROPHOTOFLUOROMETER



Scale = 1" = 1"

MRI 2976 -@

FIGURE 7.7.2 INSTRUMENT CALIBRATION WITH QUININE SULFATE



Sample: Quinine sulfate 1 µg/ml

Slits*: 1, 0, 1, 2, 0, 2

Filter: None

Peaks Ex_1 and Ex_2; scan of the excitation spectrum with the emission monochrometer set at 450 nm.

Peak Em_1; scan of the emission spectra with excitation monochrometer set at 250 nm.

Peak Em_2; scan of the emission spectra with excitation monochrometer set at 350 nm.

Background Fluorescence of Cloth (Figure 7.7.3)

Samples: A - white cloth

B - dried bloodstain on white cloth

Slits: 1, 2, 1, 2, 2, 1

Filter: 525 nm

Angle: 45^{o}

Excitation: 460 nm

Samples A and B; scan of emission spectrum. Note the marked reduction of background fluorescence at 516 nm, apparently caused by the darker, less reflective stained surface.

Effect of Application of Fluorescein Reagent to Cloth (Figure 7.7.4)

Samples: A - bloodstained cloth saturated with fluorescein-conjugated antibody

B - dried bloodstain on white cloth

* Numbers refered to size of slit openings in millimeters. Zero (0) indicated slit was wide open.

FIGURE 7.7.3 BACKGROUND FLUORESCENCE OF CLOTH

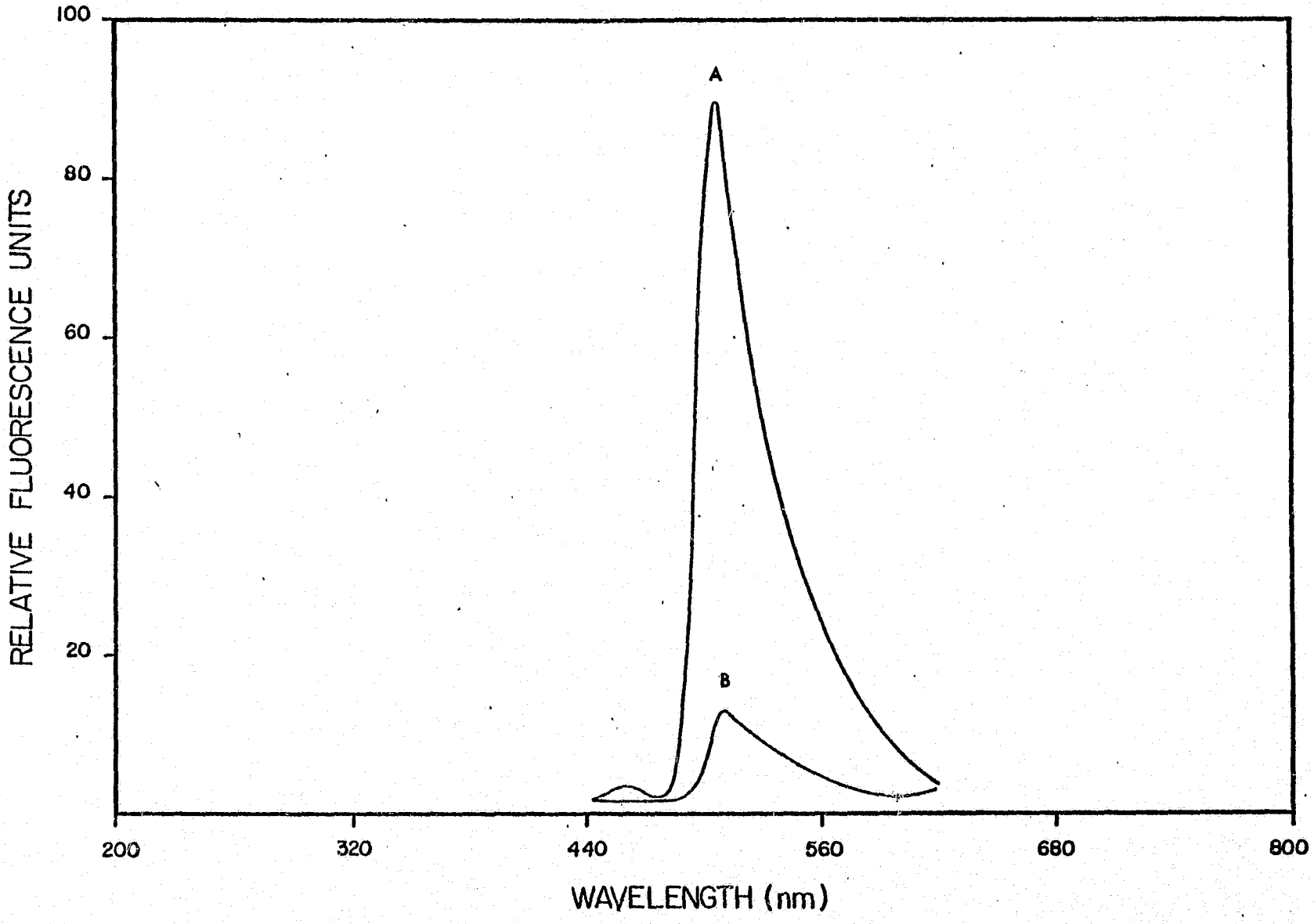
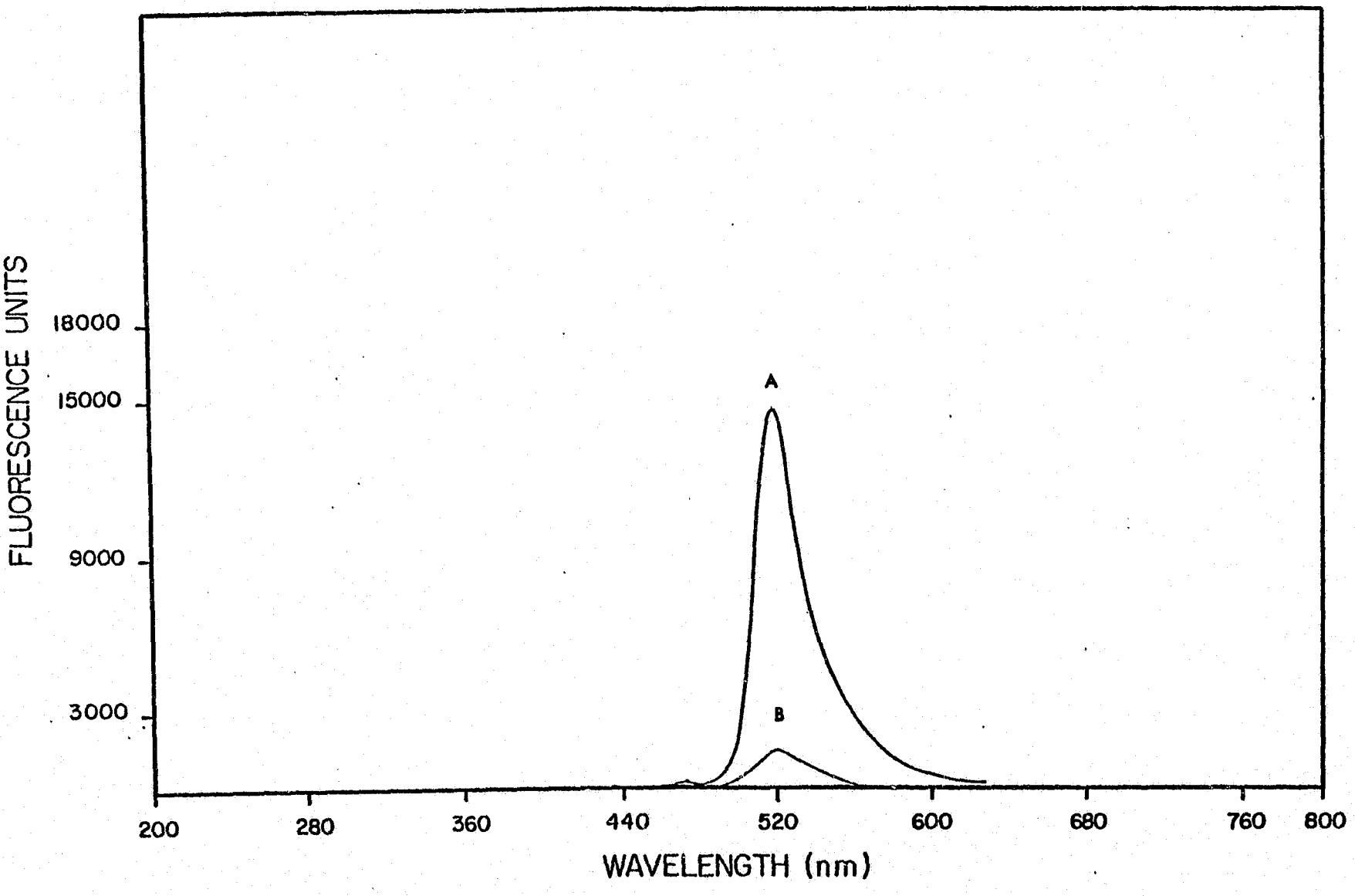


FIGURE 7.7.4 EFFECT OF APPLICATION OF FLUORESCEIN REAGENT TO CLOTH



Slits: 1, 0, 1, 2, 0, 2

Filter: 525 nm

Angle: 30^{o}

Excitation: 460 nm

This illustrated the strong signal obtained above the background surface of bloodstained fabric when concentrated fluoresceinized reagent was simply applied to the surface. The amount of tagged antibody applied was probably in greater excess than that which could be specifically bound to the available antigen.

Excitation Spectra of Fluoresceinized Antiserum (Figure 7.7.5)

Sample: FITC-Anti A (1:10)

Slits: 1, 2, 1, 2, 2, 1

Filter: 525 nm

Emmision: 520 nm

Excitation scan of labeled antibody in cuvette. Trace exhibits an excitation at 490 nm with a shoulder at 460 nm.

Excitation and Emission Spectra of Fluoresceinized Antisera Spotted On Cloth (Figure 7.7.6)

Sample: White cloth spotted with tagged Anti-A

Slits: 1, 2, 1, 2, 2, 1

Filter: None

Angle: 45^{o}

Curve A - Excitation spectra of sample, emission fixed at 516 nm. This curve exhibits three excitation peaks, 370, 468 and 495 nm. The first was attributed to the cloth background, and

FIGURE 7.7.5 EXCITATION SPECTRA OF FLUORESCEINIZED ANTISERUM

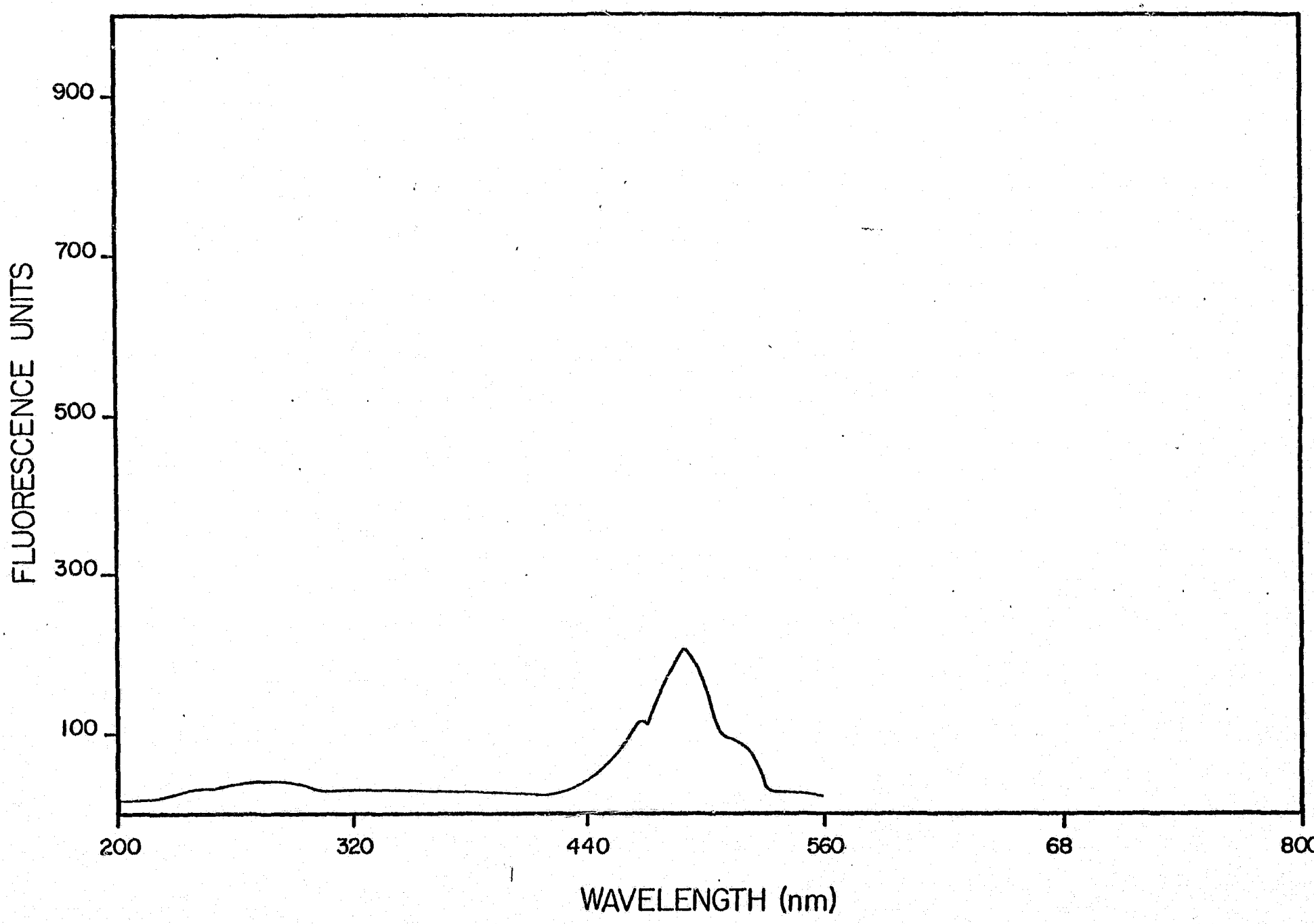
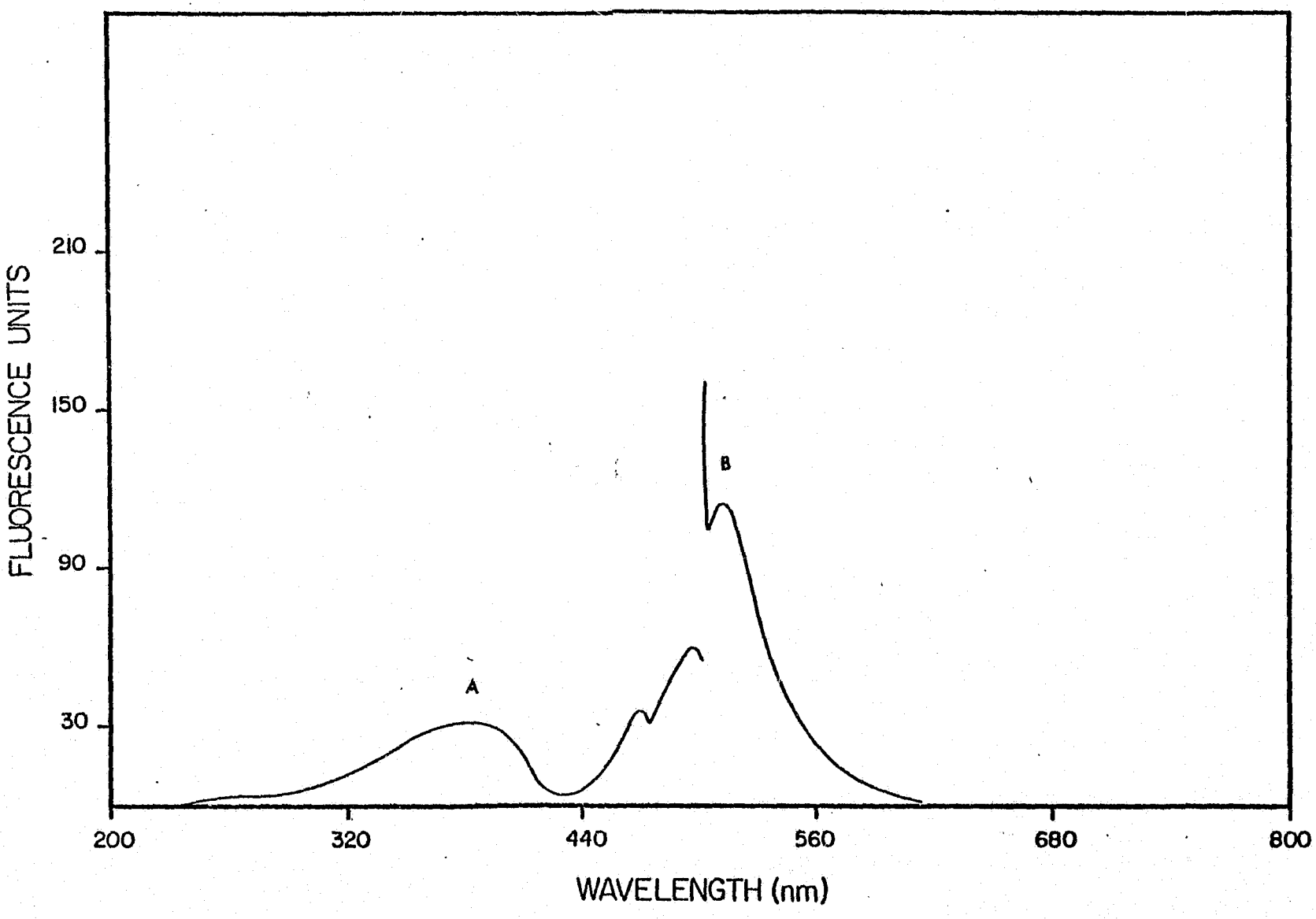


FIGURE 7.7.6 EXCITATION AND EMISSION SPECTRA OF FLUORESCEINIZED ANTISERA SPOTTED ON CLOTH



the latter two to fluorescein-conjugated antibody.

Curve B - Emission spectra of same sample with excitation fixed at 490 nm. A single emission peak was observed at 516 nm.

Emission Spectra of Bloodstained Fiber (Figure 7.7.7)

Samples: A - single bloodstained fiber (Type A), unfixed, reacted with anti-B, then FITC-goat anti-human γ-globulin.

B - single bloodstained fiber (Type B), unfixed, reacted with anti-B, then FITC-goat anti-human γ-globulins.

Slits: 4, 2, 1, 2, 2, 1

Filter: None

Angle: 45°

Excitation: 460 nm

These curves demonstrate emission peaks at 516 nm and increase in response when stain is reacted with specific antibody, using the indirect staining method.

