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WATER SYSTEM VIRUS DETECTION

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Organon Diagnostics

NAS 9-14102

124-F

November, 1975

Final Report for Period July, 1974 - September, 1975

Prepared for

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1. Report No.	2. Government Accession No.	3. Recipient's Catalog No.
NAS 9-14102		

4. Title and Subtitle	5. Report Date
WATER SYSTEM VIRUS DETECTION	November 1, 1975
	6. Performing Organization Code

7. Author(s)	8. Performing Organization Report No.
A. S. FRASER, A. F. WELLS, H. J. TENOSO	124-F
	10. Work Unit No.

9. Performing Organization Name and Address	11. Contract or Grant No.
ORGANON DIAGNOSTICS 9060 East Flair Drive El Monte, CA 91731	NAS 9-14102
	13. Type of Report and Period Covered Final Report 1 July '74 thru 1 Oct '75

12. Sponsoring Agency Name and Address	14. Sponsoring Agency Code
Richard Sauer NASA, Johnson Space Center Houston, TX 77058	

15. Supplementary Notes

16. Abstract Organon Diagnostics has developed, under NASA sponsorship, a monitoring system to test the capability of a water recovery system to reject the passage of viruses into the recovered water. In this system a non-pathogenic marker virus, bacteriophage F_2, is fed into the process stream before the recovery unit and the reclaimed water is assayed for its presence. The marker virus selected provides for a "worst-case" condition and thus provides an equal, if not greater, challenge to the water purification process than any naturally occurring human pathogenic viruses.

Detection of the marker virus in the downstream reclaimed water consists of two major components, concentration and isolation of the marker virus, and detection of the marker virus. The concentration system involves adsorption of virus to cellulose acetate filters in the presence of trivalent cations and low pH with subsequent desorption of the virus using small volumes of high pH buffer. The detection of the virus is performed by a passive immune agglutination test utilizing specially prepared polystyrene particles.

An engineering preliminary design has been performed as a parallel effort to the laboratory development of the marker virus test system. Engineering schematics and drawings present a preliminary instrument design of a fully functional laboratory prototype capable of Zero-G operation. The instrument consists of reagent pump/metering system, reagent storage containers, a filter concentrator, an incubation/detector system, and an electronic readout and control system.

17. Key Words (Suggested by Author(s))	18. Distribution Statement
Bacteriophage F_2 Marker virus Passive Immune Agglutination Viral Monitoring System Reclaimed Water	

19. Security Classif. (of this report)	20. Security Classif. (of this page)	21. No. of Pages	22. Price*
UNCLASSIFIED	UNCLASSIFIED		

*For sale by the National Technical Information Service, Springfield, Virginia 22151

NASA — JSC

JSC Form 1424 (Rev Jul 74)

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1.0 INTRODUCTION

This report describes work completed by Organon Diagnostics on Contract NAS 9-14102 for the National Aeronautics and Space Administration during the period from July 1, 1974 to October 1, 1975. Efforts during this period were directed toward the development of a viral contamination detection system to monitor waste-water reclamation during long space flights.

The program consisted of selecting a marker virus to challenge the reclamation system, developing a method for concentrating marker virus in reclaimed water, developing and evaluating several immunoassay methods for detecting the marker virus following concentration, selection and optimization of a single immunoassay method as part of the integrated system, and an engineering design of a prototype instrument. Work was continued under a program extension. The extension consisted of end-to-end testing of the marker virus detection system, including evaluation of sensitivity, reproducibility, accuracy, reliability and specificity; determining the effects of AgBr and iodine on the system; and testing of water recovery systems.

1.1 PROGRAM OBJECTIVES

a. Analyze and select a marker virus for use in a viral monitoring system;

b. Select, evaluate and develop a viral concentration and detection system; and

c. Prepare design criteria and preliminary design specifications for automation of the developed viral monitoring system.

1.2 APPROACH

The approach taken by Organon Diagnostics to complete the objectives of the program was to use a marker virus system which tests the capability of the water recovery system to reject the passage of viruses. In this system a nonpathogenic virus is added to the upstream side of the system and sought downstream. This provides certain advantages over the detection of naturally occurring viruses. First, a single virus species is added at a known concentration, which allows the use of a single reagent system for detection. Second, the marker virus is selected to give "worst-case" conditions.

"Worst-case" condition means the marker virus would provide an equal, if not greater, challenge to the water purification process than any naturally occurring human pathogenic virus.

Detection of the marker virus in the downstream reclaimed water consisted of two major tasks; concentration and isolation of the marker virus to improve detection sensitivity, and detection of the marker virus. Concentration procedures were selected for testing based on degree of specificity, concentrating ability and minimum effect on reclaimed water. Two main concentrating systems were investigated based on these criteria. Detection techniques were selected based on sensitivity, specificity and simplicity of operation. To meet these requirements, virus was detected in the sample concentrations utilizing an immunological system. Based on this approach, three techniques were investigated.

1.3 SUMMARY OF ACCOMPLISHMENTS

a. A literature survey revealed two potential marker virus candidates from the bacterial virus group (bacteriophage). These were bacteriophage ϕX174 and bacteriophage F_2. Comparison testing showed that bacteriophage F_2 had a higher resistance to selected environmental conditions than either ϕX174 or representative human pathogenic virus. Bacteriophage F_2 was selected for use as a marker virus on the basis of nonpathogenicity, resistance to environmental factors and on physical characteristics.

b. The marker virus was grown to high titer using the broth technique and was purified and concentrated using a combination of ammonium sulfate precipitation, gel filtration and ultracentrifugation. Using this technique, good yields of highly purified virus were obtained.

c. Rabbits were immunized with the purified marker virus. The antisera obtained were of high titer and contained little or no antibodies against host protein or media.

d. Two viral concentration systems were investigated. The first system involved the use of BIO-FIBERS® in which marker virus in a 400 ml sample could be concentrated to 30 ml with greater than 90% recovery.

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This gave an approximate tenfold concentration. The second concentration system involved adsorption of virus to cellulose acetate filters in the presence of trivalent cations and low pH with subsequent desorption of the virus using small volumes of high pH buffer. In this system, marker virus in 400 ml was concentrated and suspended in 3 ml with up to 100% recovery. This gave an approximate 100-fold concentration.

e. Polystyrene latex beads, 1.1 μm diameter, were used in the development of immunological detection systems.

f. An FA (Fluorescent antibody) test was investigated but could not be adapted to the detection of marker virus.

g. An RIA (Radioimmunoassay) test was developed for the marker virus which could detect $\sim 5 \times 10^8$ plaque forming unit (PFU)/ml.

h. An ELA (Enzyme labeled antibody) test was developed for the marker virus which could detect $\sim 5 \times 10^7$ PFU/ml.

i. Extensive work was done on the development of a PIA (passive immune agglutination) test. After several modifications, the finalized system had the capacity to detect $\sim 1 \times 10^9$ PFU/ml and was used in the viral monitoring system.

j. End-to-end testing of the viral monitoring system, based on engineering breadboard design, was performed. The system was shown to efficiently concentrate and detect marker virus.

k. Total system sensitivity was investigated. The system was able to detect $\sim 1 \times 10^7$ PFU/ml in starting 400 ml sample.

l. Total system reproducibility was investigated. Experiments showed that samples containing the same level of marker virus gave highly reproducible results when tested by the viral monitoring system.

m. Total system accuracy was investigated. Levels of response to various concentrations of marker virus when using the selected viral monitoring system correlated well with those obtained using other detection techniques.

n. Total system reliability was investigated. Experiments over a two-month period revealed no loss of reagent activity. The filter could be

reused at least 10 times for concentration without loss of efficiency. Peristaltic pumping of the detection reagent had no effect on reactivity.

o. Total system specificity was investigated. Experiment revealed the system detection response was due to specific interaction between marker virus and anti-marker virus antibody.

p. The volume of expendables needed for the viral monitoring system was calculated. Requirements were based on the performance of two tests a day for a six-month period.

q. The effects of AgBr and iodine on the viral monitoring system were evaluated in terms of marker virus infectivity, ability to concentrate marker virus, and ability to detect the virus.

r. A water recovery system was challenged with marker virus and tested for its ability to reject the passage of marker virus.

2.0 MARKER VIRUS SELECTION

Marker virus candidates were selected from the bacteriophage or bacterial virus group. Two viruses were selected for testing bacteriophage ϕX174 (DNA) and bacteriophage F_2 (RNA). For purposes of comparison testing, two human pathogenic viruses were chosen based on their prevalence in the population, relative resistance to environmental stress and area of the body they infect. These pathogenic viruses were adenovirus-3 (DNA) and ECHOvirus-6 (RNA).

2.1 MARKER VIRUS CANDIDATES

Selection of a marker virus was governed by several considerations. First the marker virus should not be from a mammalian source, since the possibility of human pathogenicity cannot be completely excluded. The marker virus should have characteristics similar to human viral pathogens. The use of small ($\ll$20 nm) marker viruses was a major consideration, since it would more readily pass through a purification system and thus provide a "worst-case" condition. Other factors for consideration included resistance to possible environmental conditions imposed by the water reclamation process. This resistance should be equal to or greater than the most resistant of possible viral pathogens.

The marker viruses chosen for testing were selected from two groups--those possessing ribonucleic acid (RNA) in their genetic material and those having deoxyribonucleic acid (DNA). This allowed a comparison with selected viral pathogens in both RNA and DNA groups.

Because of their nonpathogenicity to man and their ease of preparation, the bacterial viruses (bacteriophages) were prime candidates for selection as a marker virus. Two bacteriophages were selected for testing; bacteriophage ϕX174 and bacteriophage F_2.

Bacteriophage ϕX174 is one of the smallest known bacterial viruses. It is characterized as a tailless coliphage containing single-stranded DNA and infects the bacterium Escherichia coli. A second virus, bacteriophage F_2, selected for consideration, was similar in size and host range to bacteriophage ϕX174, but differed by having RNA as its nucleic acid material.

Bacteriophage F_2 occurs naturally in waters polluted with human feces and is characterized as a tailless coliphage with a relatively high chlorine resistance. It has been suggested that bacteriophage F_2 would make a good indicator virus to warn of the presence of possible pathogenic viruses in fecal contaminated water (Shah and McCamish, 1972).

2.2 PATHOGENIC VIRUSES CANDIDATES

Two mammalian viruses were ultimately selected as representatives of human pathogens, in order to assess the relative resistance of the marker virus candidates. A literature search revealed a number of papers relevant to survival of various mammalian viruses under selected conditions. These conditions included variations in ambient temperature and pH, and exposure to biocides and surface denaturing agents. This review provided information on two groups of mammalian viruses, the adenovirus group and the echovirus group.

The adenoviruses are DNA containing human pathogenic viruses, ranging in diameter from 60 to 90 nm. The members of this group are relatively resistant to temperature and pH extremes in terms of their infectivity. The ECHOviruses are small (20 to 30 nm in diameter) RNA containing human pathogenic viruses belonging to a group of viruses known as picornaviruses. The ECHOviruses are relatively stable when compared to other human pathogenic viruses and, because of their small size and similarity to several other human pathogenic viruses, including polioviruses, rhinoviruses, and coxsackieviruses, are a representative human pathogenic virus group for our purposes. One virus from each of the two groups described above was selected for testing, specifically, adenovirus type 3 and ECHOvirus type 6.

2.3 TESTING CONDITIONS

Two sets of experiments were performed to compare relative resistance of the marker virus candidates and the selected human pathogenic viruses. The first set of experiments was based on publications which related to the survival of human pathogenic viruses to physical stress including temperature and pH. The two selected marker viruses were then tested under similar conditions. The second set of experiments tested survival of both marker

viruses and the two selected human pathogenic viruses to chemical stress imposed by chemically treated urine (treated for water recovery by vapor compression distillation) and wash-water waste containing a biocide. Chemical stress conditions were selected based on information found in NASA Contract NAS 9-9191 and from communications with Mr. W. F. Reveley of the National Aeronautics and Space Administration.

2.4 RESULTS

The first set of conditions tested survival of the marker virus candidates to selected physical stress conditions. In each case, survival referred to the ability to remain infectious after treatment. The number of viruses remaining infective then indicated relative resistance to the treatment. Infectivity of the bacteriophages was determined using the agar layer plaque assay technique, with viral numbers being expressed as plaque forming units per milliliter (PFU/ml). This technique is described in Section 4.1.1. For ϕX174 bacteriophage, the host bacterium was E. coli C (ATCC No. 13708); for F_2 bacteriophage, the host bacterium was E. coli C-3000 (ATCC No. 15597). To determine the infectivity of the human pathogenic viruses tested, the micro-technique was employed. Infectivity was reported as $TCID_{50}$, which represents the dose of virus that shows a cytopathic change in 50% of the inoculated culture, and was calculated by the Reed-Muench method. Adenovirus-3 was tested in HeLa cells with ECHOvirus-6 being tested in Vero cells. Figure 1 shows the effect of distilled water at 56°C on survival of the marker virus candidates. Under these conditions, no infectious ϕX174 bacteriophage were detectable after 30 minutes; whereas, after one hour, there were still detectable F_2 bacteriophage. Under similar conditions, both adenovirus-3 and echovirus-6 showed no survival after 5 minutes.

Figure 2 shows survival of the marker viruses in distilled water (25°C) at pH 3. Under these conditions, there was no loss of infectivity by either ϕX174 or F_2 bacteriophage. Under similar conditions, both adenovirus-3 and ECHOvirus-6 also showed no loss of infectivity after 30 minutes.

Figure 3 shows survival of marker viruses in distilled water (25°C) at pH 11. Under these conditions there was no loss of infectivity of either ϕX174 or F_2 bacteriophage. Under similar conditions adenovirus-3 showed no

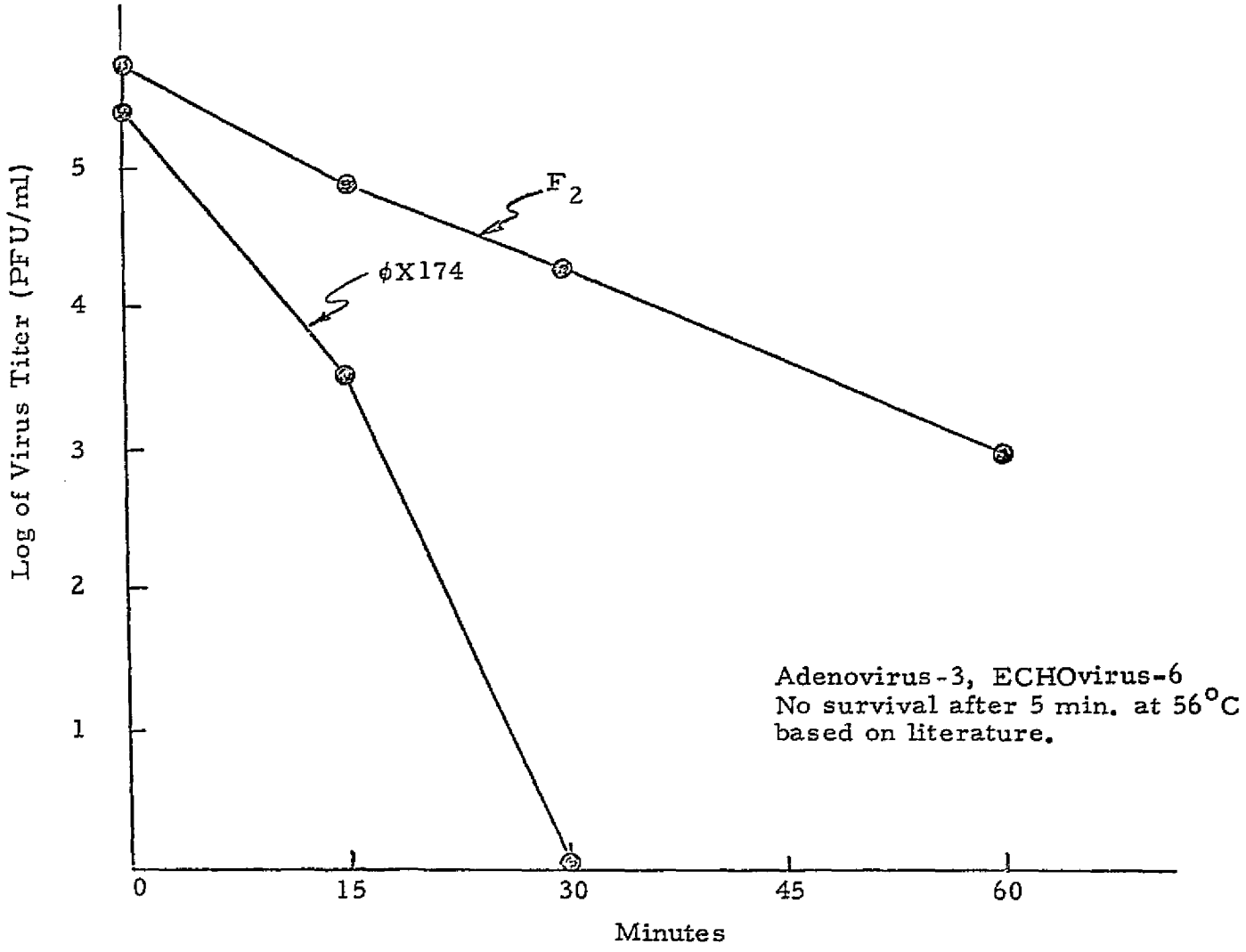


Figure 1 Survival of Marker Viruses in Distilled Water at 56°C

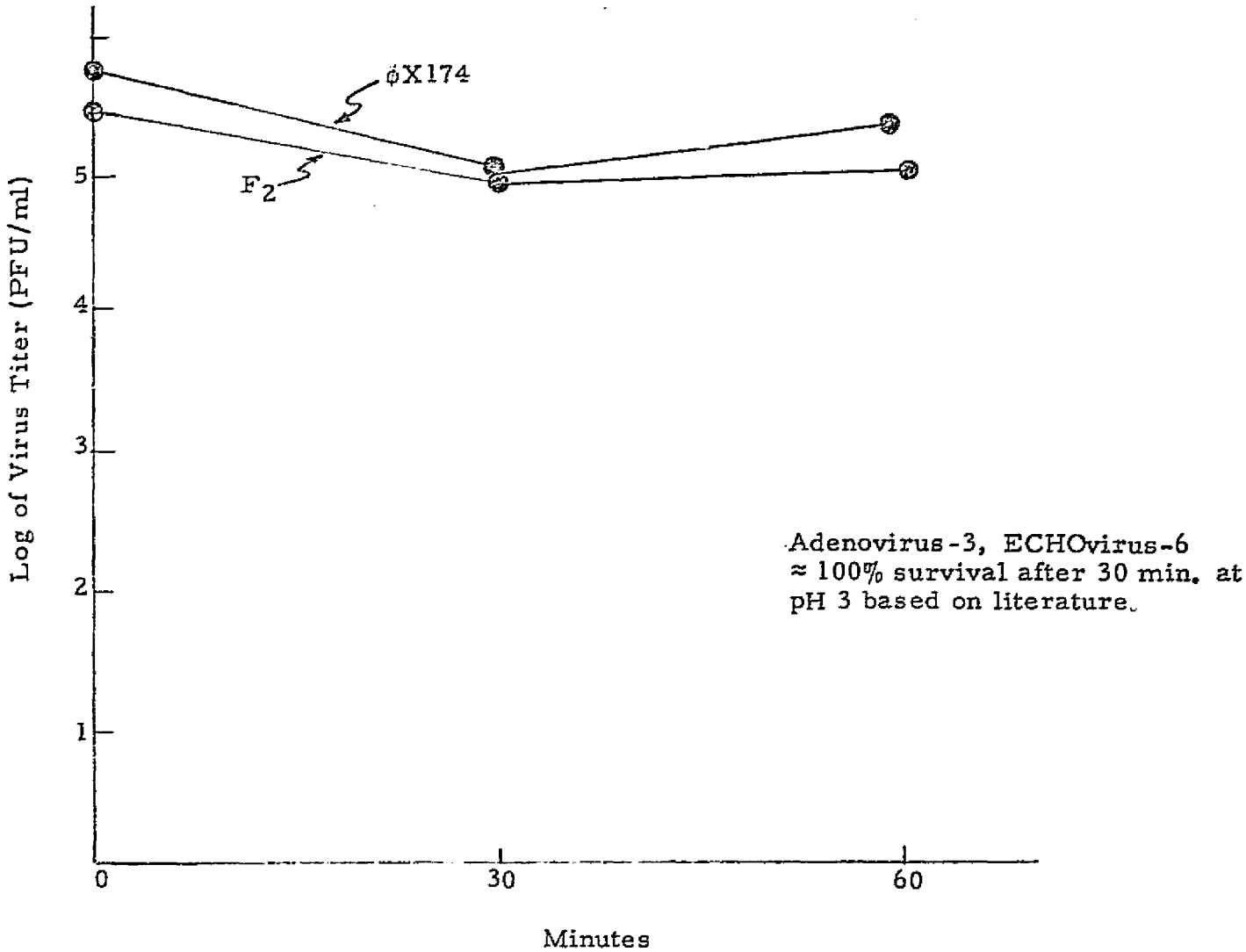


Figure 2 Survival of Marker Viruses in Distilled Water at pH 3

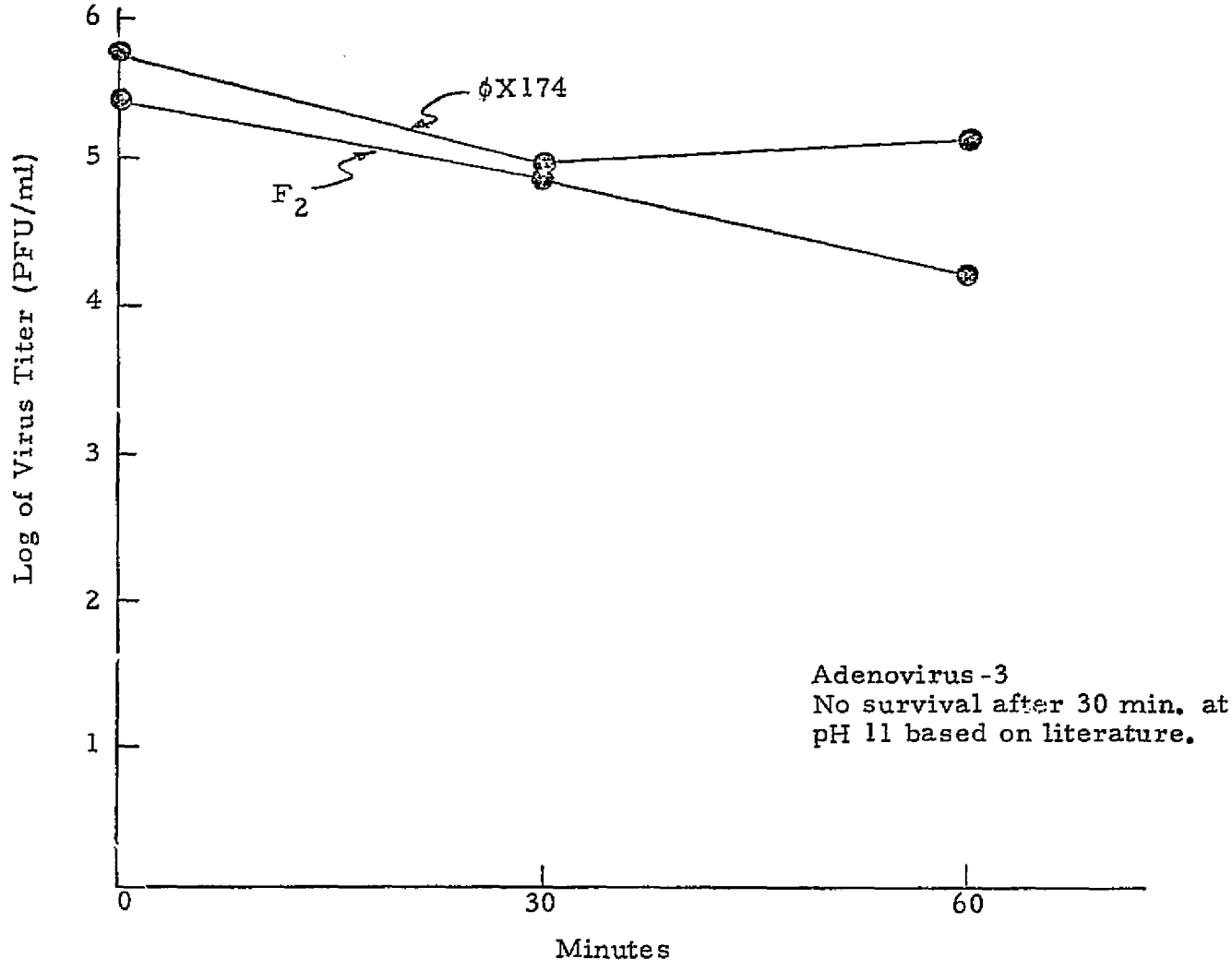


Figure 3 Survival of Marker Viruses in Distilled Water at pH 11

survival after 30 minutes at pH 11. No literature information was available on survival of ECHOvirus-6 under these conditions.

The next set of conditions tested the survival of marker virus candidates and human viruses to chemical stress, including exposure to chemically pretreated urine and wash-water waste. Table I shows the pretreatment solution for 520 gm of urine, in preparation for water recovery by vapor compression distillation (VCD). The selected viruses were exposed to this pretreated urine at 25°C.

Figure 4 shows the survival of marker viruses and human pathogenic viruses in pretreated urine. After 7 days exposure, there was no appreciable loss of infectivity of either ϕX174 or F_2 bacteriophage. In contrast, both ECHOvirus-6 and adenovirus-3 had no detectable infectious particles remaining after 7 days.

To determine if there was any difference in survival rates between the two marker virus candidates, they were tested in pretreated urine concentrated 10 times by evaporation (Figure 5). After exposure at 25°C for 7 days, there was no apparent loss of infectivity.

Virus candidates were tested in wash-water waste (Table II) at 25°C. Figure 6 shows survival of marker viruses and human pathogenic viruses in wash-water waste solution. After 7 days there was no appreciable loss of infectivity of either ϕX174 or F_2 bacteriophage. In contrast, after 2 days no detectable infectious particles of either ECHOvirus-6 or adenovirus-3 were found.

To determine if there was any difference in the survival of ϕX174 or F_2 bacteriophage, they were tested in wash-water waste with 10 times the concentration of ingredients listed in Table II. The results of this testing are shown in Figure 7. After 7 days exposure at 25°C, there was no detectable infectious ϕX174 bacteriophage whereas F_2 bacteriophage was still detectable.

2.5 CONCLUSIONS

A summary comparing sensitivity of marker viruses to human viruses can be seen in Table III. This summary compares sensitivity to test conditions which include survival at 56°C, pH 3, pH 11 and survival in urine and wash water. In general, under the conditions tested, the bacterial viruses had a higher survival rate than the human pathogenic viruses.

TABLE I URINE PRETREATMENT*

- 520 gm Urine (24 Hr)
  - Biopal VRO-20™ 1.23 gms
  - Sulfuric Acid 0.34 gms
  - Antifoam 0.13 gms
  - Water 1.30 gms

*Specifications From NASA Contract NAS 9-9191

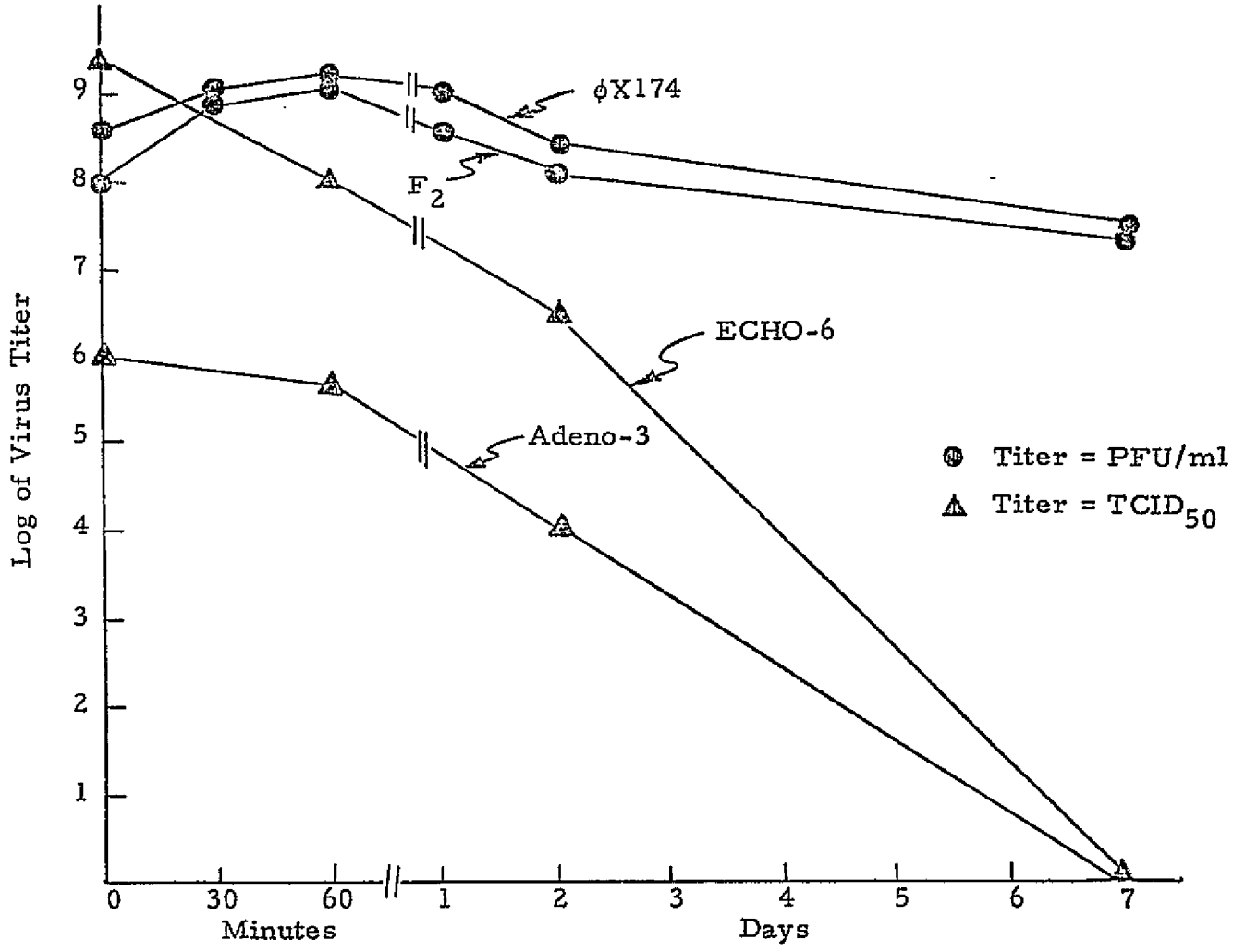


Figure 4 Survival of Marker Viruses and Human Pathogenic Viruses in Pretreated Urine