Comparison of Emission Signals: Stained Vs. Unstained, and Fluorescein Vs. Saline (Figure 7.7.8)

Samples: A - white cloth treated with PBS

B - white cloth treated with 20 µl of 1:20 dilution of FITC-anti A

C - bloodstained cloth treated with PBS

D - bloodstained cloth treated with 20 µl of 1:20 dilution of FITC-anti A

Slits: 4, 2, 1, 1, 2, 2

Filter: 525 nm

Angle: 45°

FIGURE 7.7.7 EMISSION SPECTRA OF BLOODSTAINED FIBER

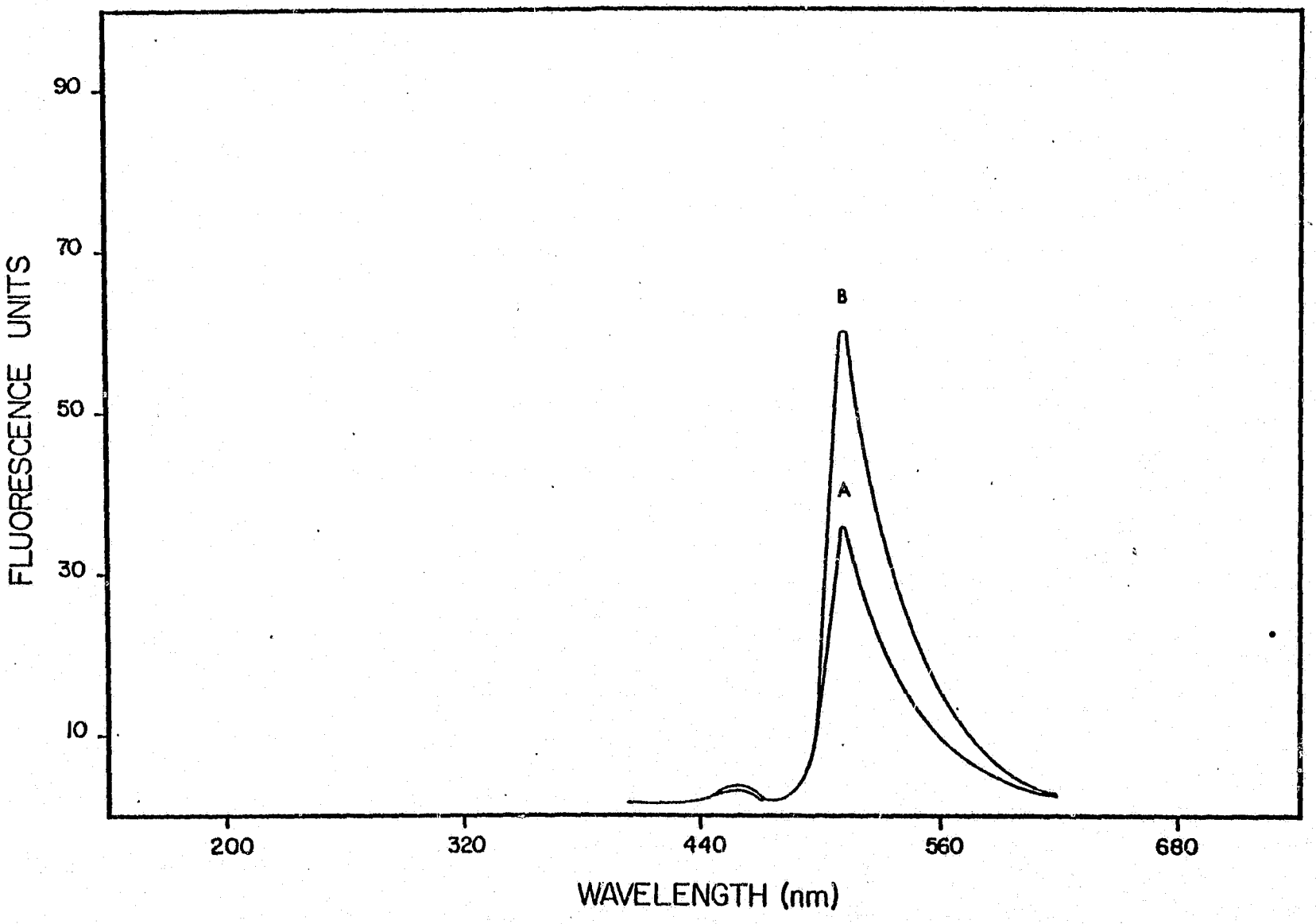
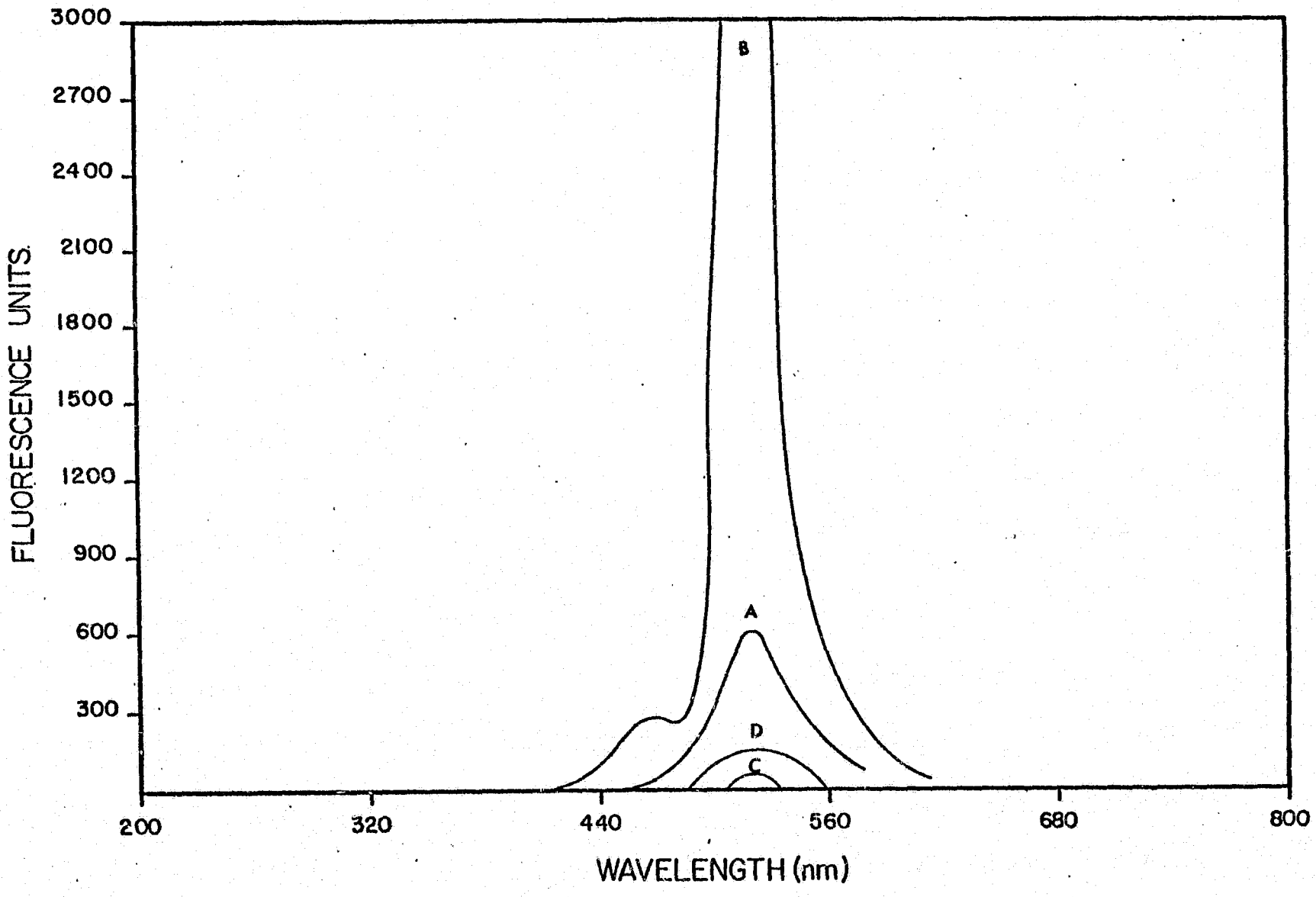


FIGURE 7.7.8 COMPARISON OF EMISSION SIGNALS: STAINED VS. UNSTAINED, AND FLUORESCEIN VS. SALINE



Excitation: 460 nm

Fluorescence Units: Sample A 600
Sample B 3250
Sample C 60
Sample D 148.5

These curves show that when equal amounts of fluorescent reagent was applied to clean white cloth and bloodstained cloth, the signal above background varied. For white cloth, the emission was 5.4 times greater; for stained cloth it was 2.5 times greater. The background signal was 10-fold less with bloodstained cloth. This data indicated that the stained surface produced less scatter and quenched some of the noise as well as the signal. Fluorescence units were determined by computation after varying attenuation. The apparent peak always present at 520 nanometers, including the negative samples (Figure 7.7.8), resulted from the breakthrough of scatter from use of the 520 nanometer cut-on filter, as well as the non-specific fluorescence from the components of the filter. The presence of a background signal was thus accepted, as the important factor was the difference in amplitude between negative and positive samples.

Effect of Lower Wavelength Excitation on Resolution of Scatter and Emission Peaks (Figure 7.7.9)

Samples: A - type A cells + F - anti-A (1:5 dilution)

Slits: 4, 2, 1, 1, 2, 2

Filter: none

Angle: 45^{o}

Excitation: 460 nm, 486 nm

FIGURE 7.7.9 EFFECT OF LOWER WAVELENGTH EXCITATION ON RESOLUTION OF SCATTER AND EMISSION PEAKS

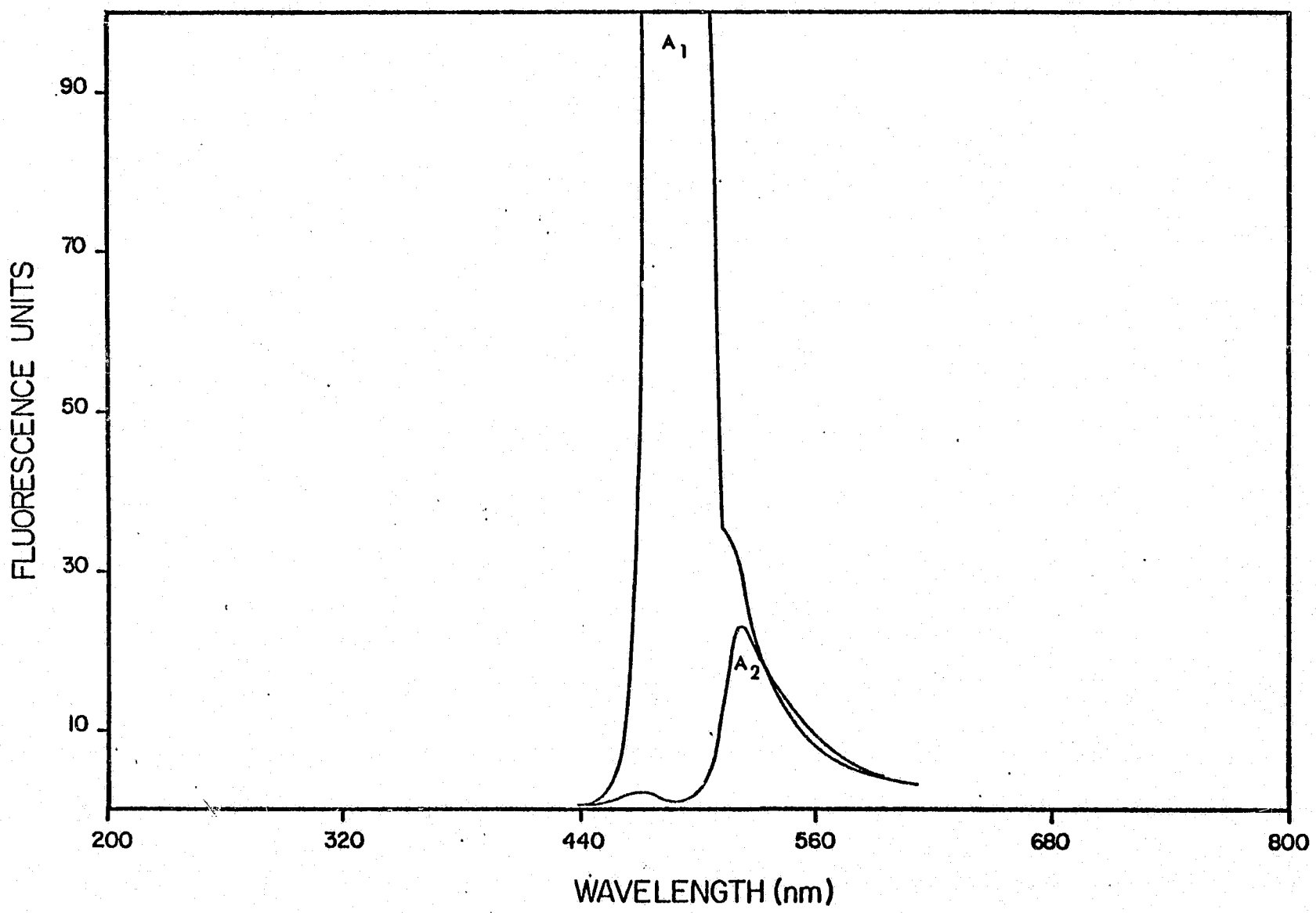
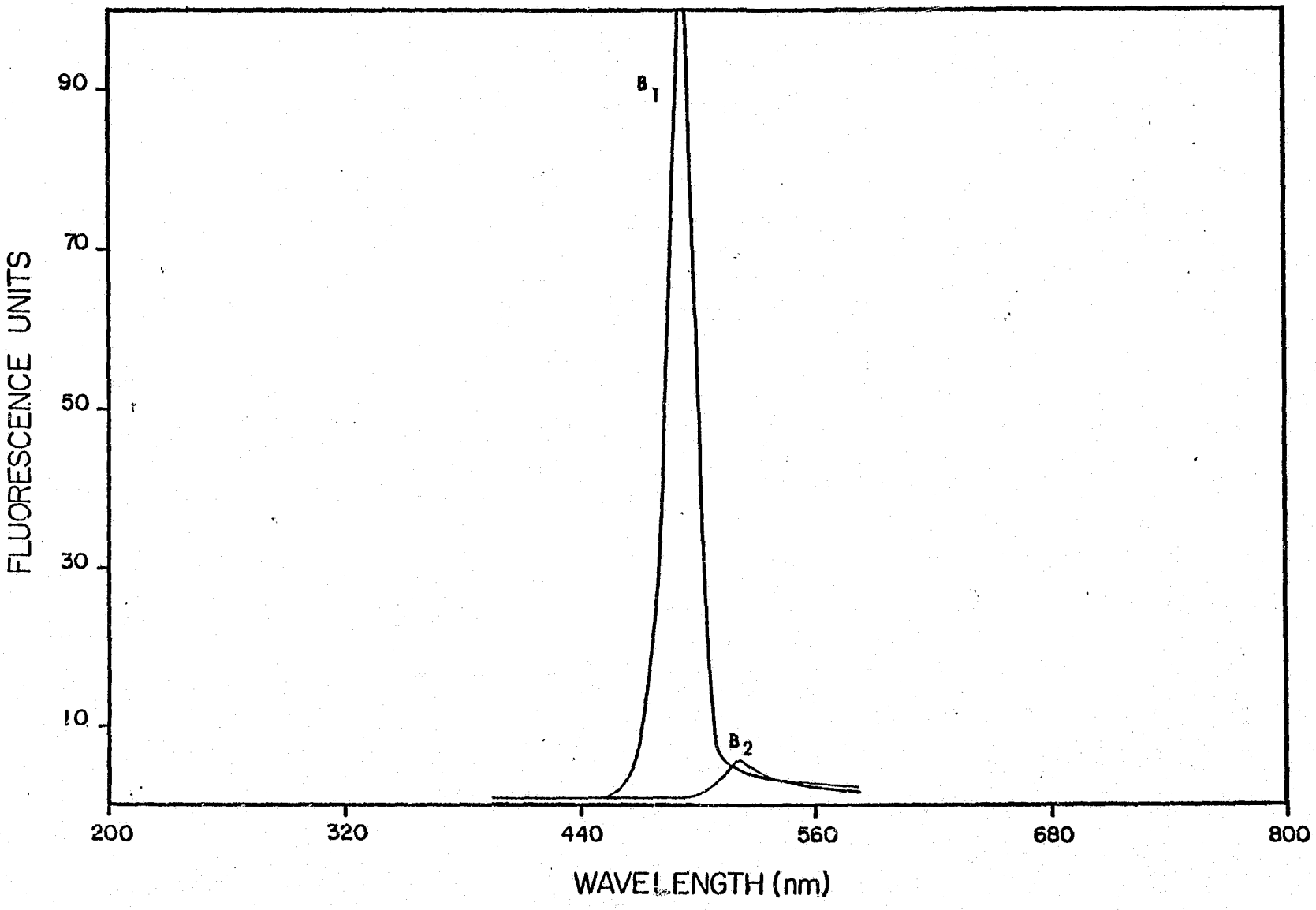


FIGURE 7.7.9 (con't) EFFECT OF LOWER WAVELENGTH EXCITATION ON RESOLUTION OF SCATTER AND EMISSION PEAKS



Washed red blood cells reacted with appropriate tagged antibodies, then sandwiched between glass slides.

Peak A_1 resulted from emission scan at 486 nm excitation, and illustrated the overlay between the scatter peak and the emission peak. Peak A_2 was obtained when excitation was decreased to 460 nm. The amplitude of the signal was decreased, but the resolution from the scatter peak was increased.

The second chart, Figure 7.7.9 (continued) illustrates the same phenomena with Sample B. Peak B_1 represents emission curve at 486 nm, peak B_2 the emission at 460 nm. Note the significantly higher signals for the positive sample (Curves A_2 and B_2).

Effect of Filtration Emission Spectra (Figure 7.7.10)

Sample: Bloodstianed cloth + tagged anti-A

Slits: 1, 2, 1, 1. 2, 1

Angle: 45^{o}

Excitation: 460 nm

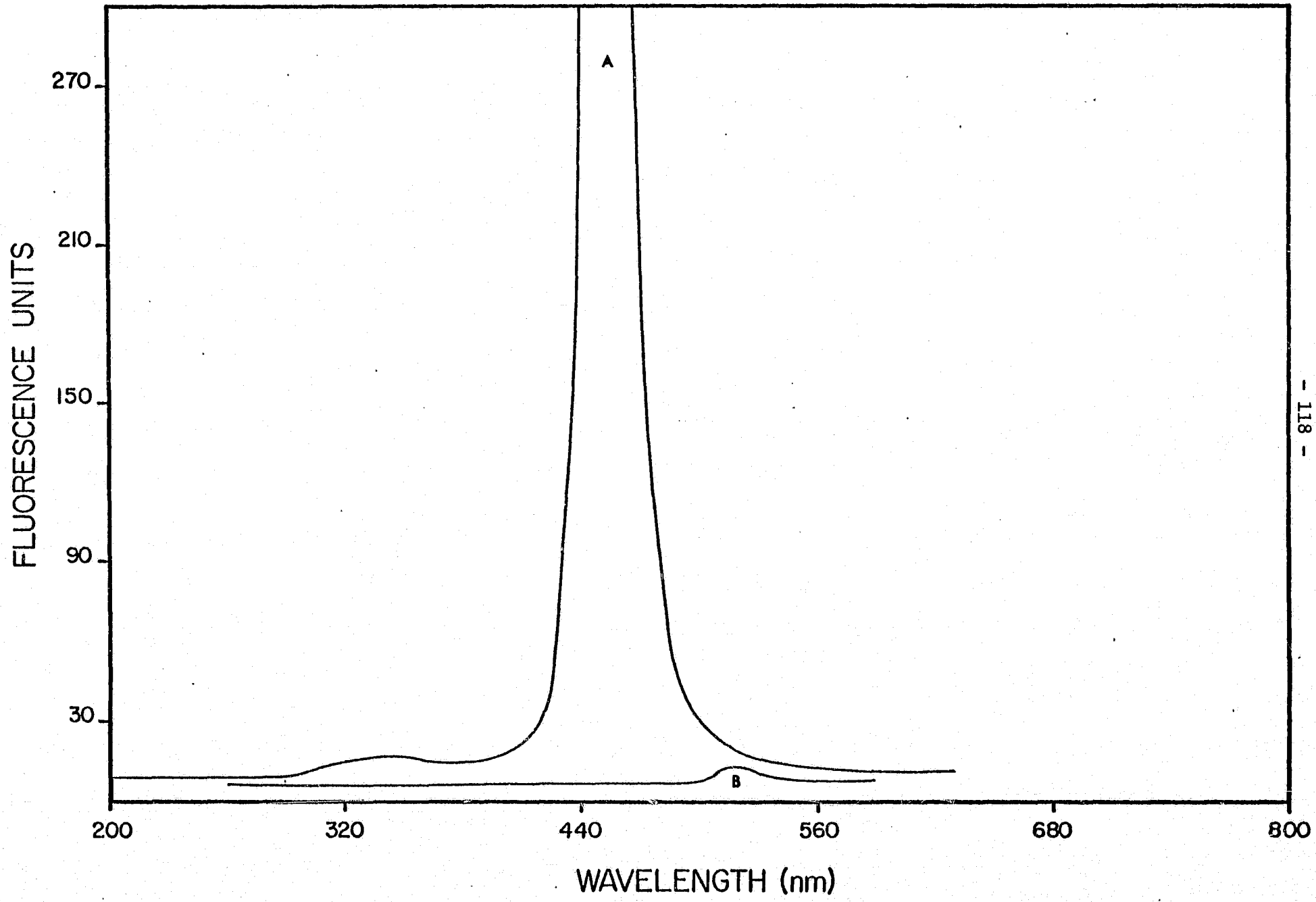
Peak A demonstrated emission spectra obtained without the filter (525 nm cut-on, Ditric Optical, Co.). The large peak is the result of light scattering. Peak B, with the filter in place. This demonstrated that the filter was effective in blocking all wavelengths below 500 nm.

Effect of Filter on Fluorescent Signal (Figure 7.7.11)

Samples: A - bloodstain (type A) reacted with 1:10 dilution of labeled anti-A.

B - white cloth reacted with anti-A as above.

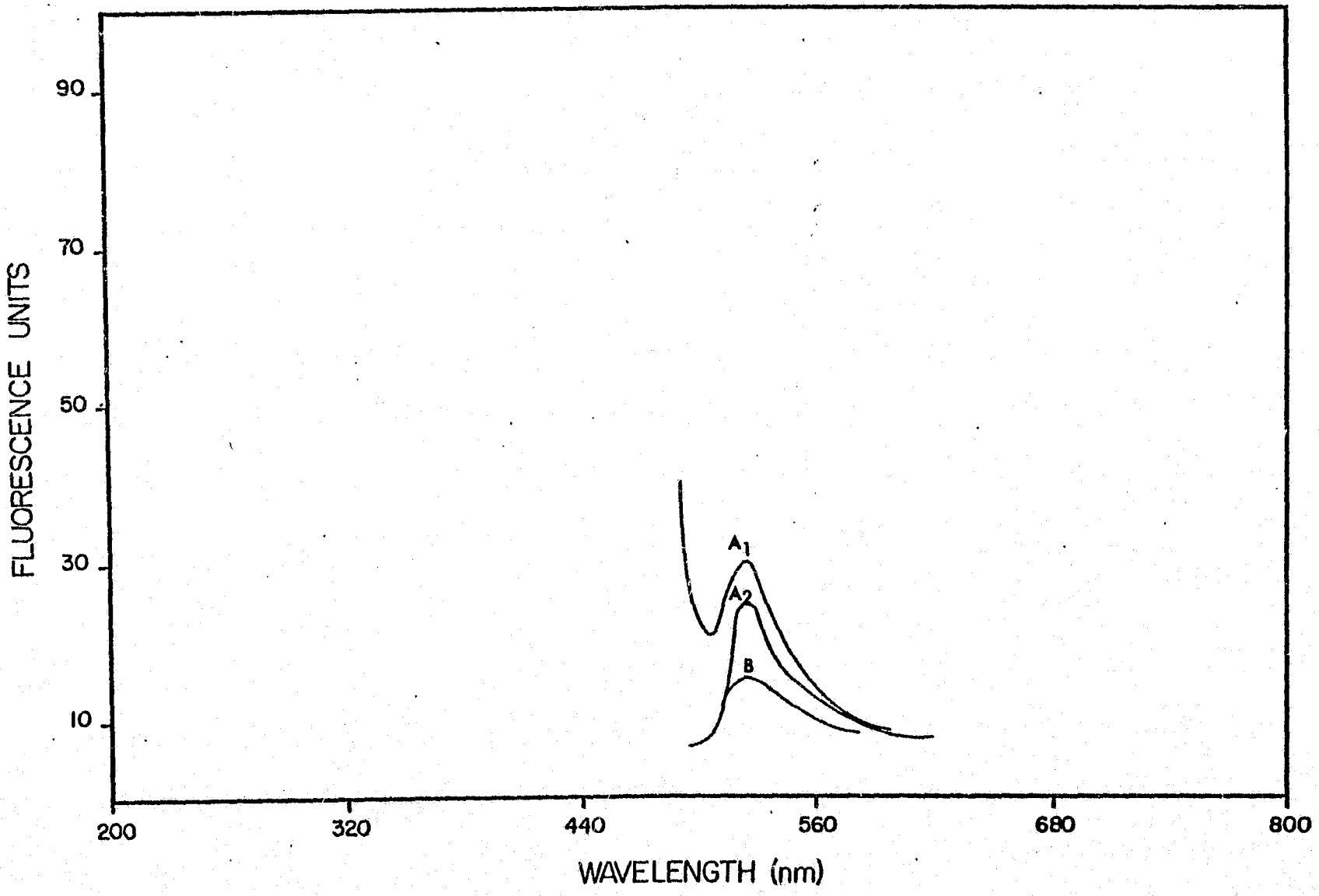
FIGURE 7.7.10 EFFECT OF FILTER ON EMISSION SPECTRA



CONTINUED

2 OF 4

FIGURE 7.7.11 EFFECT OF FILTER ON FLUORESCENT SIGNAL



Slits: 1, 2, 1, 1, 2, 1

Filter: 525 nm

Angle: 45^{o}

Excitation: 460 nm

Peak A-1 was the result of an emission scan without the 525 nm filter. The decending limb prior to the 520 nm emission maximum is the scatter peak. With the filter in place, peak A-2 resulted, while peak B was generated with the white cloth (Sample B). In addition to reducing the background noise, the filter caused some loss of signal at 520 nm.

Effect of Slit and Angle on Background Signal (Figure 7.7.12)

Sample: Plain white cloth

Slits: Set A - 4, 2, 0.5, 4, 2, 2

Set B - 4, 2, 0.5, 4, 0, 2

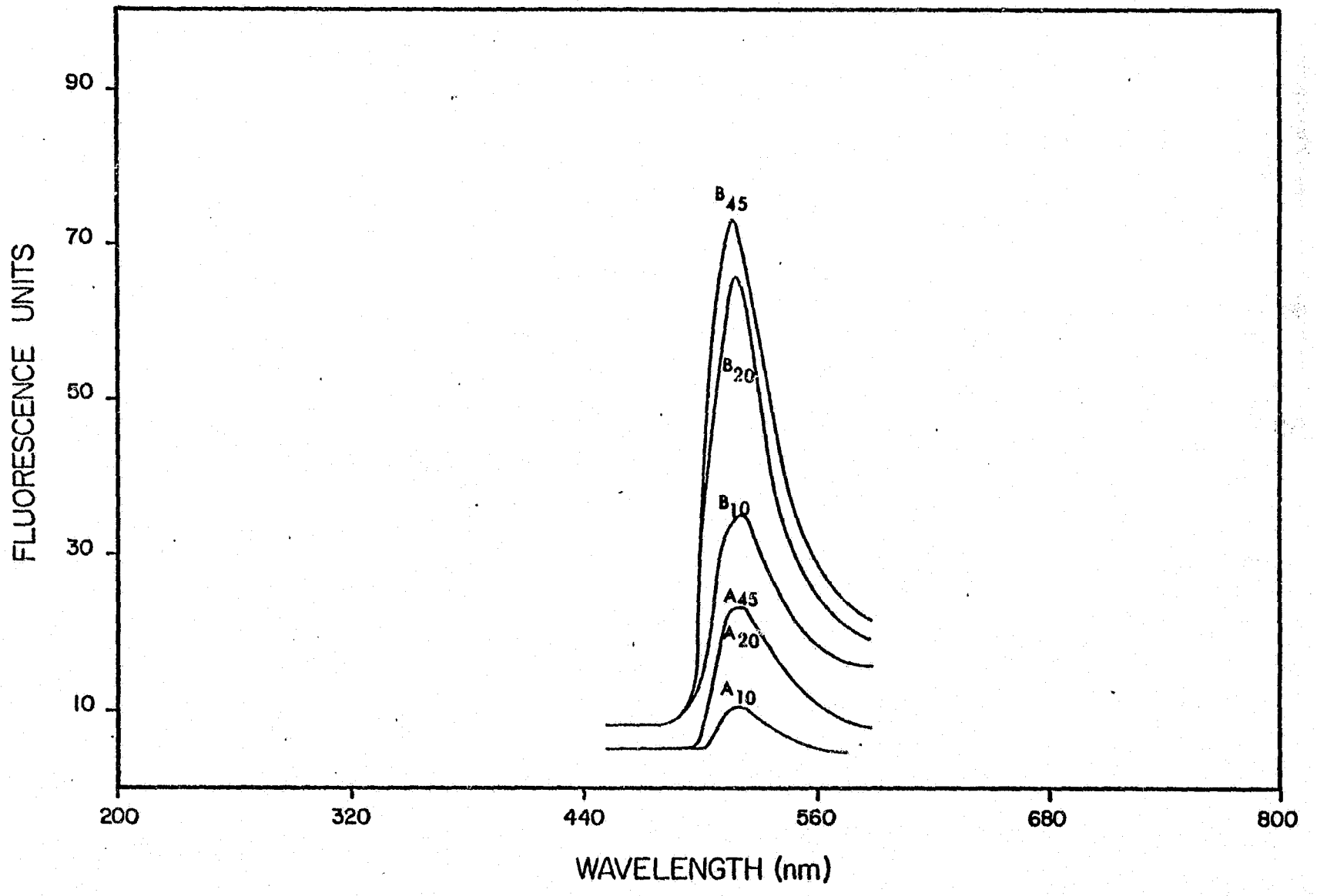
Angles: 10^{o}, 20^{o}, 45^{o}

Filter: none

Excitation: 480 nm

White cloth was scanned at 480 nm excitation. Two different sets of slit openings (Set A and Set B) were used at these different angles, generating six emission curves. Two of the curves on Set A (A_{10} and A_{20}) were superimposed. In general, the non-specific response increased in the order $45^{o} > 20^{o} > 10^{o}$. Set B curves showed a greater response at all angles because of increased slit openings in position 5 (emission slit).

FIGURE 7.7.12 EFFECT OF SLIT AND ANGLE ON BACKGROUND SIGNAL



Bloodstain Analysis (Figure 7.7.13)

Samples: Type A bloodstained cloth

Type B bloodstained cloth

Slits: 4, 2, 1, 2, 0, 2

Angle: 20^{o}

Filter: 525 nm

Excitation: 460 nm

Type A bloodstains were reacted with anti-A, anti-B and AB serum. The latter served as a negative control as the AB serum contained no detectable anti-A or anti-B antibodies. Fluorescein was introduced in the second antibody, tagged anti-human γ-globulin. The excitation energy was 460 nm. Although this is before the 490 nm excitation maximum for fluorescein, it was adequate for eliciting an emission signal at 520 nm with less scatter interference. The slit arrangement was narrow for excitation and wide for emission. The two charts demonstrate the specificity of the homologous reactions.

The excitation wavelength of 460 nanometers was chosen because of the considerable reduction of scatter at the corresponding emission peak of 520 nanometers. The loss in energy from that available at 486 nanometers (the excitation maximum) was felt to be justified because of the net gain in resolution between scatter interference and emission. Any further shift to lower excitation wavelength would reduce drastically the energy needed for fluorescein emission, as well as enhancing the contribution from the non-specific fluorescence from the optical brighteners on the cloth

FIGURE 7.7.13 BLOODSTAIN ANALYSIS

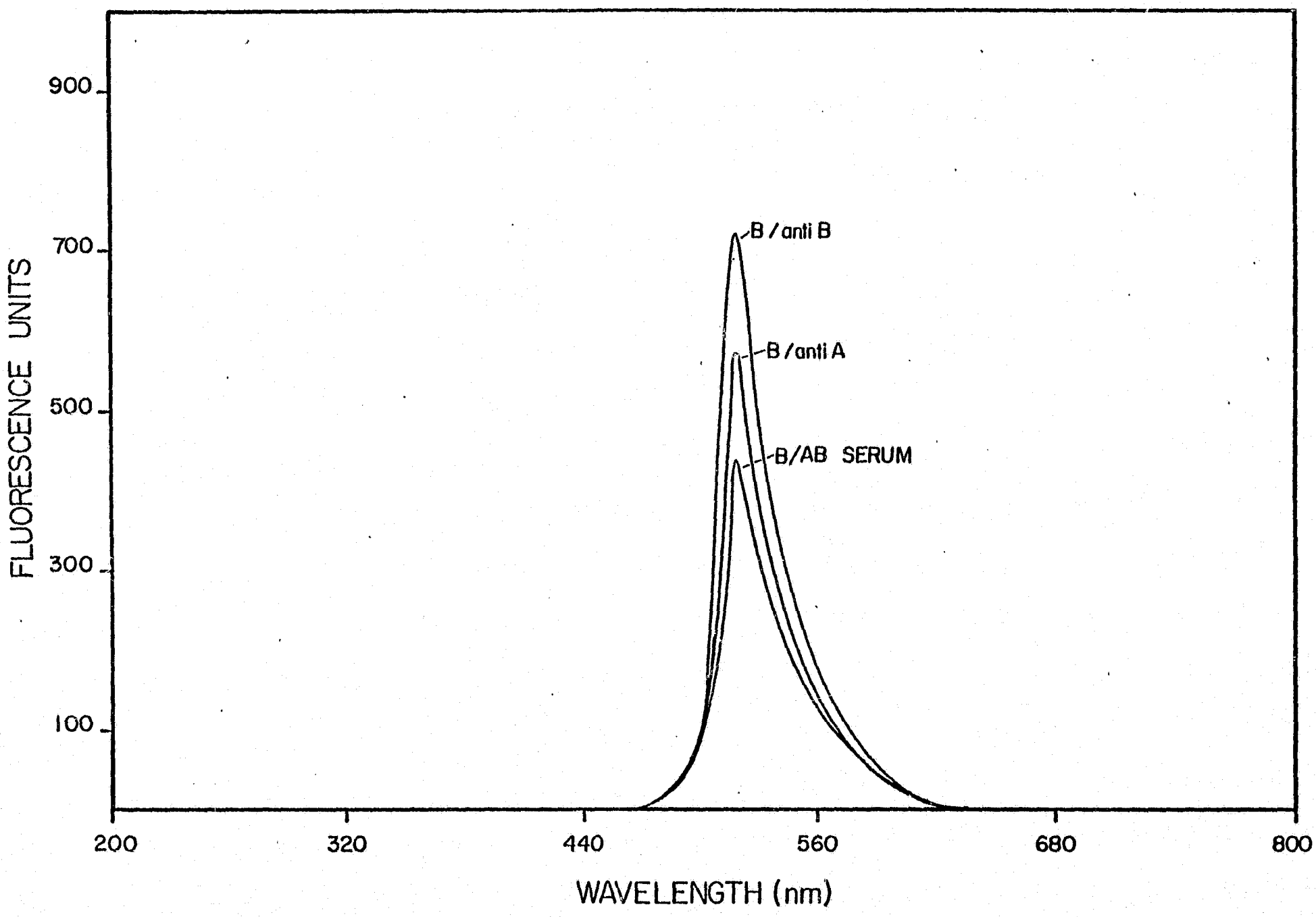
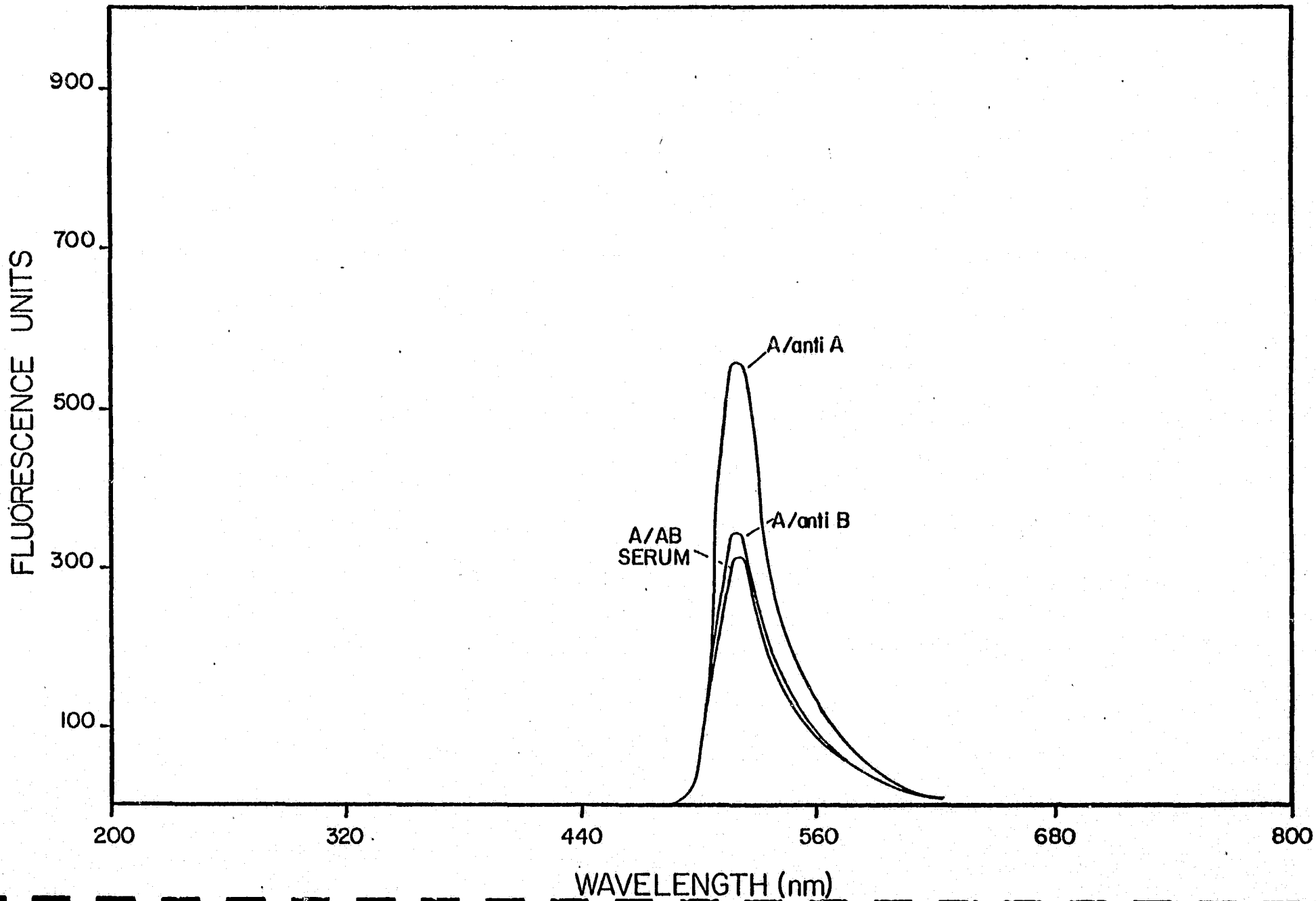


FIGURE 7.7.13 (continued) BLOODSTAIN ANALYSIS



(Figure 7.7.6, peak A). All other attempts to alter excitation and/or emission wavelengths resulted in a net reduction in sensitivity, resolution, or specificity.