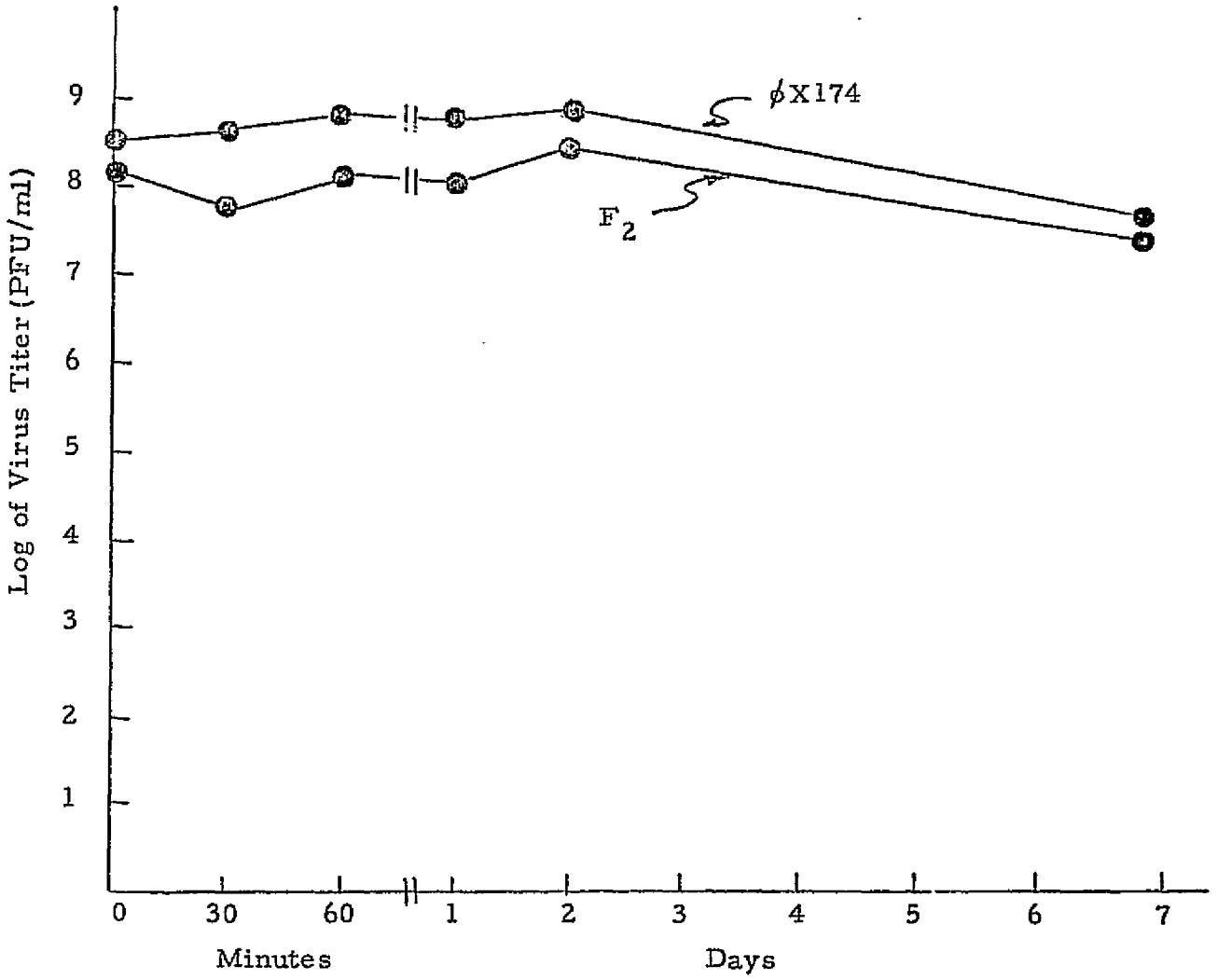


Figure 5 Survival of Marker Viruses in Pretreated Urine Concentrated 10X

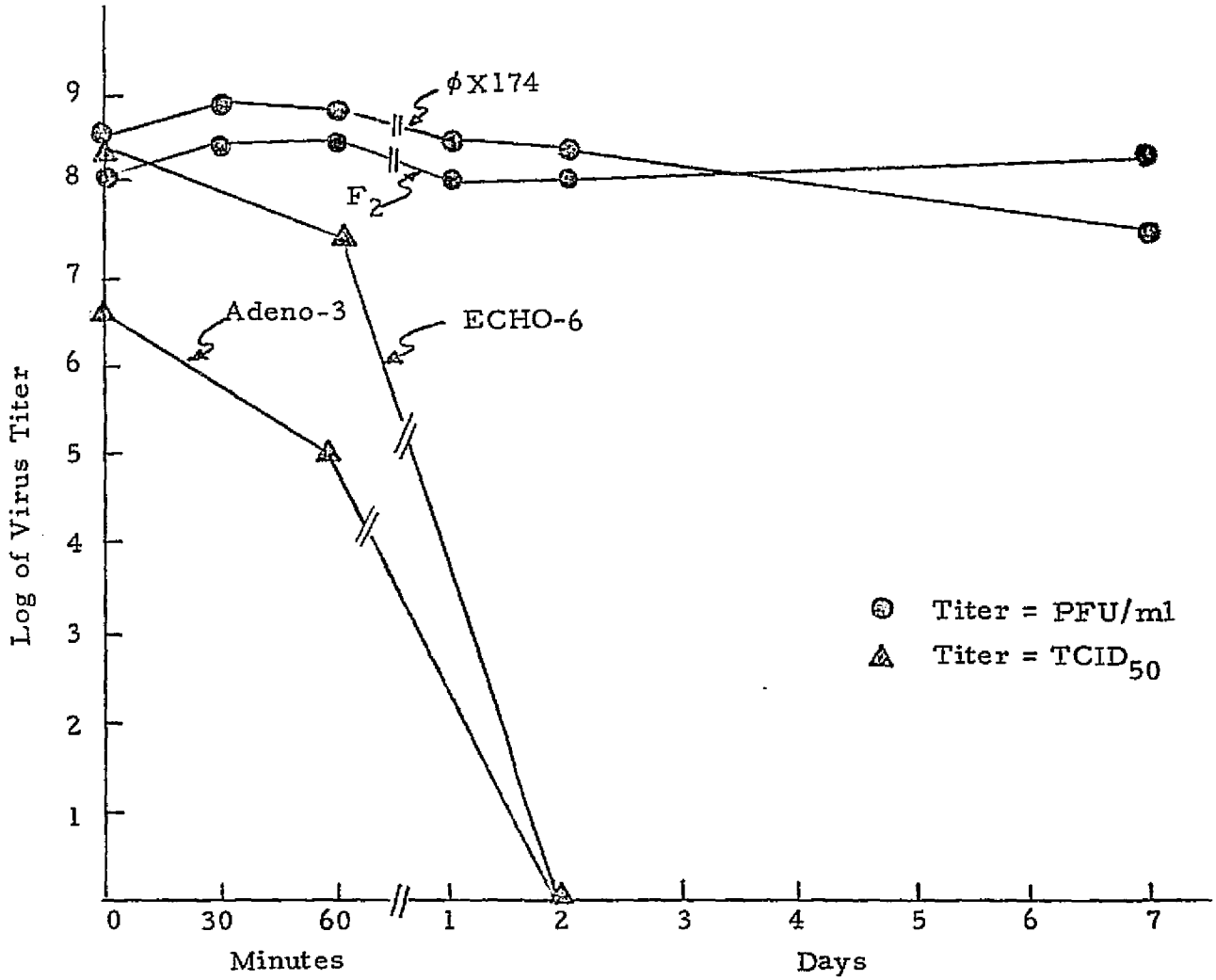


Figure 6 Survival of Marker Viruses and Human Pathogenic Viruses in Wash-Water Waste Solution

TABLE II COMPOSITION OF WASH-WATER WASTE*

- 500 ml Deionized Water
  - Neutrogena Body Soap Gel™ 1.55 gm
  - Miranol C2M™ 0.65 gm
  - Vancide BN™ 0.50 gm

*Specifications From NASA Contract NAS 9-9191

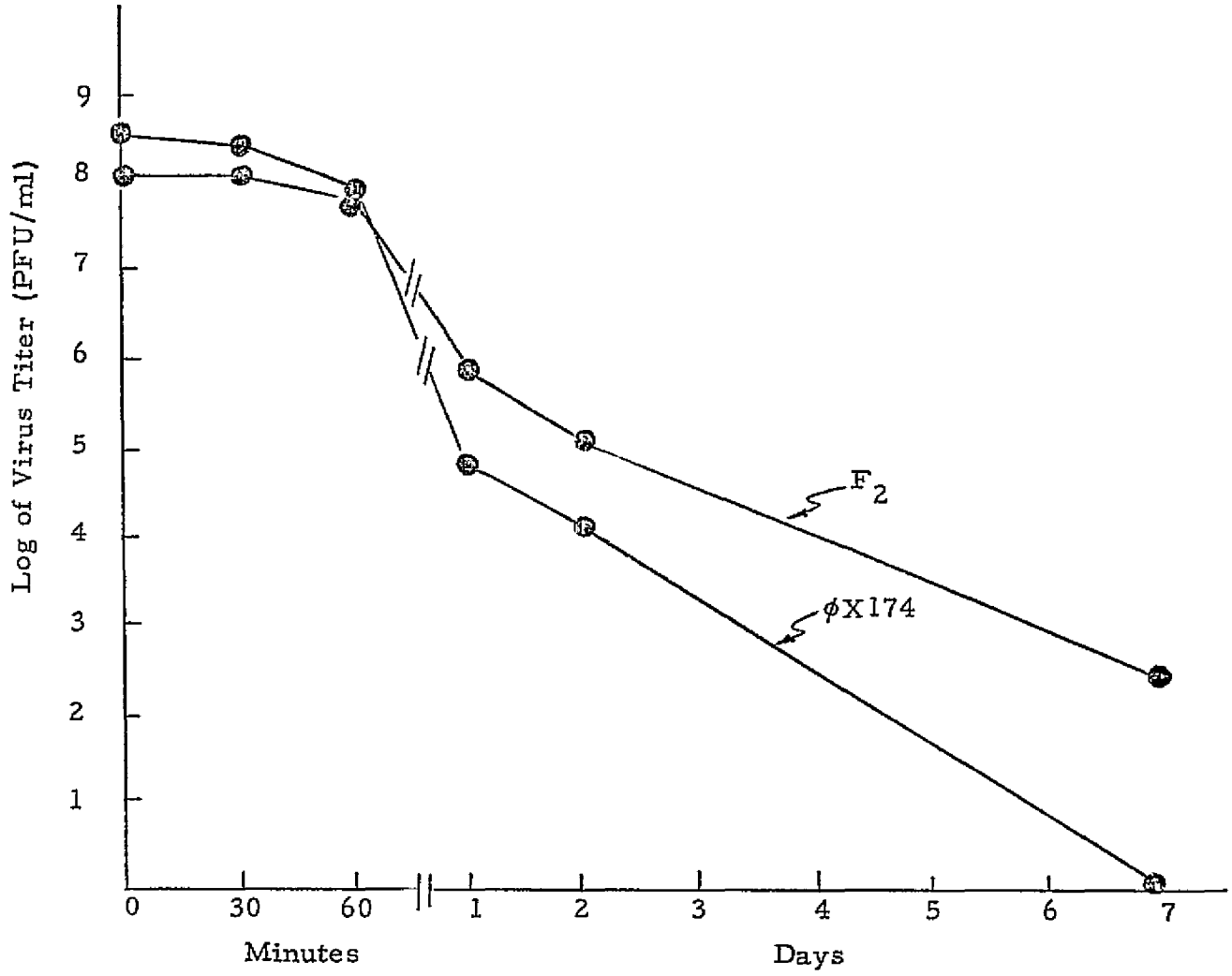


Figure 7 Survival of Marker Viruses in Wash-Water Waste Solution Concentrated 10X

TABLE III SUMMARY OF SENSITIVITY TESTING

Marker Virus vs Human Virus

TEST CONDITIONS	SURVIVAL (>0.1%)			
	F_2	ϕX174	ADENO-3	ECHO-6
56°C	Yes	No	No	No
pH 3	Yes	Yes	Yes	Yes
pH 11	Yes	Yes	No	--
Urine	Yes	Yes	No	No
Wash Water	Yes	No	No	No

A comparison was made between ϕX174 and F_2 to determine which of the two would be a better marker virus (Table IV). Ratios were determined by dividing the final virus titer by the initial virus titer. In this way a ratio of 1.00 would indicate 100% survival where a ratio of 0 would mean no survival. Based on this comparison, F_2 showed survival at 56°C and in 10 times concentrated wash solution, whereas ϕX174, under the same conditions, did not survive. Under the other conditions, both ϕX174 and F_2 bacteriophage showed an approximately equal survival rate. Based on the demonstrated superiority of F_2 to survive under the experimental conditions, it was chosen as the marker virus.

TABLE IV SUMMARY OF SENSITIVITY TESTING

F_2 Marker Virus vs ϕX174 Marker Virus

Test Conditions	Survival*	
	F_2	ϕX174
56°C	0.55	0
pH 3	0.94	0.85
pH 11	0.80	0.93
Urine	0.94	0.89
Wash	1.00	0.89
Urine 10X	0.93	0.89
Wash 10X	0.31	0

*Survival Ratio = Final log of virus titer/initial log of virus titer.

3.0 DETECTION METHOD SELECTION

3.1 GENERAL CONSIDERATIONS

Several things were considered in the establishment of a detection method. First, what were the detection considerations and, based on these considerations, what detection methods applied? Second, once the detection method candidates were selected, how did these methods compare to one another? Finally, which detection method was best suited?

Consideration of a detection technique was made with the following guidelines:

Simplicity
Specificity
Sensitivity
Reagent shelf life
Response time
Minimal effect on water purity and recovery

Based on these considerations, Table V shows the detection methods considered. Each detection procedure was based on an antibody-antigen system which generally provides high specificity and good sensitivity.

3.2 PASSIVE IMMUNE AGGLUTINATION (PIA)

The passive immune agglutination test involved the use of beads (usually polystyrene) of a certain size to which either antigen or antibody had been bound. For the detection of marker virus, either the direct or indirect PIA approach was applied. For use in the direct PIA anti-marker virus (F_2 bacteriophage) antibody was bound to latex beads. For assay, the beads were incubated with test sample. The presence of antigen (in the case of marker virus) in the test sample resulted in cross-linking between beads and aggregation, which was observed as a change in light transmission, read visually or electronically (see Figure 8). For the indirect PIA, antigen (marker virus) instead of antibody (anti-marker virus) was bound to the beads. The test was performed as follows (see Figure 9): A sample test fluid was incubated with a predetermined concentration* of antibody. Following incubation, antigen-coated latex

* The concentration of antibody required is that which is just sufficient to agglutinate the amount of antigen-coated beads used in each assay.

TABLE V PROPOSED DETECTION METHODS

- Passive Immune Agglutination (PIA)
- Fluorescent Antibody (FA)
- Enzyme Immunoassay (EIA)
- Radioimmunoassay (RIA)

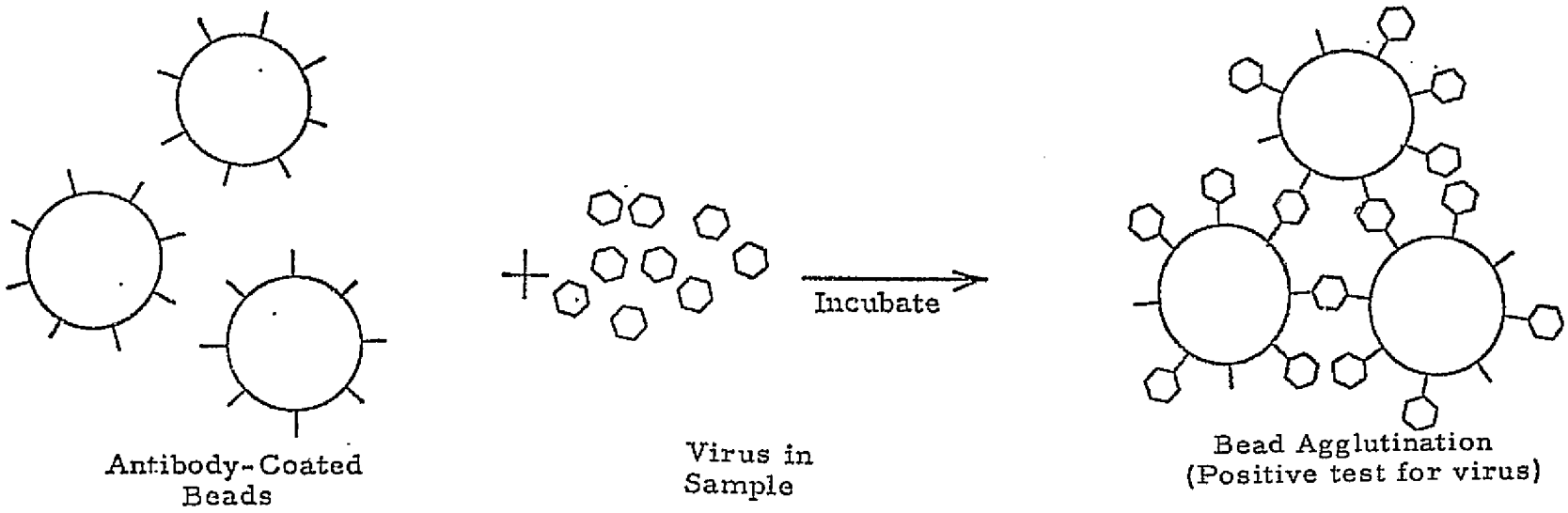
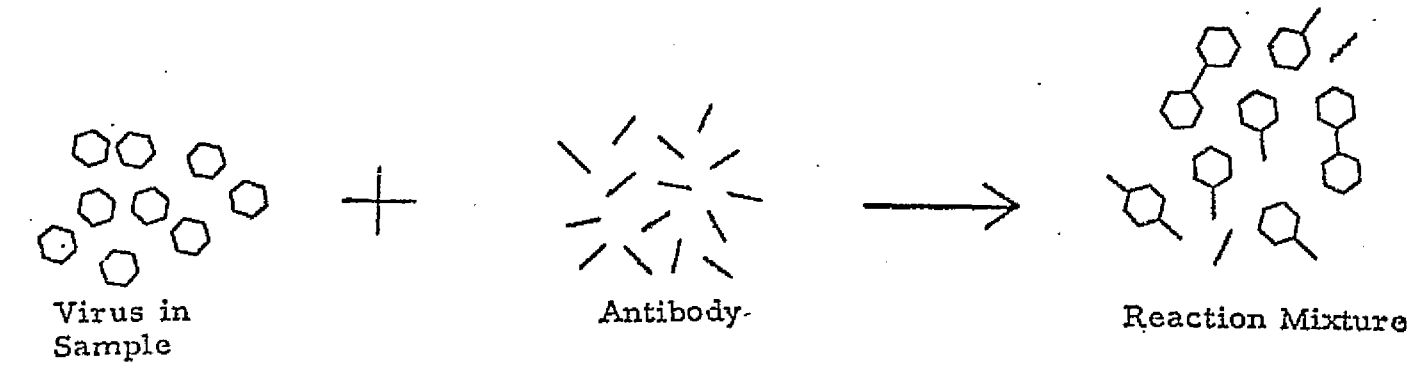