7.8 LABS-1 FLUORESCENCE DETECTION SYSTEM

The methodology developed for the detection of erythrocyte antigens was based upon the insolubility of the stromal antigens in dried bloodstains and the high proportion of corpuscular debris that remains firmly attached to fabrics. The LABS-1 was designed specifically to yield fluorescence measurements from the surface of particulates. The sensitivity required for these measurements was obtained by digital photon counting light analysis, a technique that offered significantly greater sensitivity and stability over conventional light-measuring techniques.

7.8.1 Description of LABS-1 Fluorescence Detection System

The prototype instrument was constructed from available off-the-shelf items. The design was specific for the requirements of the Low Angle, Direct Fluorescence Detecting Method within the cost constraints stipulated in the Statement of Work. The light source was a quartz halogen lamp, with variable intensity from 0-150 watts. The light passed through 3-cavity interference filter to produce a beam designed to excite fluorescein. The excitation beam illuminated the sample chamber at an angle of 28^{o}. The emitted light passed through a second 3-cavity interference filter to a photo-multiplier. The photomultiplier output was directed to an external electronic package that controlled signal amplification, amplifier discrimination and digital photon counting. Figure

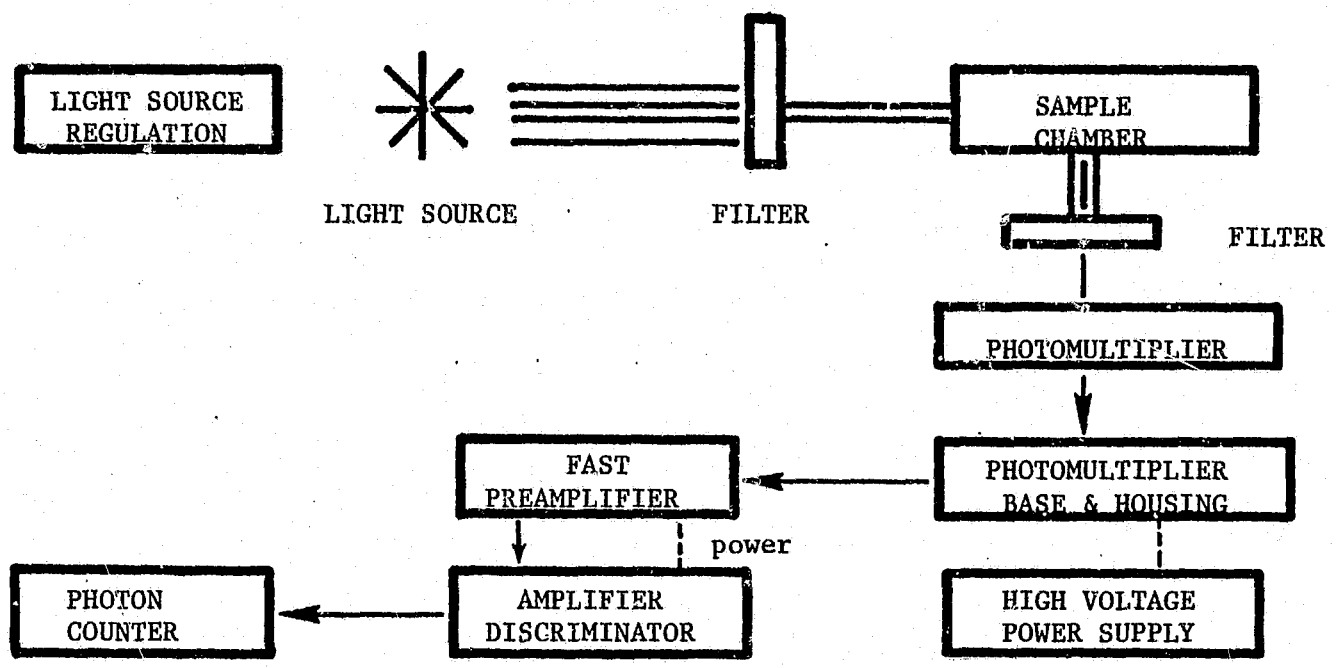
7.8.1 is an overall block diagram of the LABS-1 detection system. The optical configuration is detailed in Figure 7.8.2. The circutry design is included as Figure 7.8.4.

A sample holder was constructed from aluminum that consisted of two parts, a base and cover (Figure 7.8.3). The base measured 25 x 85 mm, and was slotted in such a way as to allow the cover to be inserted in a reproducible manner, and to allow reproducible orientation of the entire assembly in the sample chamber. The cover contained two openings. located so that bloodstains placed under the proper one would expose a known and reproducible area to the excitation beam. One opening was rectangular and designed to accept pieces of stained cloth 0.5 x 1.0 cm, the other opening circular, designed for membrane filters containing bloodstain particulates removed from the fabric.

7.8.2 Initial Testing of LABS-1

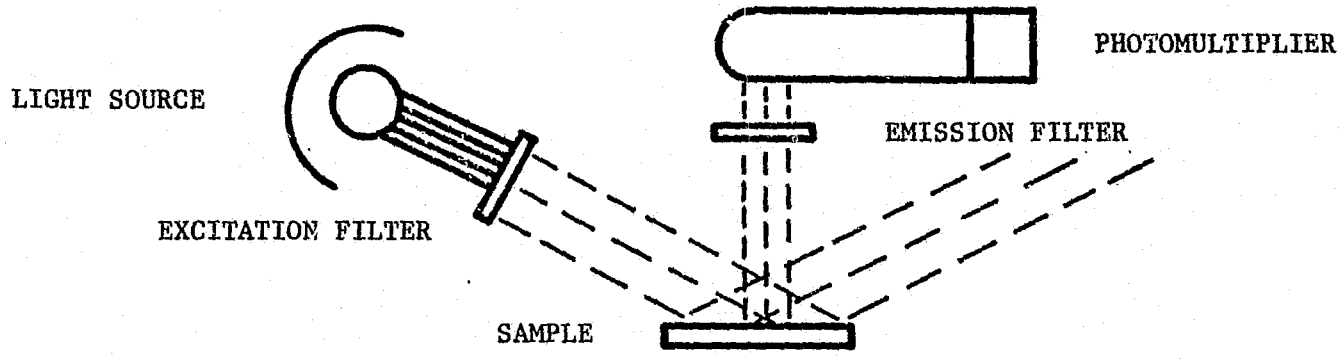
The LABS-1 was received equipped with a 470 nanometer narrow bandpass (7 nm) excitation filter and a 520 nanometer narrow bandpass (8 nm) emission filter. The fluorometer was tested for stability by taking 10 consecutive 30 second counts in the morning, at noon, and in the afternoon (Table 8.3.1). Although the values between the various time intervals varied, the successive readings were sufficiently reproducible to allow further testing. This additional testing consisted of treating

FIGURE 7.8.1



BLOCK DIAGRAM LABS-I FLUORESCENCE SYSTEM

FIGURE 7.8.2



OPTICAL CONFIGURATION LABS-I FLUORESCENCE SYSTEM

FIGURE 7.8.3

SAMPLE HOLDER FOR "LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING METHOD USED IN ORTEC FLUOROMETER

ACTUAL SIZE

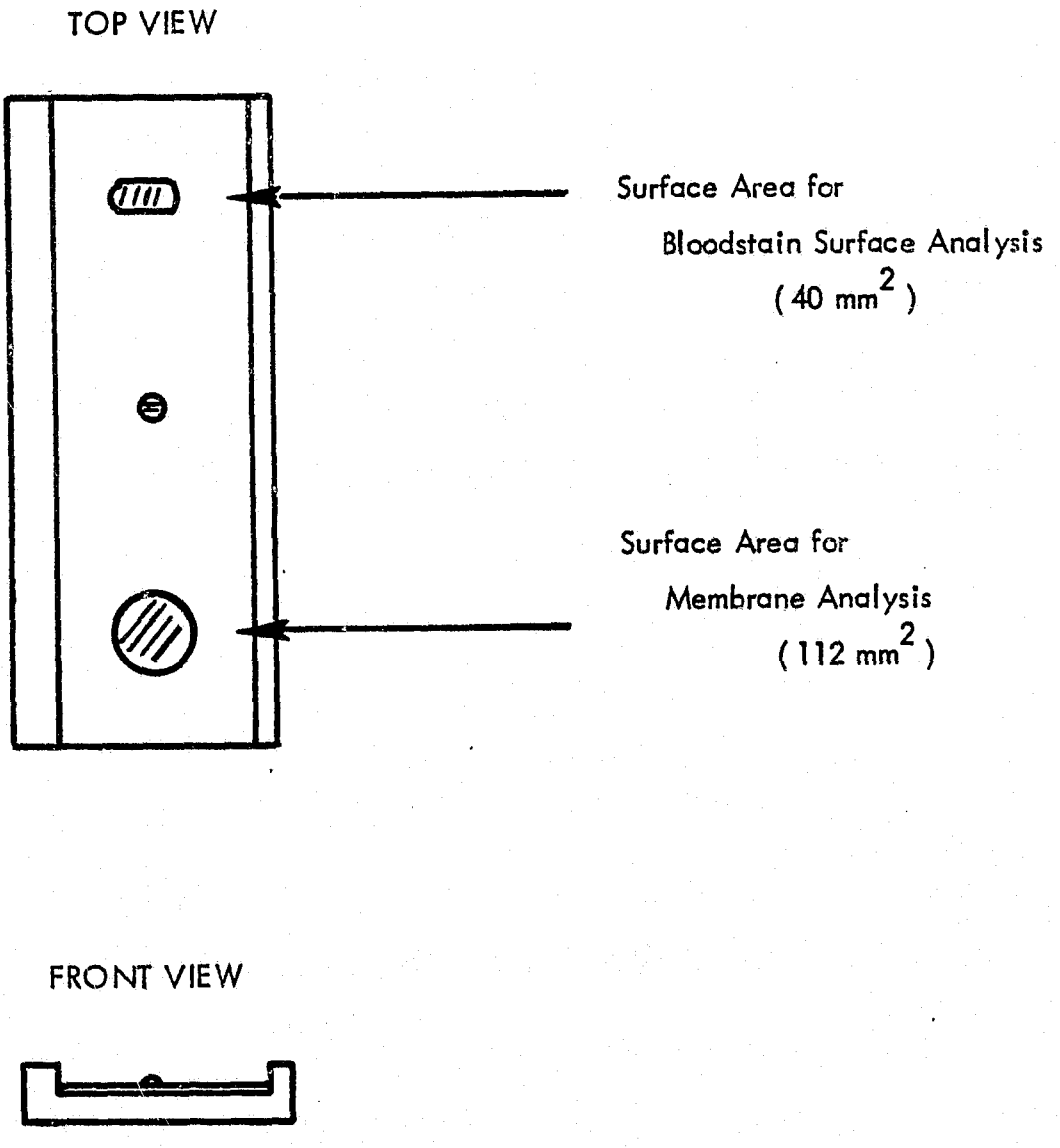
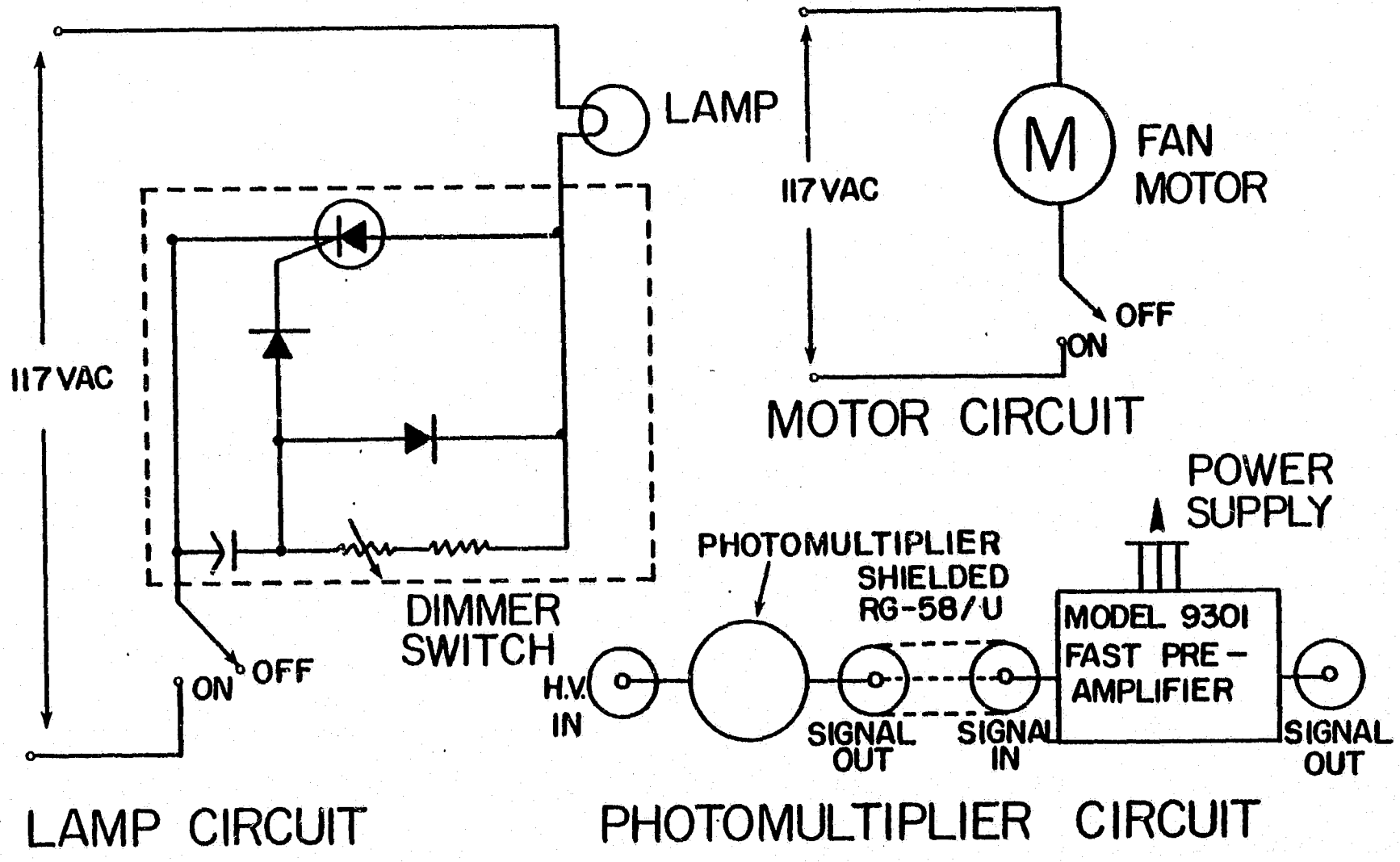


FIGURE 7.8.4

CIRCUITRY DIAGRAM: LAB-1 FLUORESCENCE SYSTEM



fixed bloodstains with either anti-A or anti-B, washing, reacting with fluorescent anti-human IgM, washing, and a single reading taken in the LABS-1. This entire process was repeated on the next day. These two sets of data are reported in Table 8.3.2. For a discussion of the statistical evaluation, the reader is refered to Section 7.4.13.2.

7.8.3 First Modification of LABS-1

All modifications of the instrumentation were performed by ORTEC, Inc. Checks and adjustments for optimizations of single photon response, noise rejection, cabling, etc., were performed in a straight forward manner. An examination of the analytical portion of LABS-1 (i.e., the light source-sample chamber-photomultiplier portion) revealed several problems. This portion of the instrument was originally hurridly constructed out of both readily available and some specially fabricated parts and was intended only for a short term of usage, before being replaced by a more appropriate configuration. It showed significant wear under the usage it was put to. Numerous light leaks were found in the sample chamber and were sealed as best as possible with black tape and silastic cement. The whole instrument also displayed what initially appeared to be an intermittent fluctuation in count rate. After some trial and error testing, this was localized to the inability of the constant voltage transformer employed to power both the light source and fan. To at least partially remedy this, a separate line cord and switch were obtained and installed so that the transformer powered only the light source. The fan was powered separately. The transformer was, however, limited and will not supply

sufficient power to allow running the light source at or near full intensity.

Eastman White Reflectance Standard and White Reflectance Coating and Sprayers were obtained from Eastman Kodak Company. Two sets of 1/16" thick aluminum rectangles were prepared; one set matching the dimensions of the LABS-1 sample chamber and the second set so that the rectangle could be positioned in the cuvette holder of the spectrofluorometer. One surface of an individual aluminum rectangle was coated with either: 1) a flat black paint; 2) the white reflectance coating; or 3) the white reflectance coating containing an arbitrary 100 micrograms per milliliter of fluorescein. Duplicates for the LABS-1 sample chamber were prepared. Spectra were run at ORTEC on the set generated for the spectrofluorometer and showed that a set of suitable reference samples had been generated; a uniform dark sample, a uniform white reflecting sample and a uniform fluorescent sample. In addition, the spectra of the fluorescent sample showed an emission maximum of 537 nanometers, while an emission spectra of the recovered, filtered, liquid supernate of the standard plus fluorescein showed an emission maximum of 520 nanometers. This shift was presumably due to the dry nature of the standard. In fact, a change in color may even be seen visually upon drying. This may be particularly relevant in considering what is the optimum status of the blood stain samples, since drying or partial or variable drying will lead to both a decrease in fluorescence intensity and to a spectral shift.

New filters were tested individually to find the combination that would give the greatest fluorescence intensity and largest ratio

of fluorescence standard reading over white reflectance standard reading. Readings were taken with a particular pair of filters with the light source at an arbitrary intensity of "11". No tests were performed with actual fluorescent-tagged blood stains, as they were not available. Readings were also taken on the black surface and with the light source off. The temperature of the photomultiplier housing was also monitored qualitatively to check for any drastic change in temperature during continued use. At the intensity selected, none was apparent. The excitation filter was opitmized first, the emission filter was held fixed, utilizing the filter originally installed in LABS-1 and the different filters substituted for the excitation filter until an optimum was found. Similarly, once the excitation filter was selected, substitutions were made for the emission filter until an optimum was selected. Representative data is given in Tables 8.3.3 and 8.3.4. The filter pair selected included a broad bandpass 500 nanometer cutoff filter for excitation and a narrow bandpass 530 nanometer filter for emission*. The broad bandpass filters obtained as alternatives for the emission filters were unacceptable; they, and probably the excitation filters to some extent as well, appear to be not completely blocked for the spectral range that the photomultiplier was sensitive to. One filter that was labeled as a narrow bandpass filter with a central wavelength of 530 nanometers was discarded during testing because, when used repeatedly, it did not act as a narrow bandpass filter. It can be seen from Table 8.3.3 that the use of the broad band cutoff filter for excitation does

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* Transmission curves for these filters are included as Figure 8.3.1

not significantly alter the ratio of the fluorescence standard to white reflectance standard reading, but does increase the absolute number of counts by about a factor of four. For this reason, the cutoff filter was selected for excitation. Similarly, because there was little difference between the 530 and 540 nanometer narrow band filters as used for emission, the 530 nanometer filter was selected for emission.

The ability of the constant voltage transformer to regulate light intensity was tested by varying the line voltage from 105-121 volts with a Powerstat Model 116 voltage regulator (Superior Electric Co., Bristol, Conn.). The constant voltage transformer did maintain constant voltage, but did not maintain constant lamp intensity, as demonstrated by the continued decrease in photons detected in all samples as the line voltage decreased (Table 8.3.5). A signal normalization study was performed on the LABS-1, utilizing three samples: a black surface, a white reflectance surface, and a fluorescein incorporated white reflectance surface. The normalized signal (S_N) was calculated as described in section 7.6.15, on a series of data collected every 45 minutes over a 5.5 hour period. Five measurements 10 seconds each were made on each of the three samples. Although there appeared to be significant variations between the measurements with respect to time, the normalization procedure yielded reproducible data. The precision of the consecutive measurements ranged from ± 0.62% to 1.78% for the black standard, ± 0.13% to 0.95% for the white reflectance standard and ± 1.81% to 2.67% for the FITC-reflectance standard (Table 8.3.6).

However, it must be stressed that LABS-1 was not a finished instrument and was not suitable for use in other than a research environment. It was constructed at a time premature to the planned instrument development, based on decisions that now appear premature, and as a model with full knowledge of its limitations and deficiencies (e.g. light source heat generation, fragility of the sample chamber, sensitivity to line voltage fluctuations). It was intended to provide a means of answering critical questions in a research environment.

7.8.4 Second Modification of LABS-1

A second visit was made on October 21 and 22 to MRI to re-examine the model fluorometer and to examine the behavior of various filters with the reference standards previously examined and with an actual bloodstain and bloodstain saturated with fluoresceinized antibody. The previous examination employed only the reference standards.

The filters examined included those already at MRI, as well as filters obtained from three different sources. After discussions with five filter vendors, filters were selected from those available, whose specifications appeared to be best suited for the bloodstain analysis.

The fluorometer was again examined for such things as light leaks, cabling, etc. The socket for the light source was repaired. Since a suitable constant voltage transformer was not available, a transformer was borrowed from ORTEC. A shield and aperture were

fashioned from a sheet of aluminum and added to the instrument, in order to minimize the heating of the sample chambers and photomultiplier housing.

The filters were examined in an empirical fashion, with each emission filter being paired with each possible excitation filter. In doing this, it became apparent that a number of the filters were not masked at the edges and transmitted unfiltered light to the photomultiplier. An interm mask was fashioned to insure that, during the selection tests, no unfiltered light reached the photomultiplier. It was necessary, after completion of the selection tests, to construct a mask using black felt that similarly blocked out the edges of the emission filter. Similarly, the aperture for the excitation filter required masking.

Some decrease in the size of the aperture from the sample chamber to the photomultiplier resulted, which will presumably mean a decrease in the intensity of the fluorescent signal.

The filters that were originally installed in LABS-1 were selected based on what appeared to be a firm decision as to the optimum excitation and emission wavelengths and bandwidths. Since this represented a forced, premature decision and since LABS-1 was built with no real provisions for the routine, easy exchange of filters, an optimization of the emission and excitation characteristics by filter substitution is not a simple straight forward procedure, but one where other variables such as potential light leaks and aperture size changes occur with each filter substitution.

The various filter pairs were examined, utilizing five samples.

These included the three reference samples employed earlier:

D = flat black surface
W = white surface
F = fluorescent surface

In addition, a bloodstained cloth and a bloodstained cloth saturated with fluorescent antibody were also used. These were labelled I and II, respectively. Replicate readings of all five samples were taken, averaged and the ratios of $\frac{W}{D}$, $\frac{F}{W}$ and $\frac{II}{I}$ calculated.

A filter pair was selected that was the best compromise between the maximization of the $\frac{II}{I}$ ratio and the minimization of the $\frac{W}{D}$ ratio. This latter, the minimization of $\frac{W}{D}$, may represent an arbitrary criteria, since these two standards are independent of the bloodstain analysis. In actual use, the ideal would be to see the maximum fluorescence from a bloodstain treated with antibody, while seeing the minimum signal from an untreated bloodstian. Optimizing for the smallest W/D ratio does not necessarily coincide with this goal (Table 8.3.7 and Figure 8.3.2).

The precision of the consecutive measurements, both preread and final, ranged for ± 0.6% to 1.7%. This indicated that the differences observed in the reactivities of A/anti-A and B/anti-A were not due to instrument variation, but rather to the fluorescence of the tagged bloodstains.

LABS-1, in its original configuration, did consistently differentiate between type A and B (Table 6.1). After the modifications, although the precision of repetitive measurements did improve, the ability to discriminate between A and B blood group antigens decreased.

7.9 RECOMMENDATIONS

7.9.1 Bloodstain Analysis: ABO System

The following is not to be considered as a detailed study plan. It is an outline of a proposed approach to the development of a bloodstain analysis system utilizing state-of-the-art immunochemical technology.

Three fluorescent antibody approaches are proposed. The common denominator between these methodologies is the isolation of purified A and B substances. Both A and B substance are commerically available, however, in order to assure the quality and purity of such antigenic material, further fractionation and purification procedures are essential.

7.9.1.1 Isolation of Specific Human Anti-A and Anti-B Antibodies

This approach is similar to the goal of this contract, as it depends on the isolation of anti-A and anti-B from human bleeds. The project reported here utilized heat elution from intact erythrocytes as a source of antibody. This technique cannot be recommended as an antibody purification method in a fluorescent detection methodology. The human erythrocyte membrane contains a plethora of antigenic moieties, as well as the ability to reversibly bind non-antibody proteins on its surface. The non-specific heat elution method from a multivalent antigen source gives rise to eluates of mixed immunological reactivity. An alternative approach involves utilizing affinity chromatography rather than the intact

human erythrocyte. Isolated A and B substance can be coupled to a support medium, producing an immunoabsorbant with a single antigenic activity (32). The appropriate immunoabsorbant can be charged with a fluorescent γ-globulin preparation and specific labeled antibody eluted. The best elution method will have to be determined experimentally. Elution is usually accomplished by heat, pH, pH change with reagents of varying hydrophobicity, chaotropic salts, or haptinic reagents. The eluted antibody can be utliized in a direct fluorescent antibody technique.

Another alternative that should be explored with this method is the indirect staining method. The anti-A and anti-B will not be fluorescent when isolated from the immunoabsorbant medium. The fluorescent label will be introduced in a second antibody, such as goat anti-human γ-globulin. This product is commercially available.

7.9.1.2 Production of Anti-A and Anti-B in Rabbits

According to Morgan (33) and Barnes and Loutix (34), the best anti-A sera are those produced by injecting purified human A substance, coupled to an antigen from Shigella shigae into rabbits. The rabbits are tested for the presence of A-like substance and naturally occurring anti-A in their serum. The rabbits are immunized with purified A and B substance by thrice-weekly intravenous injections of 1 mg of antigen. Serum from these hyperimmune rabbits require only a single absorption with human type O cells to render them specific. The reactive globulins can be

isolated by the sodium sulfate precipitation technique. Following dialysis of the globulin fraction to remove residual sodium sulfate, the antibodies can be characterized by immunoserological tests to assure the absence of any interfering substance in the reagents.

The monoreactive globulin reagent can be used in the direct staining technique by tagging fluorescent reagent, or labeled goat anti-rabbit γ-globulin can be used in the indirect staining method. A flow diagram (Figure 7.9.1) illustrates these proposals.

A new approach to labeling globulins using fluorescamine as the fluorochrome should be considered (35). This new labeling compound offers numerous advantages over FITC; its reaction with the amine functions of protein is almost instantaneous at room temperature and the labeled products are highly fluorescent whereas fluorescamine itself and its reaction products are non-fluorescent.

7.9.2 LABS-1 Instrumentation

The current design of the LABS-1 apparatus is not sufficiently flexible to permit the proper finalization of the fluorescent methodology and instrumentation requirements. The limitations of the prototype instrument have been compiled in Table 7.9.1. A detection "front end" instrument should be designed and constructed that would give complete versatility, including simple interchangeability of filters, light sources, collimating optics, focusing optics, operature sizes,

FIGURE 7.9.1

FLOW DIAGRAM OF PROPOSED STUDIES
(ABO System)

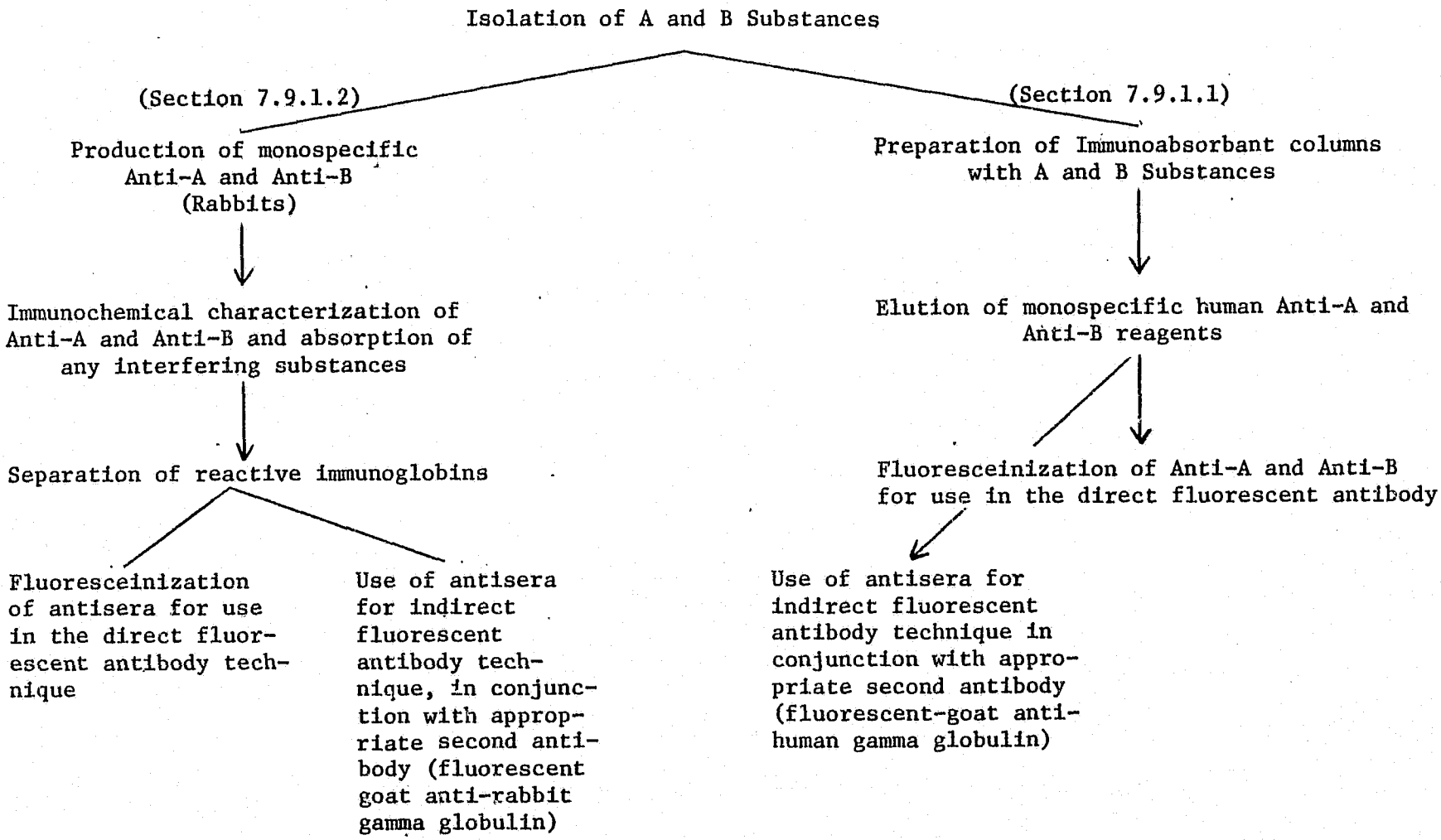


TABLE 7.9.1

PRESENT LIMITATIONS
OF LABS-1

1. Light Source Heating
2. Light Source Collimation
3. Fixed Excitation Angle
4. Fixed Excitation and Emission Apertures
5. Light Leakage from Ambient
6. Geometry
7. Inexpensive PM Tube
8. Filter Characteristics

sample configuration, etc. For the anticipated effect of these changes on the signal, see Table 7.9.2.

7.9.3 CONCLUSIONS AND RECOMMENDATIONS

At the onset of this study it was recognized that this research was of a high risk nature but with the potential of large payoff if successful. The Study Plan adopted called for simultaneous research on the primary method of fluorescent tagging and on two alternative methods. Research was to be conducted on all three of the techniques; if any of the methods proved to be infeasible they would be dropped. However, exploratory efforts directed to the simultaneous determination of feasibility of all three approaches within a four month period were discontinued in order to concentrate on development of the primary approach. Although the results supported the methodology proposed for the primary approach, they also showed that the state of the art was not sufficiently advanced to permit development, within the allocated time frame, of the accuracy necessary or desirable for application of the method to the forensic laboratory. It is felt that if sufficient resources and man-power were available to conduct the required basic research, one of these approaches could be developed into a technique of value to the forensic serologist.

If this work were reinitiated in the future, the researchers recommend the following:

- Increasing the number and scope of the consultants involved. The problem which forms the subject of this report is multifaceted requiring not only an expertise

TABLE 7.9.2

SYNOPSIS OF THE EFFECTS ON THE VARIOUS SOURCES OF SIGNAL

OF THE PROPOSED APPROACHES TO IMPROVEMENT

Proposed Approaches to Improvement	Dark Count	Background	Non-specific Fluorescence	Specific Fluorescence
1. PMT Changes				
a. Selection				
(1) Quantum Eff.	N	++	++	++
(2) Geometry		+	+	+
(3) Photocath.Size	--	+	+	+
(4) Photocath.Type	-	+	+	+
b. Utilization				
(1) Cooling	--	N	N	N
(2) Cathode-Dynode	-	+	+	+
(3) Bias Arrangement	-	+	+	+
(4) Protection	-	N	N	N
2. Filter Changes				
a. Excitation				
(1) Narrower Bandpass	N	--	-	-
(2) Higher %T	N	+	+	+
(3) Wavelength	N	+	++	++
b. Emission				
(1) Narrower Bandpass	N	--	-	-
(2) Higher %T	N	+	++	++
(3) Wavelength	N	+	++	++
3. Light Source Changes				
a. Intensity	N	++	++	++
b. Collimation	N	--	N	N
c. Cooling	--	N	N	N
4. Sample Chamber Geometry				
a. Exc. Aperture	N	+	+	+
b. Emiss. Aperture	N	+	+	+
c. Exc. Angle	N	-	+	+
d. Sample to PM Distance	N	+	+	+
e. Sample Surpace	N	-	+	+
f. Sample Size	N	+	+	+
g. Optics	N	+	+	+
5. Analysis Technique				
a. Time Gating	--	--	-	-
b. Chopped System	--	--	N	N

N No Effect
\+ Increases
\- Reduces
++ Greater Increase
-- Greater Decrease

in forensic and blood banking serology, but also in the fractionation and purification of plasma and blood group antigens and antibodies. Experience in the application of fluorescent antibody techniques to biological materials is also essential.

- Allowing sufficient flexibility in the Study Plan to accommodate creative research.
- Allowing a longer time frame for the completion of the work effort.
- Investigating at a minimum the approaches for the preparation and purification of antiserum reagents outlined in Section 7.9.1 of this report.

8. APPENDIX

This section contains the raw data associated with the reported experimentation. The data is presented in tabular form and is crossed-referenced to the appropriate subsection.

8.1 Raw Data: Development of Methodology (Section 7.4)

8.2 Raw Data: Reagent Development (Section 7.6)

8.3 Raw Data: LABS-1 Fluorescence Detection System (Section 7.8)

TABLE 8.1.1

DETERMINATION OF ANTIBODY TITER PRE AND POST FLUORESCEIN CONJUGATION

Dilution / Antisera	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	Titer**
Anti-A	4	4	4	4	4	4	4	3	3	1	±	-	512
Anti-A*	4	4	4	4	3	3	2	1	±	-	-	-	128
Anti-D	-	2	4	4	3	3	2	2	1	±	±	-	256
Anti-D*	1	4	4	4	3	3	2	1	±	±	-	-	128
Anti-B	4	4	4	4	4	4	4	3	2	2	1	±	1024
Anti-B*	4	4	4	4	4	4	3	2	1	±	-	-	256

* Indicates fluorescein conjugated antisera

** Titer is expressed as the highest antiserum dilution showing 1^{+} agglutination reaction.

See Section 7.2.1

TABLE 8.1.2

EFFECT OF TEMPERATURE ON ANTIBODY BINDING CAPACITY

Antibody Dilution	Time in Minutes 5	10	20	30
	(40°C)			
1:20	4+	4	4	4
1:40	4	3	3	3
1:80	1	1	1	1
1:160	±	±	±	±
	(42°C)			
1:20	4	4	4	4
1:40	4	3	3	3
1:80	2	2	2	2
1:160	1	1	1	1
1:320	±	±	±	±
	(45°C)			
1:20	4	4	4	4
1:40	3	3	3	3
1:80	2	2	2	2
1:160	1	1	1	1
1:320	-	-	-	-
	(48°C)			
1:20	4	4	4	4
1:40	3	3	3	3
1:80	2	2	2	2
1:160	1	1	1	1
1:320	-	-	-	-
	(50°C)			
1:20	4	4	4	4
1:40	3	3	3	3
1:80	2	2	2	2
1:160	1	1	1	1
1:320	-	-	-	-
	(Control *)			
1:20	4	4	4	4
1:40	3	3	3	3
1:80	2	2	2	2
1:160	1	1	1	1
1:320	±	±	±	±

* The dilutions were not incubated

See Section 7.4.2

TABLE 8.1.3

EFFECT OF COMMERICAL DETERGENTS COUPLED WITH TEMPERATURE ON ANTIBODY CAPACITY

Antibody Dilution	Time in Minutes 5	10	20	30
	(40°C)			
1:20	3	3	3	3
1:40	2	1	1	1
1:80	1	1	±	-
1:160	±	±	-	-
1:320	-	-	-	-
	(42°C)			
1:20	3	2	2	2
1:40	2	1	1	±
1:80	1	±	±	-
1:160	-	-	-	-
1:320	-	-	-	-
	(45°C)			
1:20	3	3	3	3
1:40	1	1	1	1
1:80	±	-	-	-
1:160	-	-	-	-
1:320	-	-	-	-
	(48°C)			
1:20	2	2	2	2
1:40	1	1	±	±
1:80	±	±	-	-
1:160	-	-	-	-
1:320	-	-	-	-
	(50°C)			
1:20	2	1	2	1
1:40	±	±	1	±
1:80	-	-	-	-
1:160	-	-	-	-
1:320	-	-	-	-
	(Control *)			
1:20	3			
1:40	2			
1:80	1			
1:160	±			
1:320	-			

* Control was not treated with detergent

** Detergent used was Cheer

See Section 7.4.3

TABLE 8.1.3 (cont.)