Figure 8 Direct Passive Immune Reaction Sequence



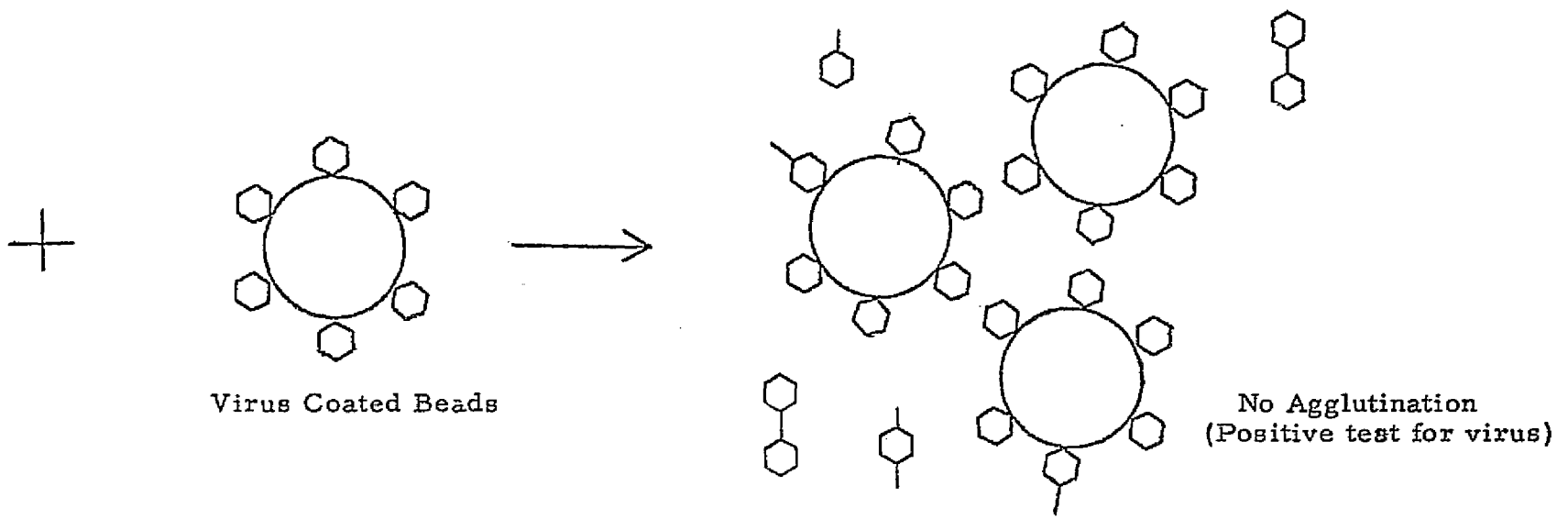


Figure 9 Indirect Passive Immune Reaction Sequence

beads were added for a second incubation. If free antibody was present at the end of the first incubation, clumping of beads occurred during the second incubation. If, however, the added antibody had been completely bound by the presence of antigen in the test sample, then no bead clumping occurs. Thus, in the direct PIA, agglutination is indicative of the presence of marker virus in the test sample, while in the indirect PIA, non-agglutination is indicative of the presence of marker virus.

3.3 FLUORESCENT ANTIBODY (FA)

The fluorescent antibody technique (Figure 10) involved the use of a fluorescent material bound to specific antibody. The tagged antibodies were incubated with specific antigen resulting in an antibody-antigen complex. Excess, non-complexed, tagged antibody was removed, generally by filtration. Complexed antibody could then be detected either visually or electronically by the intensity of light emitted from the complexes under exposure to ultra-violet irradiation. The level of light emitted was directly proportional to the amount of marker virus present.

3.4 ENZYME-LABELED ANTIBODY (ELA)

In the ELA approach, enzyme was bound to specific antibody (Figure 11). In this system, marker virus was incubated with anti-marker virus coated beads (similar to those used in the PIA). This immobilized the marker virus on the bead surface. An aliquot of enzyme-labeled antibody was then added, which attached to the marker virus, forming a sandwich. Excess free labeled antibody was removed and a colorless substrate, for which the enzyme was specific, was added. The amount of substrate converted to product was directly related to the amount of marker virus present. The substrate was chosen such that the product of the reaction was colored and therefore could be read visually or colorimetrically.

3.5 RADIOIMMUNOASSAY (RIA)

The radioimmunoassay, shown in Figure 11-a, involved the use of a radioisotope bound to specific antigen. In this system, specific antibody-coated beads were incubated with the test sample and if specific antigen was present it bound to the beads and used up some available antibody sites. Radioisotope-tagged-antigen was added in excess and incubated with the beads. This resulted

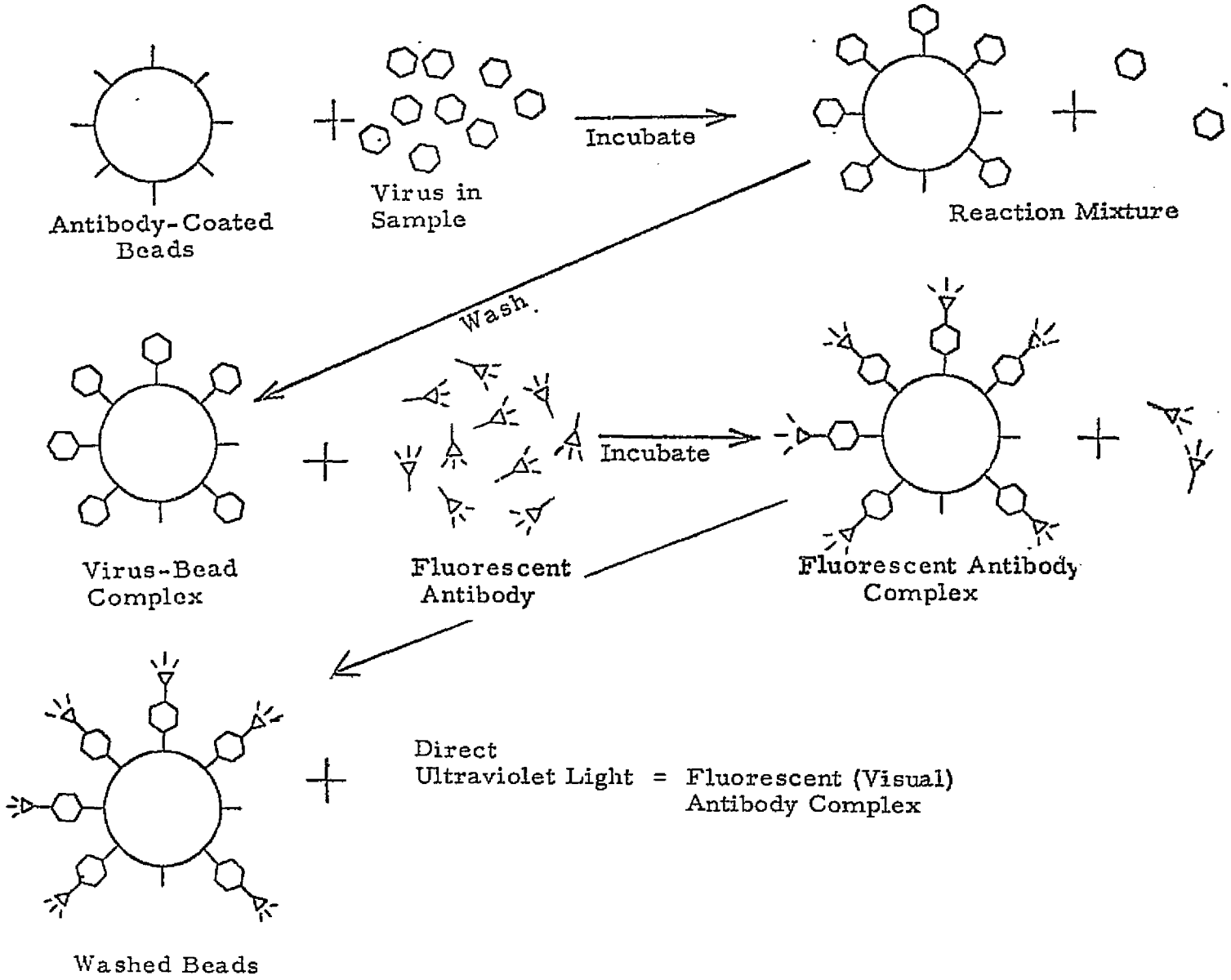


Figure 10 Fluorescent Antibody (FA) Reaction Sequence

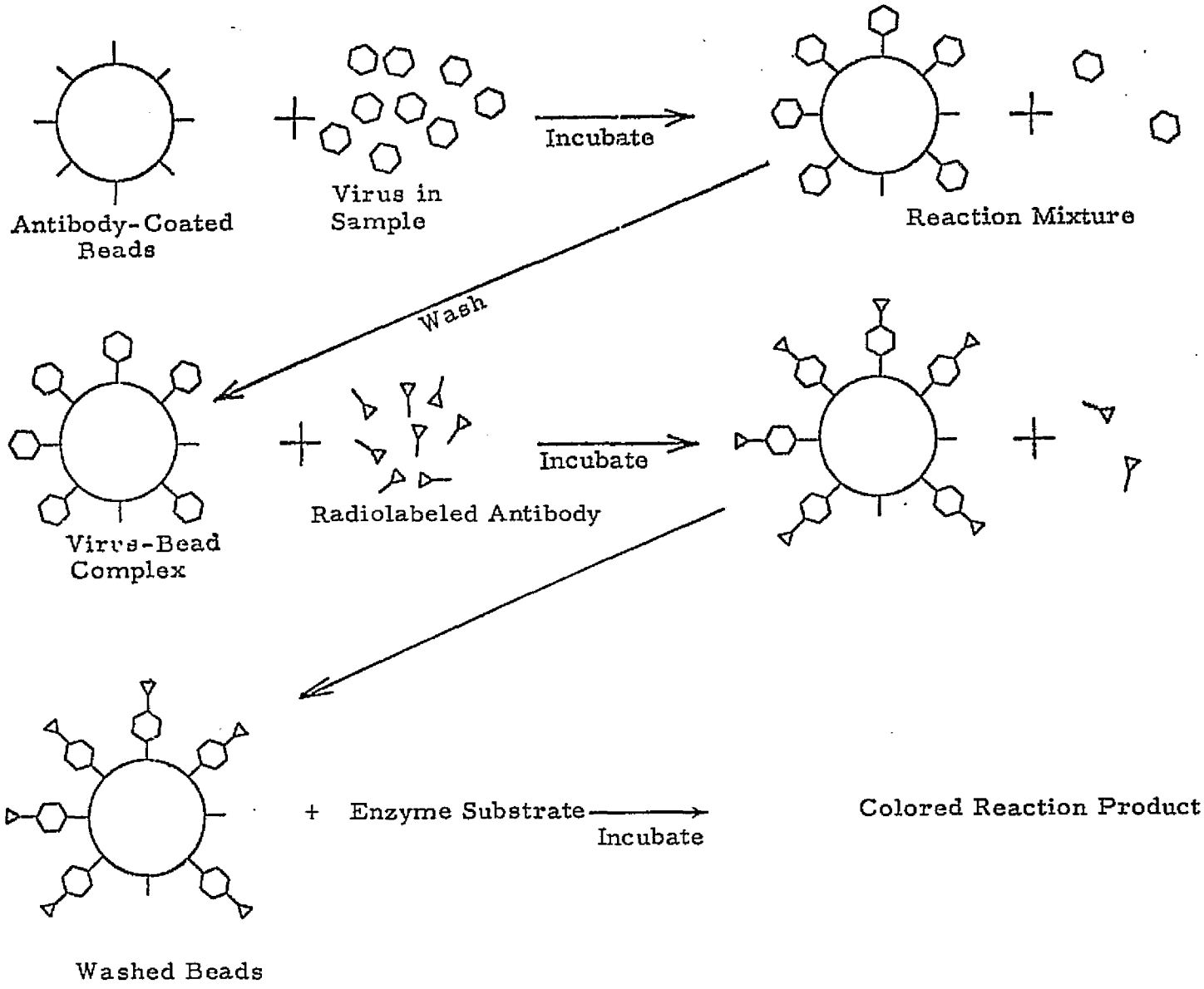


Figure 11 Enzyme Labeled Antibody (ELA) Reaction Sequence

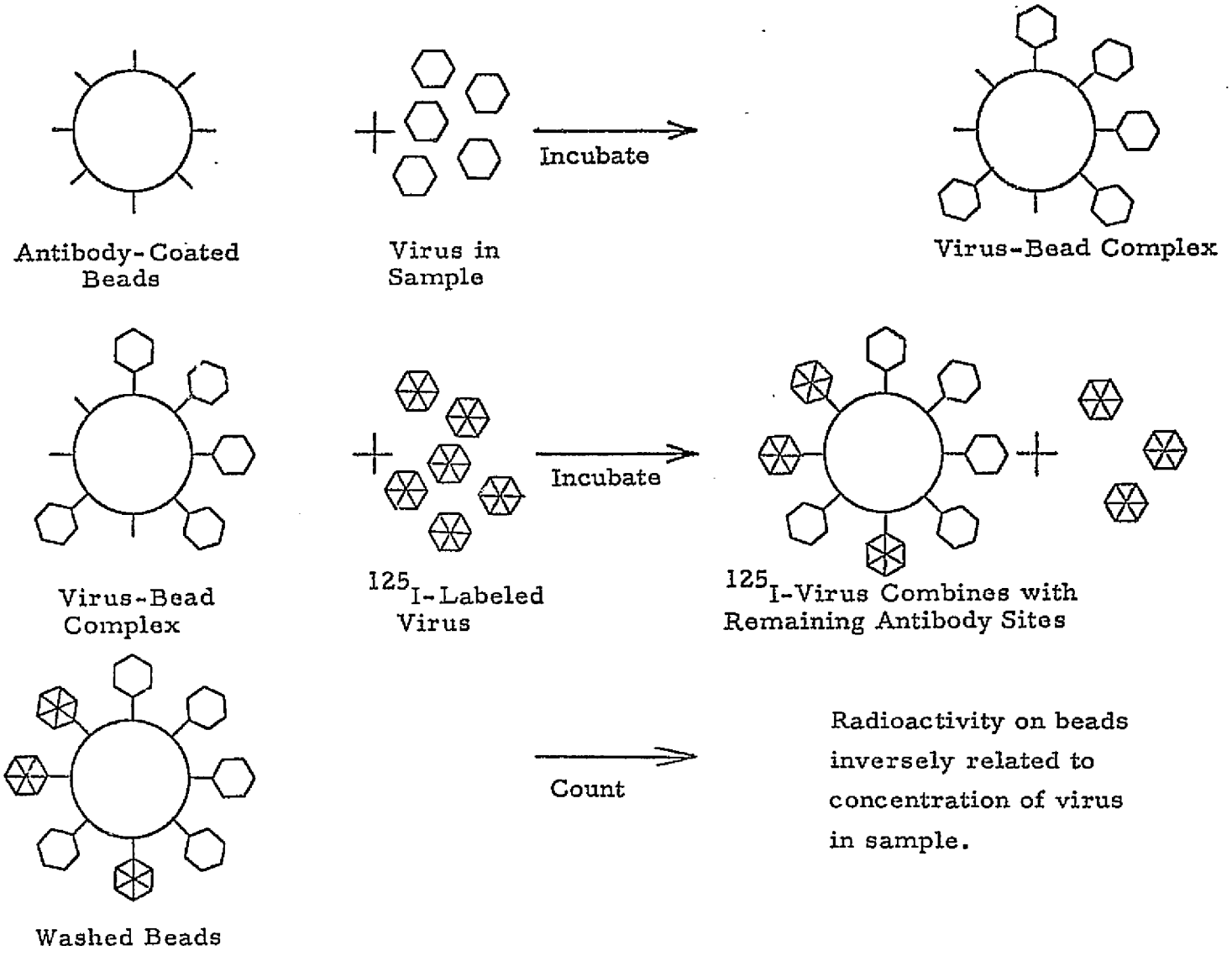


Figure 11-a Radioimmunoassay (RIA) Reaction Sequence

in the remaining antibody sites being taken up by tagged antigen. Beads were separated from free tagged antigen by filtration or centrifugation and the radioactivity of the beads counted. The amount of antigen (marker virus) present in the sample, and subsequently bound to the beads, can be calculated and is inversely proportional to the amount of radioactivity found on the beads.