EFFECT OF COMMERICAL DETERGENTS COUPLED WITH TEMPERATURE ON ANTIBODY BINDING CAPACITY

Antibody Dilution	Time in Minutes 5	10	20	30
	(40°C)			
1:20	3	3	3	3
1:40	2	2	2	2
1:80	1	1	1	1
1:160	±	±	-	-
1:320	-	-	-	-
	(42°C)			
1:20	3	3	3	2
1:40	2	2	2	2
1:80	1	1	1	1
1:160	-	-	-	-
1:320	-	-	-	-
	(45°C)			
1:20	2	3	3	3
1:40	1	1	1	2
1:80	±	±	±	±
1:160	-	-	-	-
1:320	-	-	-	-
	(48°C)			
1:20	2	3	2	2
1:40	2	1	2	1
1:80	±	±	-	±
1:160	-	-	-	-
1:320	-	-	-	-
	(50°C)			
1:20	3	3	2	2
1:40	2	1	1	1
1:80	±	±	-	±
1:160	-	-	-	-
1:320	-	-	-	-
	(Control)			
1:20	3			
1:40	2			
1:80	1			
1:160	±			
1:320	-			

Detergent used was All

See Section 7.4.3

TABLE 8.2.1

DETERMINATION OF ANTIBODY TITER

Sample	Antibody	Indicator Cells	Dilution 1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Titer
S 397	Anti-A	2% A_1	4+	4+	4+	4+	3+	+	±	512
S 398		2% A_1	4+	4+	4+	3+	2+	±	0	256
S 399		2% A_1	4+	4+	4+	4+	4+	2+	±	512
AL 377-14759		2% A_1	4+	4+	4+	3+	2+	1+	±	512
23-50000		2% A_1	4+	4+	4+	4+	3+	1+	±	512
23-46887		2% A_1	4+	4+	4+	4+	4+	3+	2+	>1024
S 400	Anti-B	2% B	4+	4+	4+	2+	±	0	0	128
S 401		2% B	4+	4+	+	±	0	0	0	64
S 402		2% B	3+	2+	+	±	0	0	0	64
BL 116-13112		2% B	4+	3+	2+	2+	1+	±	0	256
23-72165		2% B	2+	1+	±	±	0	0	0	32
40-49117		2% B	4+	4+	4+	3+	2+	1+	±	512

All readings done macroscopically

Text reference, Table 7.5.1

TABLE 8.2.2

DETERMINATION OF ANTIBODY TITER AFTER ABSORPTION FOR COLD AGGLUTININS

Sample	Procedure	Indicator Cells	Dilution 1:12	1:24	1:48	1:96	1:192	1:384	1:768	Titer
S-397	Unabsorbed	2% A_1	4+	4+	3+	2+	1+	1+	±	384
	Absorbed O+P+		4+	4+	3+	1+	±	±	0	96
S-399	Unabsorbed	2% A_1	4+	4+	4+	3+	2+	±	±	384
	Absorbed O+P+		3+	3+	2+	1+	±	0	0	96
S-400	Unabsorbed	2% B	4+	4+	3+	2+	±	0	0	96
	Absorbed O+P+		3+	3+	2+	1+	±	0	0	96

Text Reference, Table 7.6.2

TABLE 8.2.3

ANTIBODY TITER AFTER ABSORPTION

Sample	Procedure	Indicator Cells	Dilution 16	32	64	128	256	512	1024	2048	Titer
Anti-A	unabsorbed	2% A_1	4+	4+	4+	3+	2+	1+	±	0	512
	O^+P^+ (1/8)*	2% A_1	4+	4+	3+	3+	2+	1+	±	0	512
	O^+P^+ (1/4)	2% A_1	4+	4+	4+	3+	2+	1+	±	0	512
	O^+P^+ (1/2)	2% A_1	4+	4+	4+	3+	2+	1+	±	0	512
	O^+P^+ (1/1)	2% A_1	4+	4+	3+	3+	2+	1+	±	0	512
Anti-B	unabsorbed	2% B	4+	3+	2+	1+	±	0	0	0	128
	$O^{+++}P^+$ (1/8)	2% B	3+	3+	2+	1+	±	0	0	0	128
	O^+P^+ (1/4)	2% B	4+	3+	2+	±	±	0	0	0	64
	O^+P^+ (1/2)	2% B	4+	3+	2+	1+	±	0	0	0	128
	O^+P^+ (1/1)	2% B	4+	3+	2+	1+	±	0	0	0	128

*Ratio indicates volume of washed RBC's per volume of plasma

Text reference, section 7.6.2

TABLE 8.2.4

FLUOROSCEIN CONJUGATION OF ABSORBED BLEEDS

Sample	Procedure	Indicator Cells	Dilution 8	16	32	64	128	256	512	Titer
Anti-A	unabsorbed	2% A_1	4+	4+	4+	4+	3+	2+	1+	512
	absorbed O^+P^+	2% A_1	4+	4+	4+	4+	3+	2+	1+	512
	FITC-conjugated	2% A_1	1+	1+	±	0	0	0	0	16
Anti-A	unabsorbed	2% A_1	4+	4+	4+	4+	3+	2+	1+	512
	absorbed O^+P^+	2% A_1	4+	4+	4+	4+	3+	3+	1+	512
	FITC-conjugated	2% A_1	4+	4+	3+	1+	±	0	0	64
Anti-B	unabsorbed	2% B	4+	4+	2+	2+	1+	±	0	128
	absorbed O^+P^+ +	2% B	4+	3+	3+	2+	1+	±	0	128
	FITC-conjugated	2% B	1+	±	0	0	0	0	0	8

Text reference, section 7.6.3

TABLE 8.2.5

FITC-CONJUGATION OF ANTI-A BLEED

Sample	Procedure	Indicator Cells	Dilution 8	16	32	64	128	256	512	Titer
Anti-A	Absorbed O+P+	2% A_1	4+	4+	4+	4+	4+	2+	1+	512
	FITC-modif 1		4+	3+	2+	1+	±	0	0	64
	FITC-modif 2		4+	4+	2+	1+	1+	±	0	128
	FITC-modif 3		4+	4+	3+	2+	2+	1+	±	256
	FITC-modif 4		4+	4+	3+	2+	1+	1+	±	256

For definitions and conditions, see Table 7.6.3.

TABLE 8.2.6

ABSORPTION-ELUTION

Sample	Procedure	Dilution 3	6	12	24	48	96	192	384	Titer
Anti-A S-397	Absorbed O+P+	4+	4+	4+	4+	3+	1+	±	±	96
	FITC-conjugated	2+	2+	1+	±	±	0	0	0	12
	After A_2 absorption	0	0	0	0	0	0	0	0	0
	Eluate	1+	1+	±	±	0	0	0	0	6
	Eluate, conc. 10x	3+	1+	±	0	0	0	0	0	6
Anti-B S-400	Absorbed O+P+	4+	3+	3+	3+	2+	1+	±	±	96
	FITC-conjugated	2+	1+	±	0	0	0	0	0	6
	After B absorption	0	0	0	0	0	0	0	0	0
	Eluate	0	0	0	0	0	0	0	0	0
	Eluate, conc. 10x	0	0	0	0	0	0	0	0	0
	Eluate, conc. 25x	1+	1+	±	0	0	0	0	0	6
Anti-A S-399	Absorbed O+P+	4+	4+	3+	3+	2+	1+	±	±	96
	FITC-conjugated	4+	4+	3+	1+	1+	±	0	0	48
	After A_2 absorption	0	0	0	0	0	0	0	0	0
	Eluate	1+	1+	1+	±	0	0	0	0	12
	Eluate, conc. 10x	2+	1+	±	±	0	0	0	0	6

Text Reference, Table 7.6.4.

TABLE 8.2.7

ABSORPTION-ELUTION

Sample	Procedure	Dilution 4	8	16	32	64	128	256	512	Titer
Anti-A S-399	FITC-conjugated	3+	3+	2+	2+	1+	0	0	0	64
Aliquot 1	Post 1st absorption	2+	1+	±	0	0	0	0	0	8
Aliquot 2	Post 2nd absorption	3+	2+	1+	±	0	0	0	0	16
Aliquot 3	Post 3rd Absorption	3+	2+	1+	±	0	0	0	0	16
Aliquot 4	Post 4th Absorption	3+	2+	±	0	0	0	0	0	8
Eluate 1		4+	4+	3+	3+	2+	1+	±	0	128
Eluate 2		4+	4+	4+	4+	3+	2+	1+	0	256
Eluate 3		4+	4+	3+	2+	1+	0	0	0	64

Text Reference, Table 7.6.5.

TABLE 8.2.8

FLUORESCENCE MEASUREMENTS OF ANTI-A ELUATES

Sample	Photons/10 seconds	Average
Holder-empty	4547	4552
	4556	
Filter-dry	68469	68472
	68475	
Filter + H_2O (20 µl)	47638	47735
	47831	
Filter + eluate 1 (20 µl)	273394	274487
	275580	
Filter + eluate 2 (20 µl)	310850	310369
	309887	
Filter + eluate 3 (20 µl)	245655	243357
	241059	

All readings taken on LABS-1 using 2.5 cm Oxoid filter saturated with 20 µl of sample with LABS-1 standard sample holder.

Text reference, Table 7.6.5.

TABLE 8.2.9

DETERMINATION OF A AND B SUBSTANCE IN HUMAN SERUM ALBUMIN

Sample	Procedure	Dilution									Titer
		6	12	24	48	96	192	384	768	1536	
Anti-A (1:3)	5% HSA	4+	3+	3+	2+	2+	1+	±	0	0	192
Anti-A (1:3)	1% HSA	3+	3+	3+	2+	2+	1+	±	^	0	192
Anti-A (1:3)	PBS	3+	3+	3+	2+	1+	±	0		0	96
Anti-B (1:3)	5% HSA	4+	3+	3+	2+	1+	1+	±	0	0	192
Anti-B (1:3)	1% HSA	4+	4+	4+	3+	2+	2+	1+	±	0	384
Anti-B (1:3)	PBS	4+	4+	3+	3+	2+	1+	±	0	0	192

Diluted antibody preparations were incubated 20 minutes at 25°C with one volume of either HSA or PBS prior to titration.

Text reference, section 7.6.5.

TABLE 8.2.10

EFFECT OF TEMPERATURE ON HEAT-ELUTION

Sample	Anti-A plasma AL 377-14759 Dilution								Titer
	4	8	16	32	64	128	256	512	
FITC-anti-A	4+	4+	4+	3+	2+	1+	±	0	128
post A_2 absorption	4+	4+	3+	2+	1+	±	0	0	64
eluate 1 (45°C)	4+	4+	4+	2+	1+	±	0	0	128
eluate 2	4+	4+	4+	4+	3+	2+	1+	0	256
eluate 3	4+	4+	3+	2+	1+	0	0	0	64
eluate 1 (56°C)	4+	4+	4+	3+	2+	1+	1+		256
eluate 2	4+	4+	4+	3+	2+	1+	±	0	128
eluate 3	3+	2+	2+	1+	±	±	0	0	32

10 volumes FITC-anti-A plasma absorbed with 1 volume washed A_2 cells. Elution at 45° and 56°C with 1% HSA.

Text reference, section 7.6.6.

TABLE 8.2.11

PRODUCTION OF ANTI-B ELUATES

Sample	Dilution 4	8	16	32	64	128	256	512	1024	Titer
Anti-B (BL 116-13112) absorbed O^+P^+		4+	4+	4+	3+	3+	2+	1+	±	512
FITC-Anti-B		4+	4+	3+	3+	3+	2+	±	0	256
Post-absorption 10 ml	0	0	0	0	0	0	0	0		0
15 ml	0	0	0	0	0	0	0	0		0
20 ml	0	0	0	0	0	0	0	0		0
30 ml	0	0	0	0	0	0	0	0		0
45 ml	0	0	0	0	0	0	0	0		0
60 ml	3+	±	±	0	0	0	0	0		4
eluate 1 (45^oC)	4+	4+	3+	2+	1+	1+	±	0		128
eluate 2	4+	3+	2+	2+	1+	±	0	0		64
eluate 3	3+	3+	2+	2+	1+	±	0	0		64
eluate 1 (56^oC)	3+	3+	2+	1+	±	0	0			32
eluate 2	4+	3+	2+	1+	±	0	0			32
eluate 3	4+	2+	1+	±	±	0	0			16
eluate 4	3+	2+	±	0	0	0	0			8

1.0 ml of washed type B cells used for absorption. Each eluate in 1% HSA in PBS, volume 1.0 ml at indicated temperature.

Text reference, section 7.5.7.

TABLE 8.2.12

BLOODSTAIN ANALYSIS

STANDARD METHODOLOGY

Bloodstain/Antibody	Prereading	Final Reading	Difference	Average Difference
A/anti-A	3788	5305	1517	1584
	3420	4993	1573	
A/anti-A	3316	4908	1592	
	3095	4748	1653	
B/anti-A	3096	4331	1235	1300
	2977	4174	1197	
B/anti-A	2651	4016	1365	
	2839	4243	1404	
AB/anti-A	2777	4013	1236	1182
	2700	3959	1259	
AB/anti-A	2614	3712	1098	
	2736	3872	1136	
O/anti-A	2842	4260	1418	1443
	2823	4204	1381	
O/anti-A	2765	4269	1504	
	2687	4155	1468	

LABS-1 configuration - first modification

TABLE 8.2.13

BLOODSTAIN ANALYSIS

STANDARD METHODOLOGY

Bloodstain/Antibody	Prereading	Final Reading	Average Difference	Average Difference
B/anti-A	4036	6013	1977	1941
B/anti-A	4192	6097	1905	
A/anti-A	4343	6611	2268	2155
A/anti-A	4065	6106	2041	
AB/anti-A	3871	5833	1962	1934
AB/anti-A	4127	6033	1906	
O/anti-A	4014	6133	2119	1972
O/anti-A	3838	5662	1824	

LABS-1 configuration - first modification

TABLE 8.2.14

BLOODSTAIN ANALYSIS

STANDARD METHODOLOGY

Bloodstain/Antibody	Prereading	Final Reading	Difference	Average Difference
A/anti-A	6241	7255	1014	1132
A/anti-A	6295	7544	1249	
B/anti-A	6003	7257	1254	1242
B/anti-A	5986	7216	1230	
O/anti-A	5814	7251	1437	1483
O/anti-A	5772	7300	1528	
A/anti-B	5768	7034	1266	1254
A/anti-B	6036	7278	1242	
B/anti-B	5459	6836	1377	1431
B/anti-B	5381	6866	1485	
O/anti-B	5420	7028	1608	1519
O/anti-B	5468	6898	1430	

Text reference, section 7.6.10

LABS-1 configuration - first modification

TABLE 8.2.15

EFFECT OF PRETREATMENT OF STAINS WITH 5% HSA

Stain/Antibody	Preread		Final		Difference	
	A	B	A	B	A	B
A/anti-A	2530	2545	4874	4736	2344	2191
B/anti-A	2747	2712	4923	4896	2176	2184
AB/anti-A	2752	2507	4555	3046	1803	539
O/anti-A	2694	2597	4661	4633	1967	2036
A/anti-B	2615	2608	4014	3507	1399	899
B/anti-B	2644	2633	4160	3929	1516	1296
AB/anti-B	2497	2675	3577	3313	1080	638
O/anti-B	2615	2603	3721	3641	1106	1038

A = pretreatment with 5% Human Serum Albumin

B = no pretreatment

Text reference, section 7.6.10

LABS-1 configuration - first modification

TABLE 8.2.16

EFFECT OF ANTIBODY REACTION TIME ON FIXED BLOODSTAINS

Stain/Antibody	Preread		Final		Difference	
	A	B	A	B	A	B
A/anti-A	3019	2960	5274	5534	2255	2574
B/anti-A	2863	3028	4700	5167	1837	2139
AB/anti-A	2944	3176	3890	4646	946	1470
O/anti-A	3146	3051	4877	4782	1731	2006

A = FITC-anti A eluate (0.3 ml) applied for 45 minutes at 25°C.

B = FITC-anti A eluate (0.3 ml) applied for 45 minutes at 25°C, aspirated off, and reapplied for 16 hours at 4°C.

Text reference, section 7.6.10

LABS-1 configuration - first modification

TABLE 8.2.17

EFFECT OF FIXATION ON BLOODSTAIN ANALYSIS

Stain/Antibody	Preread	Final	Difference
A/anti A	8213	22264	14051
B/anti A	7853	27215	19362
AB/anti A	9507	30213	20706
O/anti A	8634	19182	10548
A/anti B	7525	28872	21347
B/anti B	9628	29294	19666
AB/anti B	8329	34850	26521
O/anti B	6744	21054	14310

Text reference , section 7.6.10

LABS-1 configuration - first modification

TABLE 8.2.18

FRACTIONATION OF ANTI A BLEED WITH AMMONIUM SULFATE

Sample	Dilution									Titer
	2	4	8	16	32	64	128	256	512	
Anti A (AL 377-14759)	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
O^+P^+ absorption	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
First $(NH_4)_2$ SO_4 precipitation	4+	4+	4+	4+	3+	3+	2+	0	0	128
Second $(NH_4)_2$ SO_4 precipitation	4+	4+	4+	4+	3+	2+	1+	0	0	128
γ-globulins at pH 9.0	4+	4+	4+	3+	3+	2+	2+	0	0	128
FITC-anti A γ-globulins	4+	4+	4+	4+	3+	2+	1+	±	0	128
After absorption with A_2 cells (1:10)	4+	4+	4+	3+	2+	1+	±	0	0	64
Eluate 1	4+	4+	4+	3+	3+	2+	1+	±	0	128
Eluate 2	4+	4+	4+	4+	4+	3+	2+	1+	0	256
Eluate 3	4+	4+	4+	3+	2+	1+	0	0	0	64

Text reference, section 7.6.13

TABLE 8.2.19

FRACTIONATION OF ANTI A BLEED WITH SODIUM SULFATE

Sample	Dilution									Titer
	2	4	8	16	32	64	128	256	512	
Anti A (23-50600)	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
O^+P^+ absorption	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
First Na_2SO_4 precipitation										NA
Second Na_2SO_4 precipitation	4+	4+	4+	4+	4+	3+	3+	2+	1+	512
γ-globulins at pH 9.0										NA
FITC-anti A γ-globulins	4+	4+	3+	3+	2+	2+	1+	1+	±	256
After absorption with A_2 cells (1:10)	4+	4+	4+	3+	3+	3+	2+	1+	±	256
Eluate 1	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
Eluate 2	4+	4+	4+	3+	3+	2+	2+	1+	±	256
Eluate 3	4+	4+	4+	3+	3+	2+	1+	1+	±	256

Text reference, section 7.6.13

TABLE 8.2.20

FRACTIONATION OF ANTI B BLEED WITH AMMONIUM SULFATE

Sample	Dilution									Titer
	2	4	8	16	32	64	128	256	512	
Anti B	4+	4+	4+	4+	4+	3+	3+	2+	1+	512
O^+P^+ absorption	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
First $(NH_4)_2$ SO_4 precipitation										NA
Second $(NH_4)_2$ SO_4 precipitation	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
γ-globulins at pH 9.0										NA
FITC-anti B γ- globulins	4+	4+	4+	4+	4+	3+	3+	2+	0	256
After absorption with B cells (1:20)	0	0	0	0	0	0	0	0	0	0
Eluate 1	3+	3+	2+	1+	±	0	0	0	0	16
Eluate 2	3+	3+	1+	0	0	0	0	0	0	8
Eluate 3	3+	3+	2+	1+	0	0	0	0	0	16

NA; data not available

Text reference, section 7.6.13

TABLE 8.2.21

FRACTIONATION OF ANTI B BLEED WITH SODIUM SULFATE

Sample	Dilution 2	4	8	16	32	64	128	256	512	Titer
Anti B	4+	4+	4+	4+	4+	3+	3+	2+	1+	512
O^+P^+ absorption	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
First Na_2SO_4 precipitation	4+	4+	4+	4+	4+	4+	3+	2+	1+	512
Second NA_2SO_4 precipitation	4+	4+	4+	4+	3+	3+	3+	2+	±	256
γ-globulins at pH 9.0	4+	4+	4+	4+	3+	2+	2+	1+	±	256
FITC-anti B -globulins	4+	4+	4+	3+	3+	2+	1+	±	±	128
After absorption with B cells (1:60)	2+	0	0	0	0	0	0	0	0	2
Eluate 1	3+	3+	3+	2+	1+	±	0	0	0	32
Eluate 2	4+	4+	3+	2+	1+	±	0	0	0	32
Eluate 3	4+	4+	2+	1+	±	±	0	0	0	16

Text reference, section 7.6.13

TABLE 8.2.22

BLOODSTAIN ANALYSIS WITH LABS-1

Bloodstain/Antibody	Pre-reading	Final reading	Difference	Average
A/anti A-γ globulin	5375	19309	13934	13572
	5647	16682	11035	
	5273	22380	17107	
	5983	18194	12211	
B/anti A-γ globulin	6338	32488	26150	19517
	7351	22575	15224	
	5865	29085	23220	
	7233	20706	13473	
AB/anti A-γ globulin	4953	25046	20093	16077
	6356	19087	12731	
	5340	20803	15463	
	5497	21517	16020	
O/anti A-γ globulin	4967	23019	18050	14724
	5659	19346	13684	
	4716	21209	16493	
	5287	15952	10666	

Numerical values are average of three consecutive readings, expressed as photons/10 seconds.