3.6 CONCLUSION

A comparison of detection methods was made based on the established criteria (Table VI). A rating system was used to grade each detection method. A rating of 0 indicated not acceptable, 2 indicated acceptable, and 4 indicated excellent. An ideal detection system would have high simplicity, high specificity, a relative sensitivity of 1, a shelf life of 1 year or more, and a test time of 3 hours or less. This system would have a maximum rating of 20. Using this rating system, a detection system based on passive immune agglutination (PIA) appeared to be the best choice.

TABLE VI COMPARISON OF DETECTION SYSTEMS

Characteristic	Rating* Scale (0-4)	Detection System Ratings RIA	PIA	FA	ELA
Simplicity	Low → High	2	4	2	2
Specificity	Low → High	4	2	4	4
Sensitivity	$10^3 \rightarrow 1$	4	2	0	4
Shelf Life	6 Mo → 1 Yr	0	4	4	2
Test Time	6 Hr → 3 Hr	4	4	4	2
SYSTEMS TOTALS	0-20	14	16	14	14

*Scale for each characteristic is 0 to 4 points, with 0 for least desirable and 4 for most desirable

4.0 MARKER VIRUS REAGENTS

The two primary reagents necessary for development of the viral monitoring system were purified antigen (virus) and antibody. It was first necessary to grow, concentrate and then purify the marker virus. Once purified marker virus was available, it was used in the production of anti-marker virus antibody. The antibody was then purified and, along with the purified marker virus, was used in the development of the marker virus detection system.

4.1 MARKER VIRUS PRODUCTION

Large quantities of marker virus were required during the program, for use in the preparation of specific antibodies as well as for testing potential virus detection systems.

4.1.1 Determination of Multiplicity of Infection

For production of bacteriophage in large numbers, the broth method was employed. This involved infecting a given number of viable host bacteria in suspension with a known number of infectious bacteriophage. The ratio of the number of infectious bacteriophage to the number of viable host bacteria is called the Multiplicity of Infection or MOI. The optimum MOI will vary depending on the type of bacteriophage being produced.

To determine the optimum MOI for preparation of high titer bacteriophage F_2 (marker virus) stocks, the following experiment was performed. Host bacteria Escherichia coli C3000 were grown overnight at 37°C in broth culture containing the following:

	Per Liter of Distilled H_2O
Tryptone	20 gms
Yeast extract	1 gm
Glucose	1 gm
NaCl	8 gms
$CaCl_2$	0.22 gms

After overnight incubation, the bacterial titer was determined using a Petroff Hauser counting chamber. The bacterial suspension was then diluted in sterile broth to 1×10^8 bacteria per ml. Bacteriophage F_2 was added to separate bacterial suspension at the following MOI: 10, 1, 0.1, and 0.01. The infected

bacteria (50 mls) were incubated for 3 hours at 37°C in 250 ml flasks and aerated using a counter top rotator (rotating at 200 rpm).

After the incubation period, 1 ml of lysozyme (50 mg) was added and reacted for 5 minutes. Following this, one part chloroform was added to 19 parts of medium and reacted for 5 minutes. The lysate was then centrifuged at 650 g to sediment cell debris. The supernate was saved and tested for viable bacteriophage using the plaque assay technique. The results of this experiment are provided in Table VII. From these results it appeared that an MOI in the range of 0.01 to 0.1 gave optimum titers of bacteriophage F_2 under the experimental conditions. The enumeration of bacteriophage using the plaque assay technique is performed as follows: About 2 mls of melted 0.7% agar is cooled to 45°C and inoculated with a drop of an overnight broth suspension of the host bacterium (E. coli C 3000). A measured volume of phage suspension is then added and the entire mixture poured over the surface of a petri dish plate filled with hardened nutrient agar. The plate is then incubated overnight at 37°C. The bacteria grow forming an opaque background called a bacterial lawn. In this lawn, clear areas are present, called plaques, each of which is assumed to have been caused by a single infective particle. A plaque count is then made and the number of plaque forming viruses present in the stock suspension calculated. The titer is then expressed as the number of PFU (plaque forming unit) present per milliliter.

4.1.2 Determination of Growth Condition

To further optimize production of bacteriophage F_2, two parameters were investigated. These were time of incubation and degree-of-aeration.

To determine optimum incubation time, host bacteria infected with bacteriophage F_2 at MOI of 0.01 and 0.1 were incubated at 37°C and aerated using a counter top rotator. Samples were taken at 1, 2, 3, and 4 hours and assayed for infectious bacteriophage by plaque assay. The results of this experiment are shown in Figure 12. Based on these results, an incubation time of 3 hours at an MOI of 0.01 was chosen as optimum. Although 4-hour incubation gave a slightly higher titer at this MOI, it was not selected due to the increased growth of resistant cells.

Three conditions were studied to determine the effect of aeration on production of marker virus. These were: aeration using a counter top

TABLE VII EFFECT OF MOI ON BACTERIOPHAGE F_2 PRODUCTION

MOI	PFU*/ml
0.01	1.0×10^{12}
0.1	1.6×10^{12}
1	6.9×10^{11}
10	5.3×10^{9}

* PFU = plaque forming unit

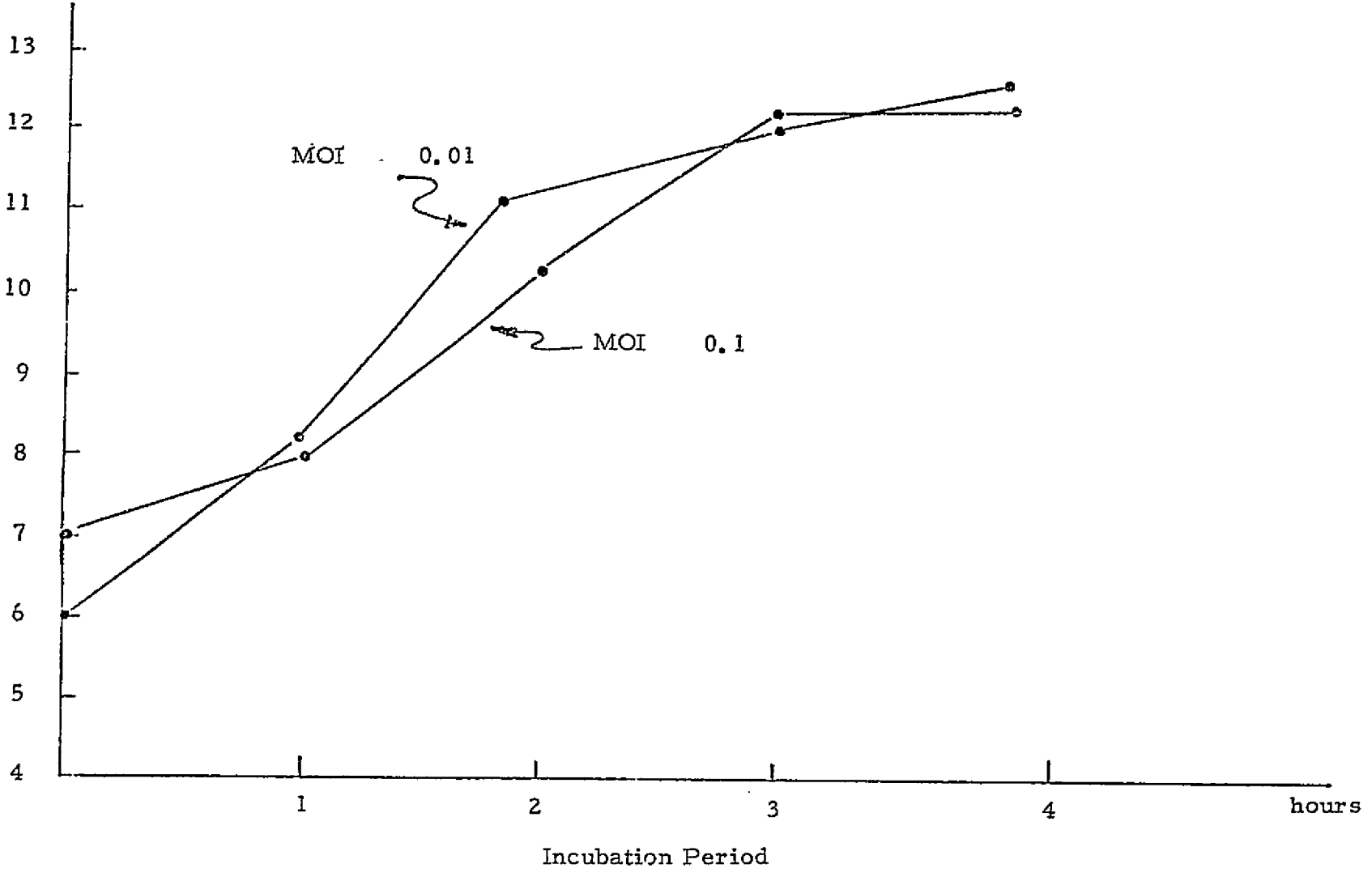


Figure 12 Timed Study of F_2 Production at MOI of 0.01 and 0.1

rotator, stirring by magnetic bar and still culture. All three conditions were tested by incubation at 37°C for 3 hours with an MOI of 0.01. The results of this experiment are shown below.

	PFU/ml
Counter top rotator	1.0×10^{12}
Magnetic stirring	7.8×10^{11}
Still culture	2.7×10^{11}

As shown, the highest titer was obtained using a counter top shaker followed by stirring using a magnetic bar. The lowest titer was obtained using still culture.

Based on the above testing, marker virus production conditions were standardized as follows: an MOI of 0.01, incubated at 37°C for 3 hours, and aeration using either a counter top rotator or magnetic stirring.

4.2 MARKER VIRUS CONCENTRATION AND PURIFICATION

A protocol for the concentration and purification of the marker virus (bacteriophage F_2) stock lysate was described by Cooper and Zinder, 1963. This technique (Figure 13) utilized ammonium sulfate precipitation in combination with low speed centrifugation for marker virus concentration, and high speed centrifugation, filtration and cesium chloride density centrifugation for marker virus purification.

Procedures for concentration and purification of marker virus were established based on this information.

4.2.1 Marker Virus Concentration

4.2.1.1 Ammonium Sulfate Precipitation and Recovery of Marker Virus

A trial run was made under the established production conditions and marker virus partially purified and concentrated by the procedure shown in Figure 14. In this test, 1200 mls of lysate having a titer of 1.03×10^{12} PFU/ml was used, thus giving a total of 1.24×10^{15} PFU. The lysate was then subjected to the described procedure, with each step being monitored for infectious

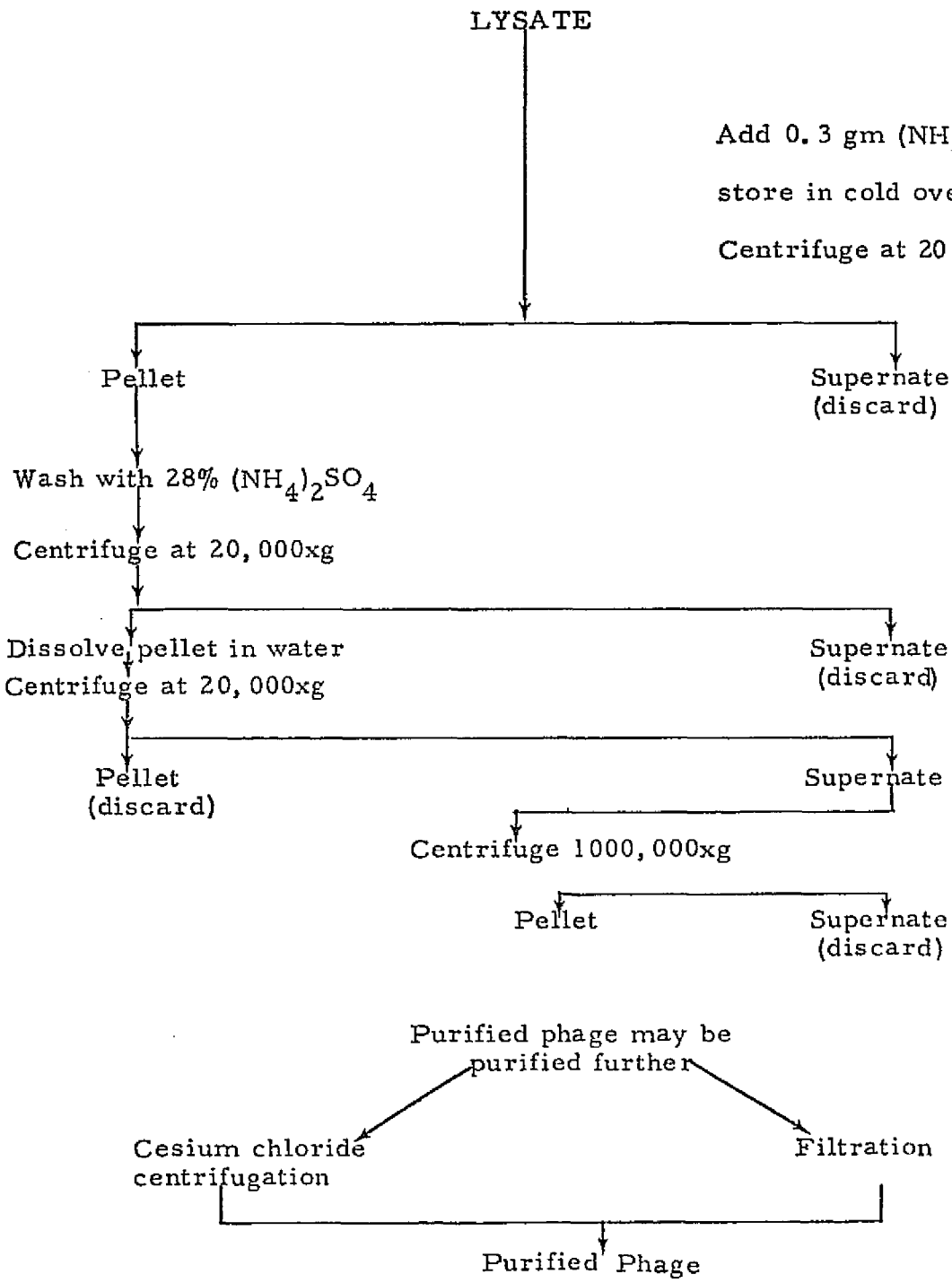


Figure 13 Purification of Marker Virus (Bacteriophage F_2) Cooper and Zinder, 1963.