Text reference, section 7.6.13

LABS-1 configuration - second modification

TABLE 8.2.22 (cont.)

BLOODSTAIN ANALYSIS WITH LABS-1

Bloodstain/Antibody	Pre-reading	Final reading	Difference	Average
A/anti B-γ globulin	5522	18615	13093	11327
	5352	15952	10600	
	5479	18861	13382	
	4682	12913	8231	
B/anti B-γ globulin	6256	20867	14611	16694
	6007	16172	10165	
	5818	18343	12525	
	5641	18421	12780	
AB/anti B-γ globulin	4582	18399	13817	14433
	3892	18044	14152	
	5007	24056	19049	
	4562	15277	10715	
O/anti B-γ globulin	4840	16496	11656	11331
	4673	15809	11136	
	4465	14074	9609	
	4649	17571	12922	

γ globulins were prepared by sodium sulfate fractionation

LABS-1 configuratión - second modification

TABLE 8.2.23

FLUORESCEIN SUBSTITUTION OF γ-GLOBULINS

%FITC added	Protein conc. (mg/ml)	OD_{495} (1/40 dilution)	F/P	HA Titer
0	-	-	-	512
2	9.01	0.302	4.0	256
3	8.27	0.370	5.4	256
4	7.63	0.410	6.4	256
5	7.09	0.425	7.2	256
6	6.62	0.414	7.5	128

Text reference, section 7.6.13

TABLE 8.2.24

ABSORPTION-ELUTION OF UNFIXED BLOODSTAINS

Bloodstain/antibody	Dilution U	2	4	8	16	32	64	Titer
A/anti A-γ	4+	3+	2+	±	0	0	0	4
B/anti A-γ	0	0	0	0	0	0	0	0
AB/anti A-γ	4+	3+	3+	2+	1+	0	0	16
O/anti A-γ	0	0	0	0	0	0	0	0
A/anti B-γ	0	0	0	0	0	0	0	0
B/anti B-γ	4+	3+	3+	1+	±	0	0	8
AB/anti B-γ	4+	4+	3+	2+	±	±	0	8
O/anti B-γ	0	0	0	0	0	0	0	0

Text reference, section 7.6.14

TABLE 8.2.25

ABSORPTION-ELUTION OF UNFIXED ALBUMIN PRETREATED BLOODSTAINS

Bloodstain/antibody	Dilution U	2	4	8	16	32	64	Titer
A/anti A-γ	4+	3+	2+	1+	±	0	0	8
B/anti A-γ	0	0	0	0	0	0	0	0
AB/anti A-γ	4+	3+	2+	1+	±	0	0	8
O/anti A-γ	0	0	0	0	0	0	0	0
A/anti B-γ	0	0	0	0	0	0	0	0
B/anti B-γ	4+	3+	3+	2+	1+	0	0	16
AB/anti B-γ	4+	3+	3+	2+	1+	±	0	16
O/anti B-γ	0	0	0	0	0	0	0	0

Pretreatment was with 5% HSA for 5 minutes at 25°C prior to reaction with antibody.

Initial titer of anti-Aγ-globulin, 256

Initial titer of anti-Bγ-globulin, 512

Text reference, section 7.6.14.

TABLE 8.2.26

ABSORPTION-ELUTION OF FIXED BLOODSTAINS

Bloodstain/antibody	Dilution U	2	4	8	16	32	64	Titer
A/anti A-γ	3+	2+	2+	±	0	0	0	4
B/anti A-γ	0	0	0	0	0	0	0	0
AB/anti A-γ	3+	2+	1+	0	0	0	0	4
O/anti A-γ	0	0	0	0	0	0	0	0
A/anti B-γ	0	0	0	0	0	0	0	0
B/anti B-γ	4+	4+	3+	2+	1+	1+	0	32
AB/anti B-γ	4+	3+	3+	1+	±	0	0	8
O/anti B-γ	0	0	0	0	0	0	0	0

Text reference, section 7.6.14.

CONTINUED

3 OF 4

TABLE 8.2.27

ABSORPTION-ELUTION OF FLUORESCENT ANTIBODY

Bloodstain/Antibody	LABS-1* Preread	LABS-1* Final	LABS-1* Difference	H. A. Titer (undiluted)	Aminco-Bowman** No. 1	Aminco-Bowman** No. 2
A/F-anti A-γ	5923 ± 56.9	8474 ± 113.6	2551	3+	3005	2985
A/F-anti A-γ	6035 ± 49.5	8662 ± 119.8	2627	2+		
B/F-anti A-γ	5867 ± 46.3	9239 ± 123.4	3372	0	4950	4960
B/F-anti A-γ	6130 ± 60.3	9879 ± 116.6	3749	0		
AB/F-anti A-γ	6005 ± 55.8	8205 ± 105.0	2200	2+	3060	3110
AB/F-anti A-γ	5841 ± 49.9	8169 ± 100.2	2328	2+		
O/F-anti A-γ	5899 ± 43.7	8376 ± 136.6	2477	0	4300	4280
O/F-anti A-γ	5952 ± 57.7	8261 ± 110.7	2309	0		

* LABS-1 readings are expressed in photons/10 seconds. Each data point is average of 5 determinations.

** Aminco-Bowman, E_x = 460 mm, E_m = 520 nm

No. 1 reading done on pool of duplicates

No. 2 rereading done on pool of duplicates after absorption with activated charcoal.

Text reference, section 7.6.14

LABS-1 configuration - second modification

TABLE 8.2.28

ANTIGEN: BLOODSTAIN TYPE A

REAGENT: FITC-A γ-GLOBULIN

Reading No. 1			Reading No. 2			Normalized Ratio
M_1	M_1-D	S_{N_1} (X10^3)	M_2	M_2-D	S_{N_2} (X10^3)	S_{N_2}/S_{N_1}
6268	3124	41	13141	9997	132	3.2
6613	3469	46	12146	9002	119	2.6
6031	2887	38	12055	8911	118	3.1
6113	2969	39	11767	8623	114	2.9
6331	3187	42	13453	10309	136	3.2
6205	3061	40	9884	6740	89	2.2
5821	2677	35	12571	9427	125	3.6
6438	3294	44	10588	7444	98	2.2
Mean Values		40.6			116.4	2.9

Data normalized according to general formula: $S_N = \frac{M-D}{F-D}$

D = 3114

F = 78799

Text reference, section 7.6.15

LABS-1 configuration - second modification

TABLE 8.2.29

ANTIGEN: BLOODSTAIN TYPE B

REAGENT: F-ANTI A γ-GLOBULIN

Reading No. 1			Reading No. 2			Normalized Ratio
M_1	M_1-D	S_{N_1} (X10^3)	M_2	M_2-D	S_{N_2} (X10^3)	S_{N_2}/S_{N_1}
6706	3562	47	14068	10924	144	3.1
6652	3508	46	11580	8436	112	2.4
6748	3604	48	13689	10545	139	2.9
6790	3646	48	14796	11652	154	3.2
7003	3859	51	17251	14107	186	3.6
6627	3483	46	13827	10683	141	3.1
6473	3329	44	12763	9619	127	2.9
6169	3025	40	14927	11783	156	3.9
6471	3327	44	13902	10758	142	3.2
6635	3491	46	13875	10731	142	3.1
Mean Values		46			144.3	3.1

Data normalized according to general formula: $S_N = \frac{M-D}{F-D}$

D = 3114

F = 78799

Text reference, section 7.6.15.

LABS-1 configuration - second modification

TABLE 8.2.30

ANTIGEN: BLOODSTAIN TYPE O

REAGENT: F-ANTI A γ-GLOBULIN

Reading No. 1			Reading No. 2			Normalized Ratio
M_1	M_1-D	S_{N_1} (X10^3)	M_2	M_2-D	S_{N_2} (X10^3)	S_{N_2}/S_{N_1}
5305	2161	29	11197	8053	106	3.7
5674	2530	33	8903	5589	74	2.2
4759	1615	21	8221	5077	67	3.2
5240	2096	28	9927	6783	90	3.2
5087	1943	26	9466	6322	84	3.2
5444	2300	30	10980	7836	104	3.5
5228	2084	28	10154	7010	93	3.3
5037	1893	25	9868	6724	89	3.6
5182	2038	27	10194	7050	93	3.4
5099	1955	26	12118	8974	119	4.6
Mean values		27.3			91.9	3.4

Data normalized according to general formula: $S_N = \frac{M-D}{F-D}$

D = 3114

F = 78799

Text reference, section 7.6.13.

LABS-1 configuration - second modification

TABLE 8.3.1

STABILITY TEST OF LABS-1

BACKGROUND READINGS*

	A.M.	NOON	P.M.
	2626	2748	2301
	2445	2907	2195
	2381	2844	2171
	2444	2717	2230
	2405	2791	2212
	2439	2762	2274
	2427	2716	2226
	2422	2885	2237
	2489	2737	2181
	2493	2751	2244
Mean	2457.1	2785.8	2227.1
Standard Deviation	± 68.41	± 69.23	± 40.26

* All numbers are photons/30 seconds

Text reference, Section 7.8.2

LABS-1 configuration - unmodified

TABLE 8.3.2

TESTING OF LABS-1 WITH BLOOD STAINS BY INDIRECT FLUORESCENT

ANTIBODY TECHNIQUE

Bloodstain Sample	Photons/10 Seconds Reading Number 1	2	3	4	5	Mean	Standard Deviation (%)	Student's t-Test P
A/anti-A	1806	1790	1712	1778	1854	1788.0	± 51.38 (2.87)	< 0.001
A/anti-B	1574	1513	1548	1571	1530	1547.2	± 26.22 (1.69)	
A/anti-A	1933	1901	1921	1983	1948	1937.2	± 30.84 (1.59)	< 0.001
A/anti-B	1789	1795	1760	1708	1725	1755.4	± 38.37 (2.19)	
B/anti-A	1466	1494	1449	1416	1474	1459.8	± 29.34 (2.01)	< 0.001
B/anti-B	1606	1677	1700	1612	1754	1669.8	± 62.17 (3.72)	
B/anti-A	1530	1452	1512	1539	1577	1522.0	± 45.76 (4.01)	< 0.001
B/anti-B	1892	1819	1890	1865	1884	1879.0	± 30.43 (1.62)	
				Repeat Study, Day 2				
A/anti-A	2086	2099	2988	2140	2059	2094.4	± 29.43 (1.41)	< 0.001
A/anti-B	1601	1701	1623	1711	1677	1662.6	± 48.44 (2.91)	
A/anti-A	2098	2149	2028	2078	1975	2065.6	± 66.67 (3.23)	< 0.001
A/anti-B	1758	1721	1830	1835	1700	1768.8	± 61.77 (3.49)	
B/anti-A	1506	1468	1580	1552	1521	1525.4	± 42.95 (2.82)	< 0.001
B/anti-B	1962	1990	1999	1911	1908	1954.0	± 42.86 (2.19)	
B/anti-A	TECHNICAL FAILURE							
B/anti-B								

TABLE 8.3.3

FILTER SELECTION

Ex	Em	F_{STD}	W_{STD}	F_{STD}/W_{STD}	F_{STD}/W_{STD}
470 N.B.	530 N.B.	203,817	8,151	12,231	25
500 Cutoff	520 N.B.	362,060	81,977	280,083	4.42
500 Cutoff	530 N.B.	716,559	37,085	679,479	19.32
500 Cutoff	540 N.B.	698,092	33,842	664,250	20.63
500 Cutoff	520 Cut-On	670,000	2,317,639	-1,647,639	0.29
500 Cutoff	540 Cut-On	898,582	2,238,428	-1,339,846	0.40

All data are expressed as counts per second and are the averages of Replicates. Ex represents Excitation Filter; Em represents Emission Filter; F_{STD} represents reference Fluorescence Standard; W_{STD} represents reference White Reflectance Standard.

Text reference, section 7.8.3

LABS-1 configuration - first modification

TABLE 8.3.4

MEASUREMENT REPRODUCIBILITY

TEST I

Sample	1st Reading	2nd Reading	3rd Reading	Average
W_{STD}	37,068	36,924	37,264	37,085
F_{STD}	699,519	726,400	723,757	716,559
D_{STD}	1,090	1,094	1,089	1,091
Lamp Off	144	156	147	149

TEST II

W_{STD}	36,084	35,584	35,834	35,834
F_{STD}	717,467	724,758	---	721,113
D_{STD}	1,144	1,144	---	1,144

Data are counts per second. The two tests were performed at different times of the same day. F_{STD} references Fluorescent Standard; W_{STD} references White Reflectance Standard; D_{STD} references Flat Black Standard. Emission filter: 530 nanometer narrow band. Excitation filter: 500 nanometer cutoff.

Text reference, section 7.8.3

LABS-1 configuration - first modification

TABLE 8.3.5

EFFECT OF LINE VOLTAGE VARIATIONS ON LABS-1 MEASUREMENTS

Line Voltage	CVT Output	Black Standard	Reflectance Standard	FITC-Reflectance Standard
121	119	7587	247438	4963708
116	119	7108	231522	4500739
111	119	6829	213750	4282348
105	119	6561	194503	3816397

All measurements are photons/10 seconds

Text reference, section 7.8.3

LABS-1 configuration - first modification

TABLE 8.3.6

EFFECT OF TIME ON LABS-1 MEASUREMENTS *

Time	Black Standard	Reflectance Standard	FITC-Reflectance Standard	SN ($X10^3$)
11:45 am	8091 ± 128	183041 ± 1286	3955293 ± 113251	44.3
12:30 pm	8657 ± 54	195867 ± 1741	4210072 ± 154089	44.6
1:15 pm	8311 ± 86	183940 ± 230	4014763 ± 114067	44.7
2:00 pm	8309 ± 91	185580 ± 909	4087538 ± 96644	43.5
2:45 pm	8911 ± 93	200125 ± 1146	4383582 ± 111941	43.7
3:30 pm	8672 ± 95	194723 ± 1314	4202544 ± 94873	44.4
4:10 pm	8828 ± 157	201015 ± 1361	4177967 ± 75627	46.1

* Each point reported is the mean of 5 consecutive 10 second readings, expressed ± standard deviation

Text reference, section 7.8.3

LABS-1 configuration - first modification

TABLE 8.3.7 REPRESENTATIVE FILTER TEST DATA

Excitation Filter	Emission Filter	D	W	F	I	II	$\frac{W}{D}$	$\frac{F}{W}$	$\frac{II}{I}$
500 Cutoff-D	530-D	474	2,477	32,507	559	8,457	5.23	13.1	15.1
500 Cutoff-D	Special-S.T.	6,849	128,071	496,525	4,887	22,831	18.67	3.87	4.67
500 Cutoff-D	530-C	3,318	69,427	559,760	2,395	69,593	20.92	8.06	29.06
500 Cutoff-D	530 D-Sp	623	1,662	35,441	517	3,706	2.67	21.32	7.17
4884 Narrow Band -C	530 D-Sp	397	2,203	50,701	387	5,772	5.55	23.01	14.92
480 Cutoff	530 D-Sp	410	4,079	162,079	849	19,897	9.72	39.73	23.44
460 Narrow Band	530 D-Sp	286	943	17,873	236	1,467	3.3	18.95	6.21
470 Narrow Band	530 D-Sp	316	882	23,861	316	2,696	2.79	27.0	8.53

Data is a partial listing of test data obtained with various filter pairs. All data are the averages of triplicates. Filters were coded as to manufacturers and special characteristics.

Test Samples:
D = Black surface
W = White surface
F = White surface plus fluorescein
I = Blood stained cloth
II = Blood stained cloth saturated with fluorescent antibody