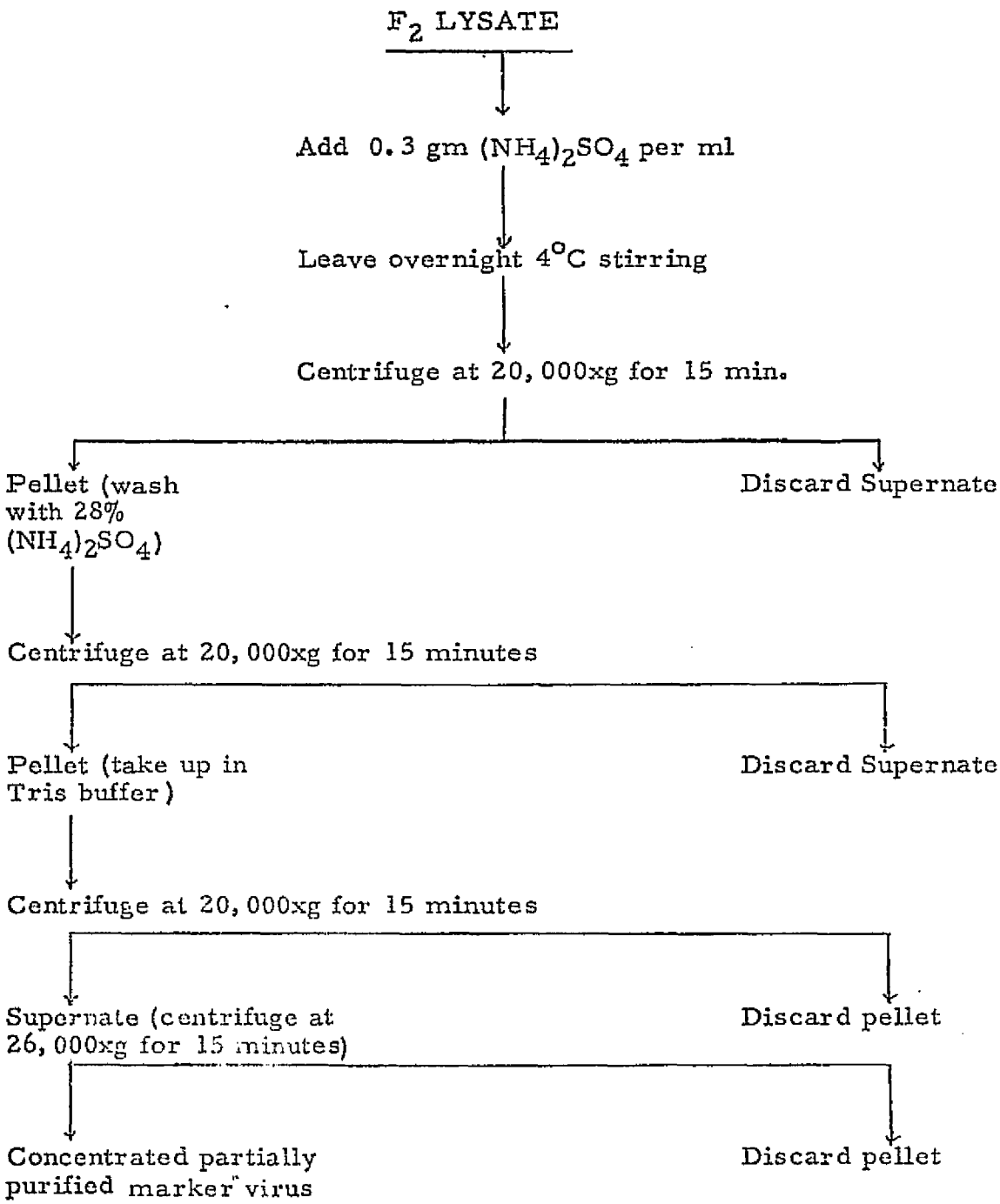


Figure 14 Procedure for Concentration and Partial Purification of Marker Virus (Bacteriophage F_2)

units. The final step resulted in 60 mls of concentrate having a titer of 1.5×10^{13} PFU/ml giving a total of 9.0×10^{14} PFU. This gave a recovery of 73%. A majority of the marker virus not recovered appeared in the supernate obtained after centrifugation of the overnight ammonium sulfate precipitation. This technique was selected as an effective purification and concentration method.

4.2.2 Marker Virus Purification

Several purification techniques were investigated, including ultracentrifugation and gel filtration chromatography. A comparison of techniques was made based on their ability to recover marker virus (bateriophage F_2) and to remove contaminating host and media protein.

4.2.2.1 Ultracentrifugation

Initial attempts to ultracentrifuge marker virus was based on the work of Cooper and Zinder, 1963, to determine if this technique would work for purification of the marker virus. Concentrated marker virus was obtained using ammonium sulfate precipitation as described in Section 4.2.1.1. This concentrated marker virus was ultracentrifuged at 113,000g for 3 hours and the pellet redissolved overnight in Tris buffer. A large part of the pellet did not dissolve and was removed by low speed centrifugation. An infectious bacteriophage assay and protein determination performed on the centrifuged sample revealed a recovery of 47% of the initial starting infectious marker virus with an 11-fold reduction in protein over the starting material (see Table VIII).

It was felt that this recovery might be improved by the use of sucrose cushioning. In this technique, a dense solution of sucrose (60% W/W) was layered at the bottom of the centrifuge tube thus providing a cushioning effect on the sedimenting virus. A less dense solution of sucrose was layered over the dense sucrose solution, providing an additional purification by restricting the passage through this layer of any contaminating protein with a density less than the second sucrose layer. In our application, the starting sample was deposited over the second sucrose layer and the tube centrifuged. The interface occurring between the two sucrose layers was collected and was expected to contain marker virus.

TABLE VIII COMPARISON OF PURIFICATION TECHNIQUES

Technique	Relative* Recovery %	μg Protein per PFU**	Purification Factor
None (starting material)	100	3.39×10^{-10}	___
Gel Filtration	77	1.01×10^{-11}	33.6
Pelleting	47	3.08×10^{-11}	11.0
Sucrose cushion	16	5.25×10^{-11}	6.5

*
** As assessed by retained infectivity
PFU = plaque forming unit

Specifically, an experiment was set up using marker virus concentrated by the ammonium sulfate precipitation procedure as the starting material. The marker virus was ultracentrifuged at 113,000 g for 3 hours at 4°C using a sucrose cushion with the interfaces occurring between the sucrose layers collected, dialyzed and tested for protein and infectious marker virus.

The results shown in Table VIII revealed that sucrose cushioning was less effective than pelleting in both recovery of starting viable marker virus with only a 16% recovery, and in removal of contaminating protein, with a 6.5-fold reduction in protein.

4.2.2.2 Column Chromatography

The use of gel filtration chromatography was investigated as a possible method for purification of marker virus. In this technique, proteins can be separated according to their molecular weights. An attempt to purify marker virus was made utilizing Bio Rad A-5m, which is an agar gel with the ability to separate proteins with molecular weights ranging between 5,000,000 and 10,000. A column was poured and a sample of marker virus concentrate added. Three peaks showing absorbance at 254 nm were observed (see Figure 15). These were collected and assayed for protein and viable phage. This testing revealed that the second peak contained a majority of the starting viable marker virus. As seen in Table VIII, this technique allowed for a recovery of 77% of the initial infectious marker virus with a 33.6-fold reduction in protein.

Based on this information, it was decided that column chromatography in combination with pelleting by ultracentrifugation would be used for the purification of marker virus.

4.2.3 Finalized Procedure for Marker Virus Concentration and Purification

Table IX summarizes the results of the concentration and purification of marker virus (bacteriophage F_2) following the procedure shown in Figure 16. Each major step of this protocol was monitored for infectious marker virus and protein to determine recovery and purity.

Marker virus was produced under established production conditions yielding 1000 ml of lysate containing a total of 1.0×10^{15} plaque forming units. A Lowry protein determination revealed that there were 2.4×10^{-9} μg of protein

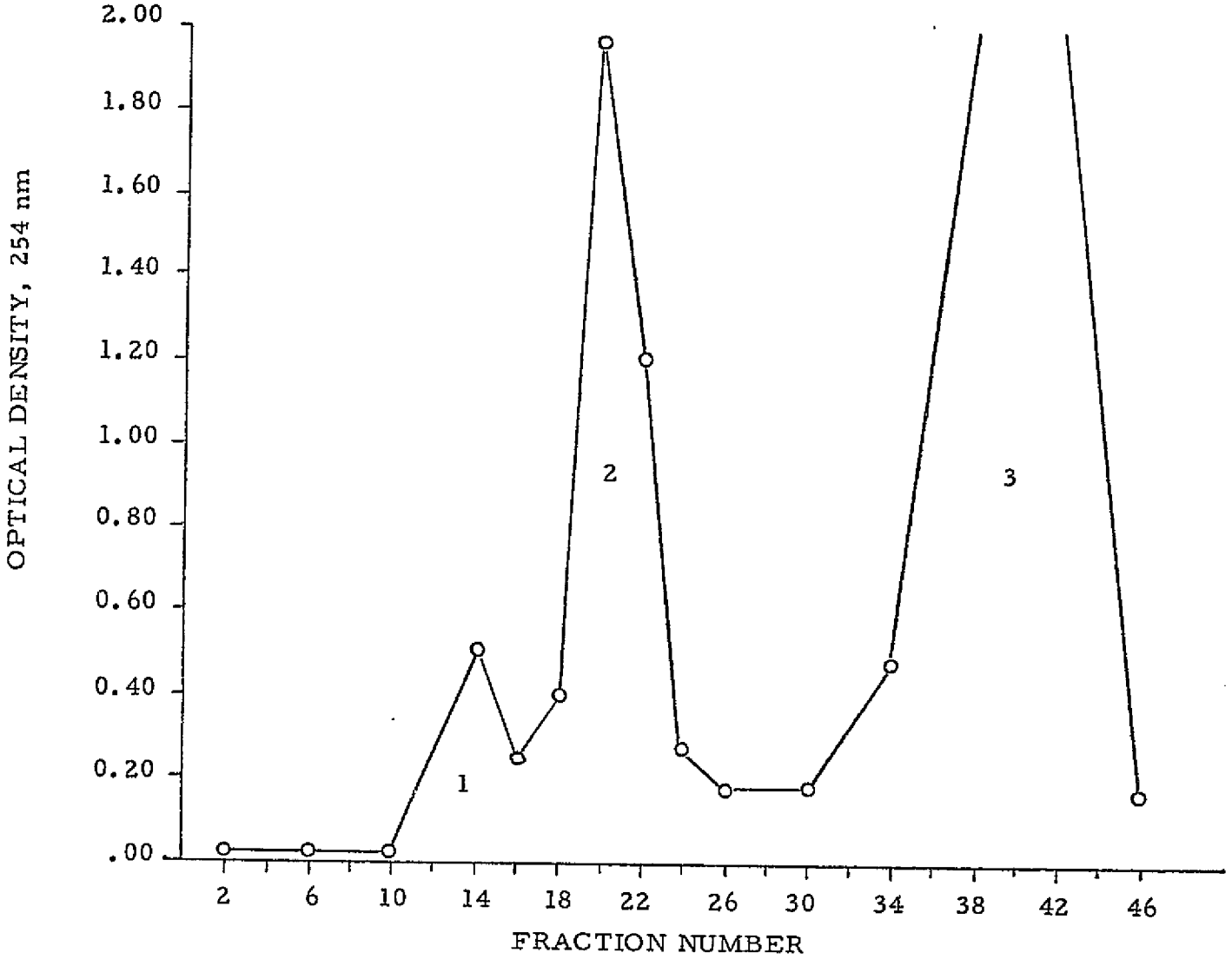


Figure 15 Purification of Marker Virus Using Bio-Rad A-5m

TABLE IX RECOVERY AND PURITY OF MARKER VIRUS (BACTERIOPHAGE F_2)

Sequential Purification Steps	Total PFU Recovered	Recovery %		μg Protein Per PFU	*Purity
		Single Step	Overall		
Lysate	1.0×10^{15}	100	100	2.4×10^{-9}	—
$(NH_4)_2SO_4$ Precipitation	7.5×10^{14}	75	75	6.0×10^{-11}	4.00×10^{1}
Gel Filtration	5.1×10^{14}	68	51	1.22×10^{-11}	1.97×10^{2}
Ultra-centrifugation	2.6×10^{14}	51	26	6.55×10^{-12}	3.66×10^{2}

*Ratio: $\frac{\text{Amount of protein in lysate}}{\text{Amount of protein following purification step}}$

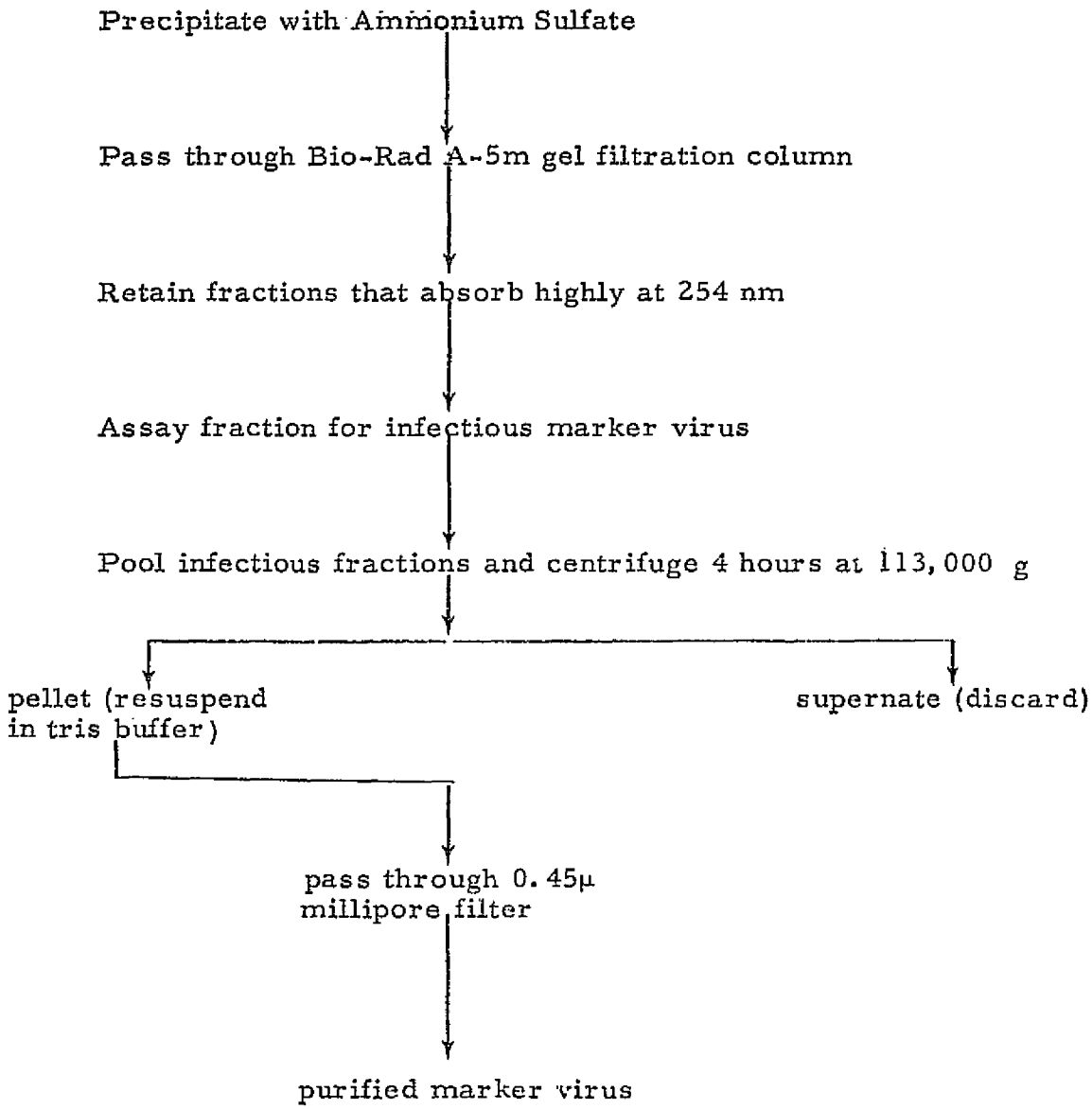


Figure 16 Procedure for the Final Purification of Marker Virus (Bacteriophage F_2)

per infectious virus particle. The lysate was then subjected to the ammonium sulfate precipitation procedures. This resulted in a reduction of volume to 10 ml containing 7.5×10^{14} infectious virus particles or 75% of the original total. A protein determination revealed 6.0×10^{-11} μg of protein per infectious virus particle, this represented a 4.00×10^{3}-fold reduction of protein. This 10 ml was subjected to gel filtration on an A-5m column and the 2nd peak, containing the viable phage, collected. A total volume of 40 ml was recovered containing 5.1×10^{14} infectious virus particle or a 51% recovery of the original starting number. A protein determination showed a protein to infectious virus particle ratio of 1.22×10^{-11} μg and represented a 1.97×10^{4}-fold reduction in protein when compared to the original lysate. The 40 ml was ultracentrifuged for 4 hours at 113,000 g and the resulting pellet 0.45 HA Millipore filter and contained a total of 2.6×10^{14} infectious virus particle or a recovery of 26% of the original. A protein determination showed there was 6.55×10^{-12} μg protein per infectious virus particle which represented a 3.66×10^{4} times reduction in protein per infectious virus particle below that in the original lysate.

It was calculated that, based on a molecular weight of 3.6×10^{6}, one infectious virus particle, assuming 100% protein, would be equivalent to 5.8×10^{-12} μg. Since the final purification step approached this level of protein per plaque forming unit, within the errors of the measurement techniques, no further purification was performed and inoculation of rabbits was initiated.

4.3 MARKER VIRUS ANTIBODY PRODUCTION

New Zealand female white rabbits were selected for use in the production of specific antibodies. Before inoculation was begun, each rabbit was trial bled and the sera tested for anti-marker virus activity. This was performed by mixing a 1/10 dilution of rabbit sera with a known titer of infectious marker virus and incubating for 30 minutes at 37°C. A marker virus assay was then performed utilizing the plaque assay technique, to see if any inactivation had occurred. Once it was established that the sera contained no anti-marker virus activity, as indicated by no loss of viral titer, the inoculation schedule was begun. Two groups of rabbits were inoculated with purified marker virus. One group (Group I-A), consisting of five rabbits, was inoculated with commercially purified bacteriophage F_2 (marker virus) purchased from Miles Laboratory, and the other group

(Group II), consisting of 12 rabbits, was inoculated with "in-house" purified marker virus. Another group (Group I-B), consisting of five rabbits, was inoculated with control antigen.

4.3.1 Immunization Schedule

The immunization schedule consisted of a seven-week program. During the first six weeks, a series of three intramuscular injections (I.M.) were given at two-week intervals. The volume, amount and compositions of these infections are described in sections 4.3.2 and 4.3.3. After the sixth week, a booster injection of material was given intraperitoneally. Trial bleedings were made after the 4th, 6th and 7th week. Group I-A and II rabbits were sacrificed after the seventh week and Group I-B rabbits, after the eighth week.

4.3.2 Production of Anti-Marker Virus Antibody

Purified marker virus suitable for antibody production was derived from two sources. A Miles Laboratory purified marker virus was used to inoculate one group of rabbits (Group I-A) with a second group of rabbits (Group II) started on the inoculation schedule using marker virus purified "in-house".

Intramuscular injections at each time period consisted of two 1.0 ml injections, one in each hind leg, of a 50/50 suspension of marker virus, at a titer of 1×10^{11} PFU/ml, and Freunds complete adjuvant. Intraperitoneal injections consisted of marker virus, at a titer of 1×10^{11} PFU/ml and were suspended in 0.05 M tris + 0.1 M NaCl, pH 7.6

4.3.3 Production of Anti-Control Antigen Antibody

Production of antibodies against control antigen was performed using material consisting of host bacteria and growth media preparation. The control antigen was prepared following the previously described marker virus production protocol, with the single exception that no marker virus was added. In this way the control antigen contained all the protein material found in the lysate except for the marker virus. With antibodies formed against this material, it would then become possible to check the purity of the marker virus preparations.

When it was established that no marker virus was present in the control antigen preparation by the plaque assay technique, inoculation of rabbits was initiated.

Intramuscular inoculation of control antigen at each time period consisted of two 1.0 ml injections, one in each hind leg, of a 50/50 mixture of control antigen and Freunds complete adjuvant, Intraperitioneal injections consisted of 1.0 ml injections of control antigen.

4.3.4 Antibody Response

Trial bleedings from rabbits inoculated with marker virus (Group I-A and Group II) were tested for specific antibodies by three procedures; Ouchterlony gel diffusion, complement fixation and marker virus inactivation. Trial bleedings from rabbits inoculated with control antigen were tested for specific antibody response by Ouchterlony gel diffusion and by complement fixation.

4.3.4.1 Ouchterlony

The Ouchterlony agar gel diffusion technique can be used to determine if an antibody response has occurred. In this procedure, wells are cut in an agar plate with antibody (rabbit sera) placed in one well and antigen (purified marker virus) placed in a neighboring well. The antibody and antigen diffuse towards each other until an optimum ratio of antibody to antigen is reached where a visible precipitation occurs. By this procedure, it is possible to detect the presence of a specific antibody when tested against a specific known antigen or vice versa. The Ouchterlony technique was used to monitor antibody production during the inoculation schedule, in all three groups of rabbits.

In all rabbits inoculated with marker virus (Group I-A and Group II), specific antibody was detected in sera from the fourth week trial bleeding. In the rabbits inoculated with control antigen (Group I-B), no specific antibody was detected in any of the trial bleedings. The absence of detection of specific anti-control antigen antibody could indicate one or more of the following: First, it is possible that antibody against control antigen had not yet been produced in high enough titer to produce a precipitation line and a more sensitive antibody detection method would have to be employed. Second, the control antigen may not have elicited an antibody response.

Finally, there may have been a good antibody response, but the condition may not have been correct for the precipitation reaction to occur.

4.3.4.2 Complement Fixation (CF)

Complement fixation can be used to determine if antibody response has occurred and at what level. The procedure is based on the ability of an antibody-antigen complex to bind complement. The test involves two steps. In the first step, serum containing the antibody to be tested (anti-marker virus) is serially diluted. To each dilution of serum, a constant amount of antigen marker virus and complement is added. If antibody-antigen complexes occur, the complement is bound. The second step involves the addition of an indicator system to show if there is free complement present. This system uses sheep red blood cells (SRBC) complexed with anti-sheep cell antibody (hemolysin). When this complex is reacted with free complement, the SRBC lyse. Therefore, when this system is added as the second step of the complement fixation test, and lysis of the SRBC occurs, it indicates that no antibody was present in the first step. If, however, lysis does not occur, it indicates that the complement needed for lysis had become bound to the antigen-antibody complex that was present in the first step. The amount of antibody present in a sample can be semi-quantitated by determining the highest dilution of antibody which, when complexed with antigen, will still bind complement. Once this has been determined, the inverse of this dilution is then taken as the CF titer. Although this is a rather complex testing system, it can be very useful, when used with the proper controls, in determining the presence and level of specific antibody or antigen. The greater the CF titer, the greater the level of either antibody or antigen. Sera obtained from trial bleedings during the 4th, 6th and 7th weeks, from groups I-A and II, were tested by CF. The results obtained from group I-A are shown in Table XII. With group I-B (control antigen), an additional inoculation consisting of an intravenous injection (IV) was given, due to the low CF titer following the 7th week. No further increase in CF titer resulted. The results from this group of rabbits are shown in Table XIV. The CF titers from group I-A rabbits did not increase after the intraperitoneal (IP) injection. With group II rabbits, however, there was a general increase in CF titer the week following the IP injection. The results from this group of rabbits are shown in Table XIII.

TABLE XII COMPLEMENT FIXATION TITERS AND K VALUES FOR ANTI-SERA (ANTI-MARKER VIRUS) FROM GROUP I-A RABBITS AFTER THE FOURTH, SIXTH, AND SEVENTH WEEKS OF THE INOCULATION SCHEDULE

Rabbit	4th Week		6th Week		7th Week	
	CF Titer	K Value	CF Titer	K Value	CF Titer	K Value
1	64	2321	128	7920	128	7920
2	64	3281	128	5198	64	7291
3	128	2739	128	4600	128	7917
4	32	1266	128	4600	128	7539
5	64	912	128	4600	128	7291

TABLE XIII COMPLEMENT FIXATION TITERS AND K VALUES FOR ANTI-SERA (ANTI-MARKER VIRUS) FROM GROUP II RABBITS AFTER THE FOURTH, SIXTH AND SEVENTH WEEKS OF THE INOCULATION SCHEDULE

Rabbit	4th Week		6th Week		7th Week	
	CF Titer	K Value	CF Titer	K Value	CF Titer	K Value
12	64	2,807	254	7,796	64	4,810
13	64	3,216	512	8,102	256	14,008
14	32	4,579	256	7,995	256	15,631
15	64	2,405	128	9,087	256	15,631
19	32	1,608	64	2,993	256	15,631
21	32	841	128	6,018	256	10,633
22	32	2,992	64	3,447	256	14,008

TABLE XIV COMPLEMENT FIXATION TITERS FOR ANTI-SERA (ANTI-CONTROL ANTIGEN) FROM GROUP I-B RABBITS AFTER THE 7TH AND 8TH WEEKS OF THE INOCULATION SCHEDULE

RABBIT	CF TITER RECIPROCAL	
	7TH WEEK	8TH WEEK
6	8	8
8	4	4
9	4	8
10	8	8

4.3.4.3 Marker Virus Inactivation

The presence of antibody can also be determined by marker virus inactivation. In this procedure, the ability of the antisera to inactivate the infectivity of a given number of bacteriophage is indicative of the presence of specific antibodies. Antibodies against bacteriophage (and virus in general) have the property of reacting with the bacteriophage to form an antibody-bacteriophage complex that is non-infectious for the host cells due to the fact that adsorption of the bacteriophage to the host cells is prevented. The ability of an antisera to inactivate bacteriophage can be expressed as the K value of that antisera. This K value is determined by mixing bacteriophage and antisera and incubating at 37°C. Samples are removed at defined intervals and tested for the ability of the bacteriophage to form plaques. In a range of 90-99% inactivation of bacteriophage by a given dilution of antisera, a K value can be determined by using the following equation:

$$K = 2.3\, D/t \log (Po/P)$$

Po = Bacteriophage assay at a 0 time

P = Bacteriophage assay at time t

D = Final dilution of serum in the bacteriophage-serum mixture

K = Velocity constant

With this system the higher the K value, the higher the level of specific antibody present in the sera. K values calculated for anti-sera from the trial bleedings of group I-A and II are shown in Table XII and XIII. As shown, there were significant increases in K values in both groups following the intrapertoneal injections.

Although no K values have been found in the literature for bacteriophage F_2, K values have been reported for other bacteriophage. When testing was performed on antisera obtained against the T phage group, a K value of 3000 was reported as a good antibody response (Adams, 1959). Based on these results group I-A and II rabbits were sacrificed after the seventh week of the inoculation schedule and their sera frozen and stored. Since no increase in CF titer was observed with group I-B rabbits following the addition IV injection of control antigen, it was decided that they had reached their antibody production peak and were sacrificed. The sera were stored frozen.

4.4 MARKER VIRUS ANTIBODY PURIFICATION

Anti-marker virus antibody (IgG) was isolated from high titered sera of group II rabbits. The isolate was obtained by three successive precipitations with 35% saturated ammonum sulfate. The final precipitate was redissolved in distilled water at one-half the original serum volume. After dialysis overnight against 0.0175 M phosphate buffer, pH 7.6, the IgG was purified by passage through a DEAE Sephadex column. Under the conditions employed, only IgG passed through; the other serum proteins were retained by the gel. Each fraction which contained protein was tested by electrophoresis and immunoelectrophoresis to determine purity.

Electrophoresis involves the separation of proteins according to their mobility in an electrical field. The proteins migrate towards the anode or cathode at a rate dependent on their mass and net charge. Whole serum contains two major groups of proteins, albumin and globulins. The globulins are separated further into alpha, beta, and gamma. The purity of the isolated gamma globulin can be determined by the electrophoresis pattern obtained.

Immunoelectrophoresis, as the name implies, combines the technique of electrophoresis with an immunological, antigen-antibody reaction. In this system, the rabbit sera is subjected to electrophoresis in a soft agar support. After migration has occurred, anti-rabbit serum containing antibodies to all the protein components found in normal sera is added to the system. The separated rabbit proteins and the anti-rabbit sera diffuse throughout the gel and, where they contact each other at appropriate concentrations, a visible line of precipitation is produced for each unique protein. This procedure is more sensitive than single electrophoresis, with more components of the rabbit sera being revealed. This purification and test program was applied to pooled sera from group II. High titered sera from rabbits # 14, 15 and 19 were pooled and their IgG purified and designated IgG 14-15-19. Testing by electrophoresis and immunoelectrophoresis confirmed the purity of the IgG preparation. Further testing of the preparation by complement fixation and marker virus inactivation was performed to determine the reactivity of the IgG preparation. Testing revealed the IgG 14-15-19 preparation had a CF titer of 256 and a K value of 5424. Additional complement fixation tests showed no antibodies present against control antigen. This purified IgG was then used as the antibody in developing the marker virus detection technique.

5.0 DEVELOPMENT OF A VIRAL MONITORING SYSTEM

The viral monitoring system, as envisioned, involved two major steps. The first step consisted of a marker virus concentration system which was needed to increase the effective sensitivity of the detection system. The second step involved the development of a detection system to quantitate the presence of marker virus in the concentrate.

5.1 MARKER VIRUS CONCENTRATION

A number of marker virus concentration techniques were investigated. Two techniques, involving the use of Bio-Fibers®and viral adsorption, were studied extensively. Several other techniques, based on viral precipitation and ultrafiltration, were looked at briefly.

5.1.1 Concentration of Marker Virus Utilizing Bio-Fibers

A concentration system has been marketed by Bio-Rad Laboratories utilizing a bundle of semi-permeable hollow fibers. The fibers can be considered as molecular sieves, with a fixed effective pore size, available in a variety of sizes depending on the size of the molecules to be retained. For our experimentation, Bio-Fiber 80 was employed with a M.W. cutoff of 30,000. A diagram of the basic system is shown in Figure 17. Using this approach, a sample containing virus to be concentrated is pumped and recycled through the fibers. All molecules, such as water, that have a molecular weight less than 30,000, are removed from the fiber bundles by either creating a vacuum on the outside of the fibers or positive pressure on the inside of the fibers. All molecules with molecular weight greater than 30,000, such as the selected marker virus with a molecular weight of 3,000,000, are then concentrated inside the fibers.