Text reference, section 7.8.4

LABS-1 configuration - second modification

FIGURE 8.3.1 TRANSMISSION CURVES FOR EXCITATION AND EMISSION FILTERS INSTALLED IN LABS-1:FIRST MODIFICATION

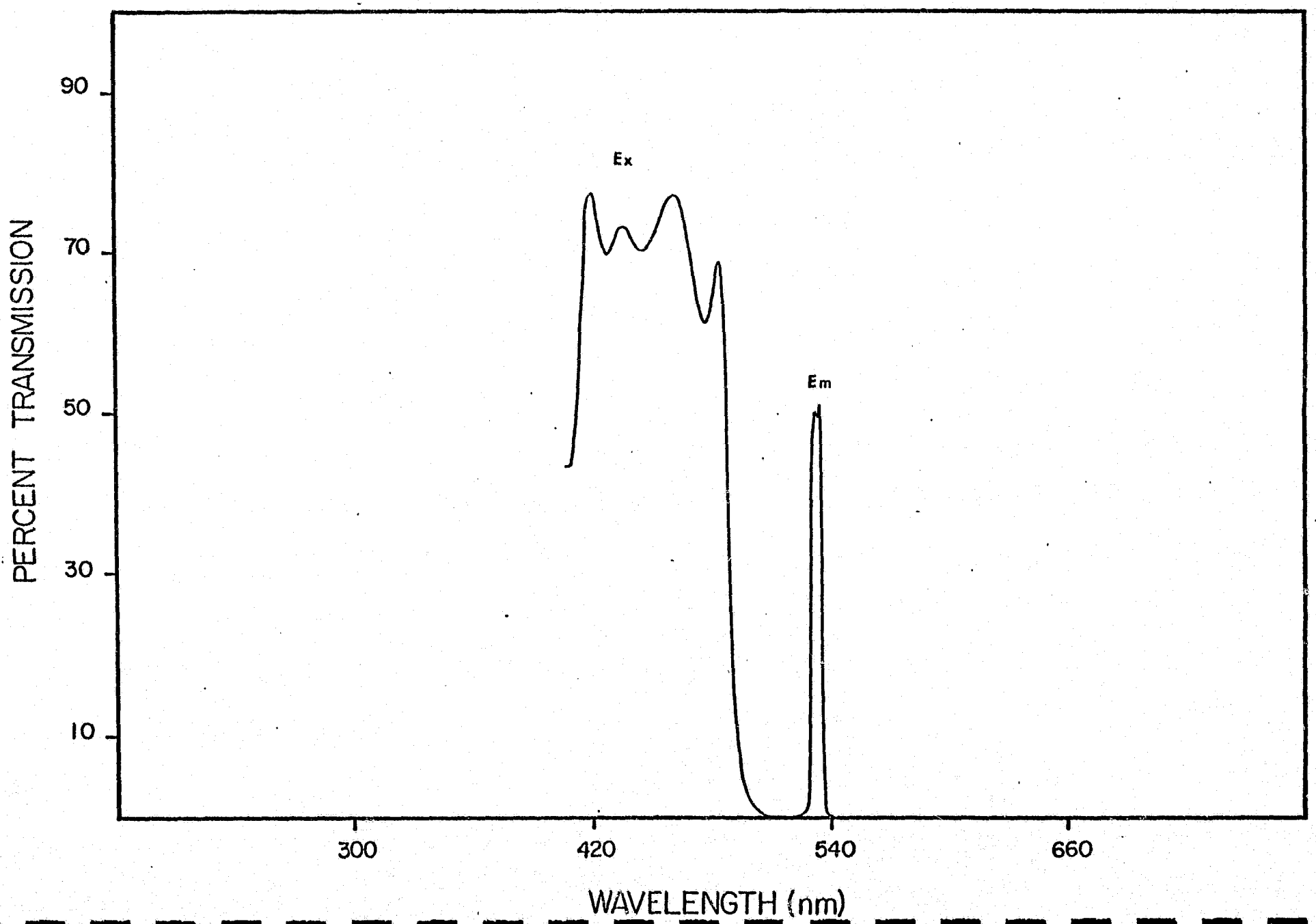
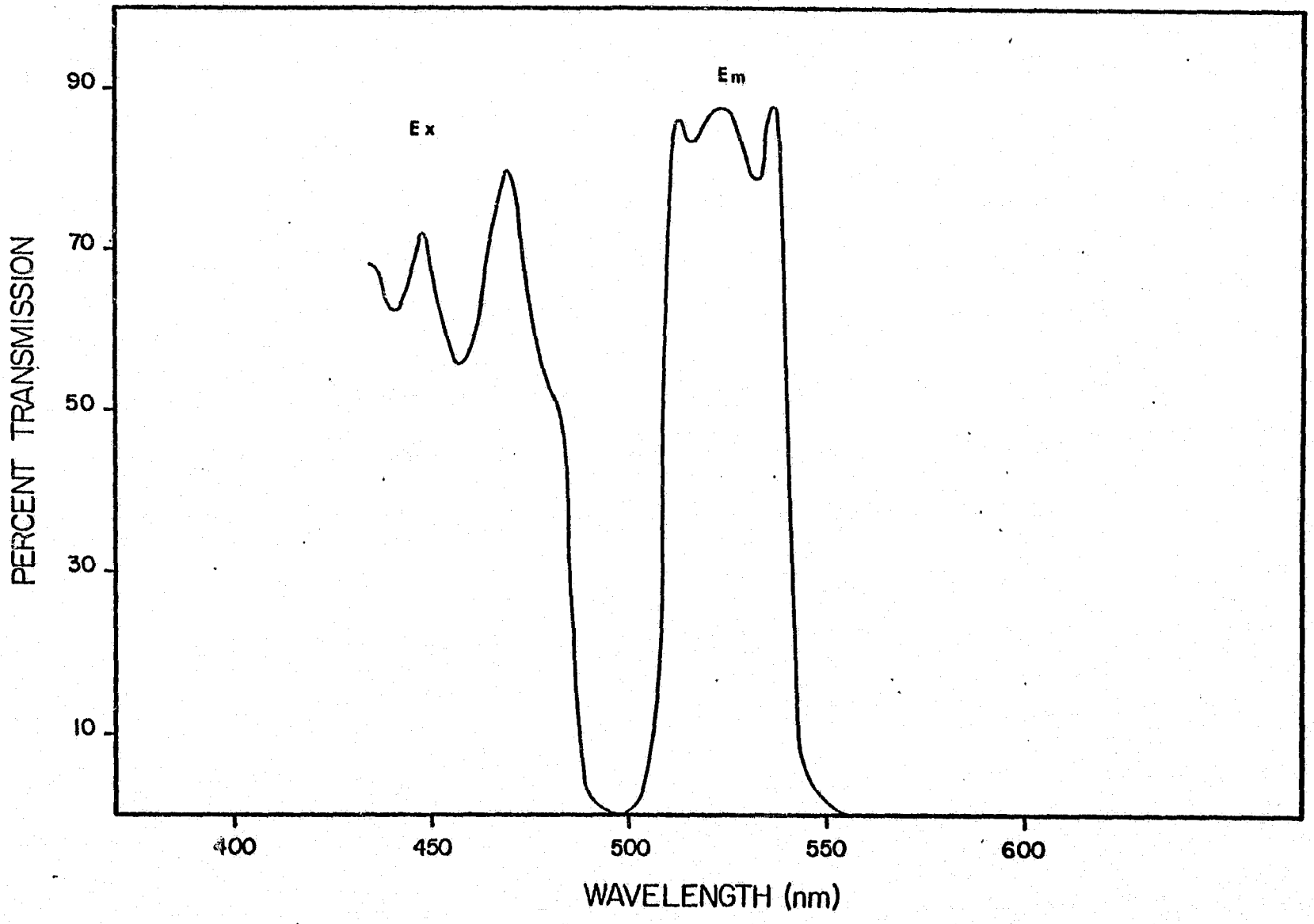


FIGURE 8.3.2 TRANSMISSION CURVES FOR EXCITATION AND EMISSION FILTERS INSTALLED IN LABS-1: SECOND MODIFICATION



8.4 PROCEDURE:

DIRECT FLUORESCENCE ELUTION METHOD

Introduction: This procedure is still under development. However, it utilizes fluorescein tagged goat anti-human IgG as a common fluoresceinized indicator system negating the necessity of individually fluoresceinizing a battery of specific blood typing sera.

Prodedure: 1. Cut a bloodstained cloth sample approximately 1-2 cm^2.

2. Fix in 2.0 ml of an equal mixture of ether/ethanol (v:v) for 10 minutes at room temperature. Then remove excess by aspiration.

3. Fix additionally in 2.0 ml of 95% ethanol for 20 minutes at room temperature. Then remove excess by aspiration.

4. Wash twice with 3.0 ml phosphate buffered saline (PBS), and add 0.4 ml of 22% albumin (DADE). Incubate at room temperature for 20 minutes. Then remove excess by aspiration.

5. Add 0.3 ml of undiluted specific antiserum (non-fluoresceinized) and incubate at room temperature for 45 minutes.

6. Wash 3 times with 3.0 ml PBS.

7. Add 0.4 ml of 22% albumin (DADE) and incubate for 20 minutes at room temperature. Then remove excess by aspiration.

8. Add 0.4 ml of fluorescein conjugated goat anti-human IgG (1:10 dilution) and incubate in the dark at room temperature for 45 minutes.

9. Recover supernatant and place in clean cuvettes (1 x 0.4 cm) (Sarsted, Princeton, N.J.) for observation of fluorescence.

10. Wash reacted bloodstain 3 times with 3.0 ml PBS.

11. Elute fluoresceinized antibody by adding 0.5 ml PBS and heating at 50° for 30 minutes. Recover supernatant and place in clean cuvettes for observation of fluorescence.

12. Read for fluorescence in Aminco-Bowman Fluorometer.

Excitation 486 nm

Emission 520 nm

Slits 4,2,1,1,2,1

525 nm cut-on filter in place

Positive Test: (Supernate) - A decrease in fluorescence of the goat antiserum after reacting with human IgG adsorbed to antigenic sites on bloodstain.

(Eluate) - An increase in fluorescence over a negative control.

8.5 PROCEDURE:

LOW ANGLE, SOLID PHASE, DIRECT FLUORESCENCE TAGGING METHOD

INTRODUCTION: The following procedure has not been optimized in terms of duration of reaction times. Although it has been used satisfactorily in identifying A, B, C, D and e bloodstains, it should be considered as still developmental. Importantly, it utilizes fluorescein tagged goat anti-human IgG as a common fluorescenized indicator system eliminating the necessity for individually fluoresceinized specific blood typing sera.

PROCEDURE: 1. Cut a bloodstained cloth sample approximately 0.8 x 2 cm square.

2. Fix in a 2.0 ml of an equal mixture of ether/ethanol (v:v) for 10 minutes at room temperature. Then, remove excess by aspiration.

3. Fix additionally in 2.0 ml of 95% ethanol for 20 minutes at room temperature. Then, remove excess by aspiration.

4. Wash twice with 3.0 ml phosphate buffered saline (PBS) then add 0.5 ml of 22% Albumin (DADE) and incubate at room temperature for 20 minutes. Then, remove excess by aspiration.

5. Add 0.2 ml of specific antiserum (non-fluoresceinized) and incubate for 45 minutes at room temperature.

6. Wash 3 times with 3.0 ml PBS

7. Add 0.3 ml of 22% albumin (DADE) and incubate for 20 minutes at room temperature. Then, remove excess by aspiration.

8. Add 0.2 ml of fluorescein conjugated goat anti-human IgG and incubate for 45 minutes in the dark at room temperature.

9. Wash 3 times with 3.0 ml PBS.

10. Mount bloodstained cloth on stainless steel cylinder, with or without oxoid filter background, and cover with glass coverslip. Seal coverslip edges with paraffin to prevent drying.

11. Read for fluorescence in Aminco-Bowman Fluorometer

Excitation 486 nm
Emission 520 nm
Slits 4, 2, 1, 1, 2, 2
525 nm cut-on filter in place

NEGATIVE CONTROL: 1. Cut a bloodstained cloth sample approximately 0.8 x 2 cm square.

2, 3, 4 } Same as above

5. Add 0.2 ml of a suitably absorbed (non-reactive) human serum and incubate for 30 minutes at room temperature.

6. through 11. Same as above.

POSITIVE TEST: An increase in fluorescence of the bloodstain as a result of fluoresceinized goat anti-human IgG reacting with human IgG antibodies adsorbed to blood group antigens on the bloodstain surface.

8.6 PROCEDURE:

BLOODSTAINED FABRICS (A, B, AB System)

1. Cut a bloodstained cloth sample from an area of bloodstain that has a homogeneous appearance. (3 cm x 1.5 cm)

2. Divide sample into 3 equal pieces, 1 cm x 0.5 cm, using a sharp razor blade. Mark top left corner with pencil point to indicate the face or surfaces opposite of that to be compared. There should be no other pencil markings on the bloodstained fabric.

3. Fix in 2.0 ml of an equal mixture of ether/ethanol (v:v) for 5 minutes at room temperature while rotating. Then, remove fixative by aspiration.

4. Fix additionally in 2.0 ml of 95% ethanol for 10 minutes at room temperature while rotating. Then remove fixative by aspiration.

5. Wash twice with 10 ml phosphate buffered saline (PBS) and mix by inverting 5 times each wash. Then, add 0.4 ml of 22% albumin (DADE) and mix on automatic rotator at room temperature for 5 minutes. Remove supernate by aspiration.

6. Wash 3 times with 10 ml PBS inverting 3 times for each wash. Revove supernate by aspiration.

7. Add 0.4 ml of specific antiserum (nonfluoresceinized) and incubate for 90 minutes at room temperature while mixing on rotator with face down. (NOTE: 1 piece will be treated with anti-A, one with anti-B, and one with anti-AB serum).

8. Wash 3 times with 10 ml of PBS for 10 minutes each, using an automatic mixing rocker.

9. Add 0.4 ml of 22% albumin (DADE) and incubate for 10 minutes at room temperature while on rotator. Then, remove albumin solution by aspiration.

10. Add 0.4 ml of fluorescein conjugated goat anti-human IgG (1:10) and incubate 90 minutes on a rotator at room temperature.

11. Wash 3 times with 10 ml PBS, allowing samples to mix 10 minutes per wash on automatic mixing rocker. Remove excess PBS by aspiration and stopper tube to prevent sample from drying.

12. Mount bloodstained cloth in the center of the sample holder with pencil point identified top left corner face down.

13. Read fluorescence in ORTEC apparatus.

8.7 PROCEDURE:

BLOODSTAINED FABRICS (C, D, e System)

1. Cut a bloodstained cloth sample from an area of bloostain that has a homogeneous appearance. (3 cm x 1.5 cm)

2. Divide sample into 3 equal pieces, 1 cm x 0.5 cm, using a sharp razor blade. Mark top left corner with pencil point to indicate the face or surfaces opposite of that to be compared. There should be no other pencil markings on the bloodstained fabric.

3. Fix in 2.0 ml of an equal mixture of ether/ethanol (v:v) for 5 minutes at room temperature while rotating. Then, remove fixative by aspiration.

4. Fix additionally in 2.0 ml of 95% ethanol for 10 minutes at room temperature while rotating. Then remove fixative by aspiration.

5. Wash twice with 10 ml PBS and mix by inverting 5 times each wash.

6. Add 0.4 ml of 22% albumin (DADE) and mix on automatic rotator at room temperature for 5 minutes. Remove supernate by aspiration.

7. Add 0.4 ml undiluted specific antiserum (DADE, anti-C, anti-D and anti-e). Allow to react for 90 minutes at room temperature while mixing on rotator with face down (pencil marked face up).

8. Ascertain that pencil marked face is turned down and place in refrigerator at $4^{\circ}C$ to react overnight.

9. Next morning aspirate supernatant serum and wash 3 times with 10 ml of PBS for 10 minutes each, using an automatic mixing rocker.

10. Add 0.4 ml of 22% albumin and mix on rotator for 10 minutes.

11. Add 0.4 of fluorescein conjugated goat anti-human IgG (1:10 dilution) and allow to react for 90 minutes at room temperature on automatic rotator.

12. Wash 3 times with 10 ml of PBS allowing samples to mix 10 minutes per wash on automatic mixing rocker. Remove excess PBS by aspiration and stopper tube to prevent sample from drying.

13. Mount bloodstained cloth sample with pencil marked face down.

14. Read fluorescence in ORTEC apparatus.

8.8 PROCEDURE:

BLOODSTAINED THREADS OF FIBERS

1. Carefully tease nine threads form a homogeneous area of the bloodstained fabric, each approximately 2 inches in length.

The threads will be treated in sequence as follows, i.e., thread to be used as a control will be that immediately adjoining thread to be used for test.

THREAD NO.	TESTED AGAINST
1	anti-A
2	anti-B
3	anti-AB
4	anti-C
5	AB Serum Control
6	anti-D
7	AB Serum Control
8	anti-e
9	AB Serum Control

2. Bloodstained threads will be treated with the same timing and procedures used for bloodstained cloth, i.e., according to those procedures specific for the A, B and AB system and for the C, D and e system.

NOTE: Reactions will be carried out in glass tubes using an automatic mixing rocker.

8.9 PROCEDURE:

SONICALLY DISRUPTED, MEMBRANE EMBEDDED BLOODSTAIN PARTICULATES (A, B, AB, C, D and e Systems)

When a bloodstain is too irregular to obtain samples for surface comparisons, e.g., cloth and threads or fibers, the following procedure is recommended.

1. For each blood group to be tested remove a piece of fabric 1 cm x 2 cm and cut into 2 mm square pieces.

2. Place in a glass vial with 2 ml of cold phosphate buffered saline (PBS) and sonicate for 15 minutes.

3. Aspirate supernatant PBS mixture and place into glass tube.

4. Add fresh 2 ml cold PBS to cloth pieces and sonicate for an additional 15 minutes. Then aspirate supernate and add to PBS mixture.

5. Rinse cloth pieces with 2 ml cold PBS and add rinse to PBS supernate mixtures.

6. Transfer the combined sonicate washes to Millipore apparatus and impinge on membrane filter by applying vacuum.

7. Wash with 10 ml cold PBS.

8. Transfer membrane filter to small petri dish and treat as follows:

<u>A, B, AB System-</u>

9. Add 1.0 ml (1:1 PBS dilution) of the specific nonfluoresceinized antiserum and incubate at 37°C for 60 minutes and then at 4°C for

60 minutes.

10. Return membrane filter to the Millipore apparatus and wash e times with 10 ml of cold PBS.

11. Add 0.5 ml (1:10 dilution) of fluoresceinized goat anti-human IgM serum and allow to react at room temperature for 90 minutes. Keep filter surface covered with antiserum. Add additional anti-IgM serum if necessary.

12. Wash 4 times with 10 ml of cold PBS and read in fluorometer while wet. Membranes may be kept in PBS damp petri dishes.

C, D, e System-

9. Add 1.0 ml (1:1 22% albumin dilution) of the specific non-fluoresceinized antiserum and incubate at 37°C for 2 hours and then overnight in the refrigerator at 4°C.

10. Next morning return membrane filter to Millipore apparatus and wash 4 times with 10 ml cold PBS.

11. Add 0.5 ml (1:10 dilution) of fluoresceinized goat anti-human IgG serum and allow to react at room temperature for 90 minutes. Keep filter surface covered with antiserum. Add additional anti-IgG serum if necessary.

12. Wash 4 times with 10 ml of cold PBS and read in fluorometer while wet. Membranes may be kept in PBS damp petri dishes.

8.10 GOALS AND REQUIREMENTS OF THE METHODOLOGY TO BE DEVELOPED FOR A BLOODSTAIN ANALYSIS SYSTEM

From the "Statement of Work for a Bloodstain Analysis System" prepared by The Aerospace Corporation, 5 February 1974, Annex B, Paragraphs 3.1.3.2 - 3.2.6.

. Accuracy of Analysis

The goal for reliability of detection of the blood constituents selected by the contractor for bid package BI shall be 99% at the 90% confidence level. This goal applied only to uncontaminated stains, with the actual reliability of analysis of contaminated stains to be determined during development and prototype tests. The reliability goals for bid packages BII and BIII shall be set as high as practicable, keeping in mind the end-use of the blood analysis data, that of evidence in a court of law.

. Skill Requirements

It is desired that the method to be developed shall be capable of being learned in two weeks and reliably used by personnel with no less than two years of college level chemistry, including organic or biochemistry, plus one year of applicable serological experience.

. Cost

A goal for the cost of analysis of a single stain using the method to be developed shall be $50.00 including the cost of reagents, operator time, equipment amortization, and maintenance

requirements.

. Hazards

Reagents used by the method to be developed shall be nontoxic on skin contact or on vapor inhalation. They shall not introduce radiological hazards under prescribed procedures and currently practiced applicable safety provisions. Any deviations from the requirements established by the Health and Safety Act of 1970 must be brought to the attention of the Program Manager.

. Bloodstains

The method to be developed must be capable of analyzing bloodstains found on a variety of substrates such as textiles, glass, plastics, cement, plaster of paris, etc.

The method used for bid packages BI and BII must be capable of performing the complete analysis on stain sizes equivalent to 25 microliters of fresh blood without consuming all of the evidence. This stain size becomes a goal for bid package BIII.

. Reagent Characteristics

Reagents required by the method to be developed shall be storable at laboratory freezer temperatures for periods up to six months. They must be nonhazardous as described above. As a goal, these reagents should be available from more than on U.S. source at a cost not exceeding $5.00 per stain analyzed. If required, standards and methods of calibrating these reagents against

such standards must be provided.

. Requirements and Objectives Pertaining to Equipment Development Size

Because of the need to conserve bench space in criminalistic laboratories, it is desired to miniaturize the equipment to the maximum extent. In addition, electrophoreses tanks, if used by the new method to be developed, should also be designed to permit stacking of tanks so that several separations can be performed concurrently with minimum utilization of bench space. Similarly, power supplies should be minimum in size and should be capable of serving several tanks simultaneously.

. Power

The equipment shall be capable of operation from a 110 volt AC power source and draw no more than 10 amps of current.

. Operating Characteristics

It is desired that as much automation as feasible be introduced into equipment operation and that the skill level required to operate the equipment not exceed that specified for the methodology in Section 3.1 of this Annex.

. Reliability

It is required that the equipment perform the analyses associated with the performance level of bid package BI at an equipment

reliability of 99% at the 95% confidence level. Reliability goals for the analyses required under bid packages BII and BIII should approach the numbers above. Standards of performance, and methods of calibrating the equipment against such standards must be provided.

. Hazards

The equipment must be designed to prevent human contact with electrical energy sources with open circuit potential of 30 volts or more and with capability of delivering 2.5 milliamperes into a short circuit. Cutouts must be provided to assure the absence of current whenever human manipulation of the equipment is required in the course of the analysis procedure. Radiological hazards must be avoided. Any deviation from the requirements of the Health and Safety Act of 1970 must be brought to the attention of the Program Manager.

. Costs

The upper limit of cost permitted for equipment capable of performing the analyses for any of the three bid packages is $10,000.00. This upper limit represents the allowable cost for fully automatic equipment. Manual equipment performing only the analyses required for bid packages BI should not exceed $500.00 in cost.

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