A number of experiments were performed to evaluate and optimize this system for use as a marker virus concentrator. Testing included studies on the effect of flow rate and backwashing on the recovery of marker virus. Testing was also performed to evaluate the overall recovery and concentration of marker virus by either producing a vacuum on the outside of the fibers or by creating a pressure inside the fibers to remove water. Once optimum concentration

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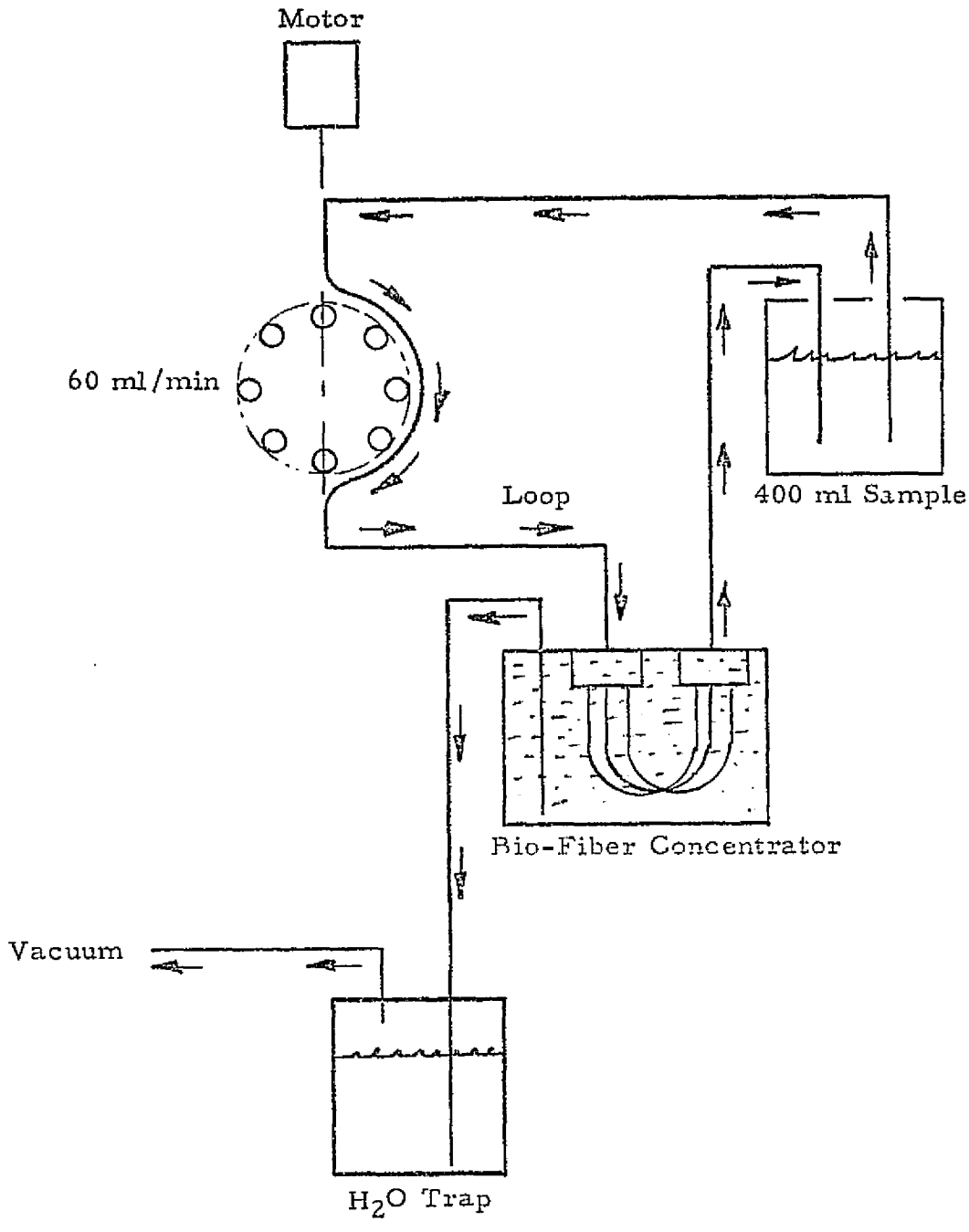

Figure 17. Diagrammatic Representation of Marker Virus Concentration Utilizing Bio-Fibers (Applied Vacuum)

conditions were established, experiments were performed to modify the system for ease of automation.

5.1.1.1 Effect of Flow Rate and Backwashing

An experiment was set up to determine the effect of flow rate of the sample through the fibers on recovery of virus. Sample flow rates of 35 ml/minute and 70 ml/minute, using a peristaltic pump, were investigated. In each case, the initial starting volume was 400 ml of distilled water. A marker virus concentration of approximately 1×10^8 PFU/ml was used. The sample was concentrated to approximately 30 ml utilizing a pressure differential across the fibers of approximately 0.5 atm. At a flow rate of 35 ml/minute, a 400 ml distilled water sample containing a total of 3.46×10^{10} PFU was concentrated to 27 ml in one hour A plaque assay on this concentrated sample revealed a total of 1.8×10^{10} PFU or a 52% recovery. This low recovery, it was felt, was due to virus building up on the inside wall of the fibers. Thus, a higher flow rate was tested in hopes that the shear forces created would prevent this buildup. When testing at a flow rate of 70 ml/minute, a 400 ml distilled water sample containing a total of 5.8×10^{10} PFU was concentrated to 34 ml in approximately one hour. Testing revealed that this concentrated sample had a total of 3.9×10^{10} PFU or represented a 67% recovery.

It was felt that the recovery of the remaining 33% of the marker virus could probably be accomplished by a backwash procedure. To do this after the sample has been concentrated, a pressure differential is set up to reverse the flow of solute. In other words, instead of using a vacuum, for example, to pull water out of the fiber bundles, a pressure differential is exerted to push water into the fiber bundles and thus free marker virus adhering to the inside of the fibers. An experiment was set up in which a 400 ml sample containing marker virus was concentrated using a sample flow rate through the Bio-Fibers of 70 ml/minute with a pressure of 0.5 atm. on the outside of the fibers. Under these conditions, the sample was concentrated to 31 ml in approximately one hour. A backwash procedure was employed by attaching a vacuum to the inside of the fibers and drawing the solute into the fibers. An additional 10 ml was collected which contained 27% of the initial

starting marker virus. When combined with the original 31 ml concentrate, this resulted in 41 ml of concentrate containing approximately 100% of the initial starting marker virus. Thus for maximum virus recovery, using the Bio-Fiber system, a flow rate of 70 ml/minute is needed combined with a backwash procedure.

5.1.1.2 Comparison of Vacuum vs Pressure

Water may be removed from the fiber bundles, either by decreased pressure on the outside of the fiber or by increased pressure on the inside of the fibers. As described in Section 5.1.1.1, good concentration and recovery were obtained when using the hollow fibers with sample recycled at 70 ml/minute, with a vacuum pulling water out of the fiber bundles, then applying a backwash procedure. Using the hollow fibers in this manner, a 400 ml sample was concentrated to 31 ml in less than an hour with approximately 100% virus recovery, as determined by plaque assay.

A different approach was tried using the pressure created by the pump. In this system, the need for a vacuum pump was eliminated (See Figure 18). A back pressure of 10 lb psi was created by the use of a metering valve in the pumping line. Utilizing this approach, a 400 ml sample containing marker virus was concentrated to 20 ml in approximately 40 minutes. The back pressure was released and a positive pressure of 10 lb psi was applied to the outside of the fibers by the use of nitrogen under pressure. A 10 ml volume of water was pumped into the fibers releasing any adherent marker virus. This resulted in a final concentrate volume of 30 ml. To test the recovery of marker virus in the concentrate, a radioimmunoassay (RIA) test was performed (as described in Section 5.2.2) on the sample to quantitate marker virus before and after concentration. An RIA test was also performed on a positive control in which the same amount of marker virus added to the 400 ml sample was placed in a 30 ml sample. In this way it would be possible to determine the per cent recovery in the concentrate by comparing RIA ratios between the concentrated sample and the positive control. Using this new approach, the unconcentrated sample gave a ratio of 0.49, the concentrated sample gave a ratio of 0.25 (the lower the ratio, the higher the concentration of marker virus) and the positive control gave a ratio of 0.14. The results showed a higher RIA ratio

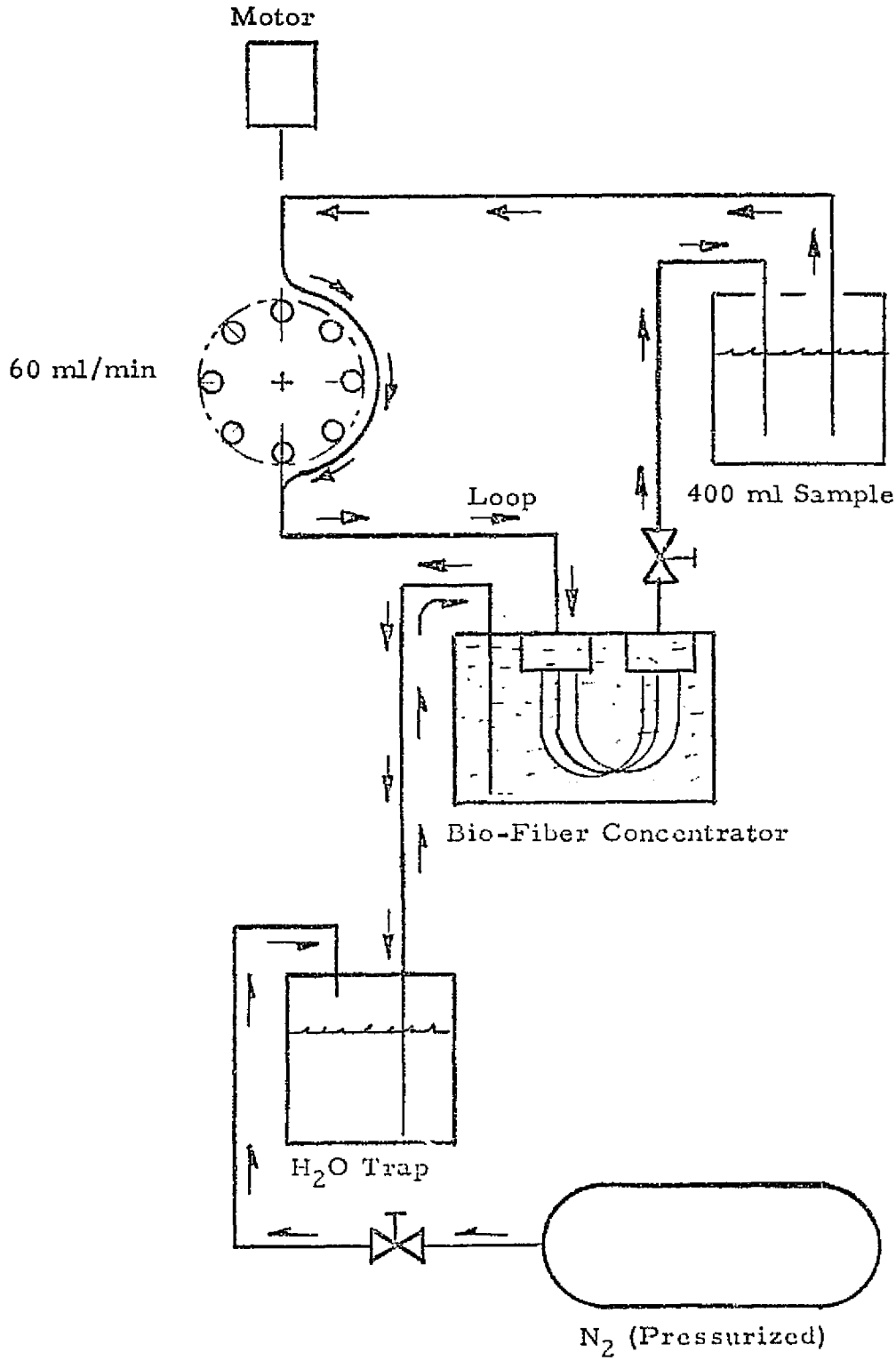

Figure 18. Diagrammatic Representation of the Marker Virus Concentration System Utilizing Bio-Fibers (Applied Pressure)

with the concentrated sample than with the positive control indicating that there was some loss of marker virus with an approximate 70% recovery.

5.1.1.3 Final Design Using Bio-Fiber System

As described in Section 5.1.1.2, the approach employing back pressure in the Bio-Fiber was not as effective in concentrating and recovering virus as when vacuum was applied to the exterior of the Bio-Fibers. Since the vacuum approach appeared to give better results, a procedure was worked out in which the peristaltic pump was used both to pump the sample and create a reduced pressure, thus eliminating the need for a vacuum pump.

To utilize the peristaltic pump, as both a pump and source of reduced pressure, three lines were used to pump the sample while the fourth line was used to create a reduced pressure. This final modification can be seen in Figure 19. When operating at full speed, the pump, with three lines, pumped the sample at approximately 60 ml/minute with a vacuum ranging from 10-13 inches of Hg. created in the remaining line. Utilizing this approach, a 400 ml sample containing marker virus was concentrated to approximately 35 ml. The vacuum was then released and a positive pressure of 10 lb psi was applied to the outside of the fibers and 14 ml of water pumped through. This resulted in a final concentrate volume of 49 ml. To test the concentration and recovery, using this system, an RIA test was performed on the concentrate and on a positive control in which the same amount of marker virus added to the 400 ml sample was placed in a 49 ml volume. Results from the RIA gave a ratio for the concentrate, when divided by the negative control, of 0.40, with the positive control giving a ratio of 0.38.

The results show a difference of only 0.02, when comparing the concentrate with the control. The difference is probably not significant. In any event, recoveries were greater than 95%. This approach, when compared to the pressure-in-line system, gave a better overall recovery in approximately the same time with no additional equipment required.

5.1.2 Other Concentration System

The Bio-Fiber concentration system, as developed, concentrated the marker virus in 400 ml to approximately 30 ml in one hour with greater than 95% recovery yielding a 10-fold concentration. Several other concentration

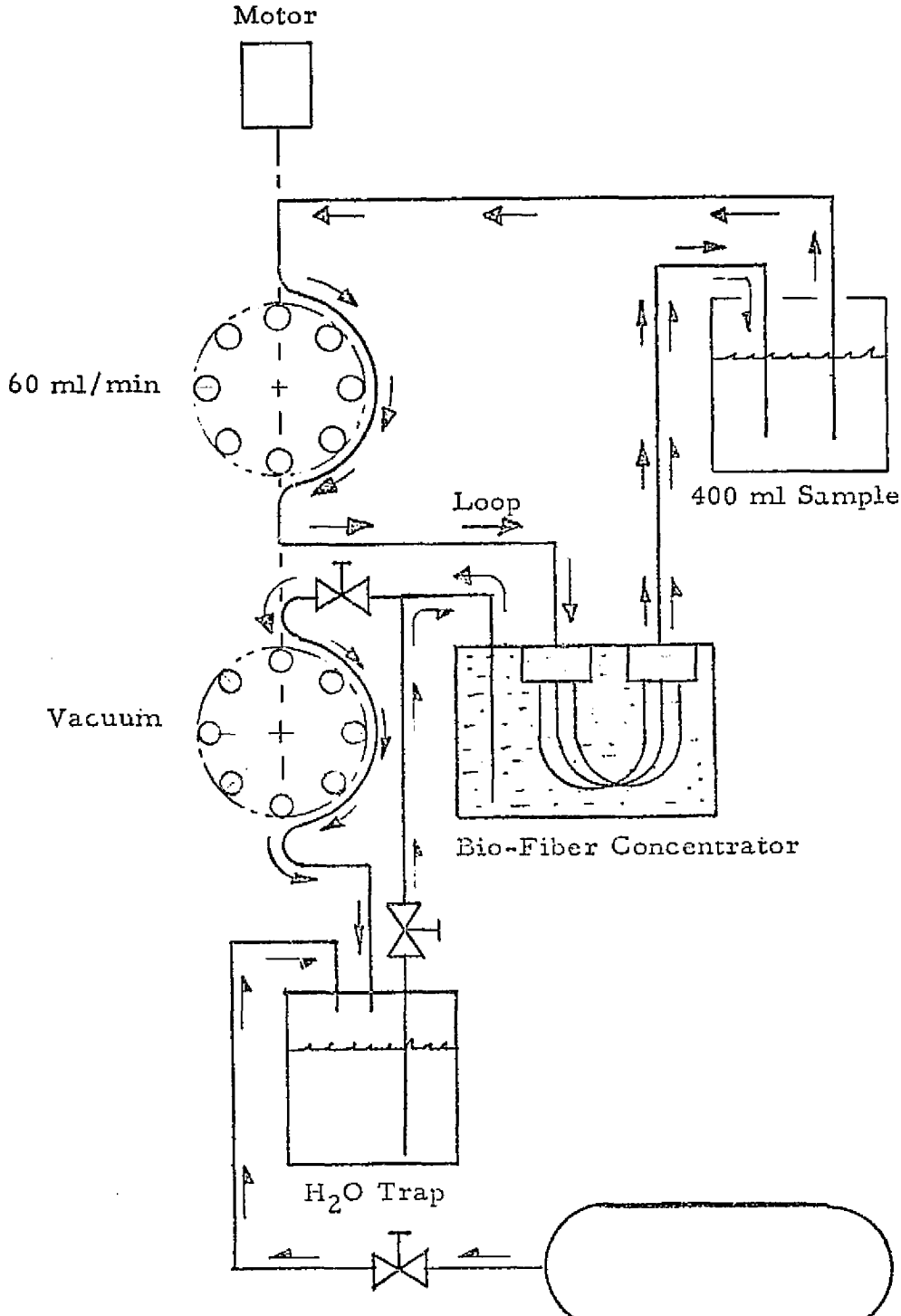

Figure 19. Diagrammatic Representation of the Bio-Fiber Concentration System Utilizing Vacuum Created by Peristaltic Pump

systems were looked at to further concentrate the marker virus in the 30 ml to approximately 3 ml. This would then result in an overall 100-fold concentration. These systems involved marker virus precipitation with polyethylene glycol or ammonium sulfate, ultrafiltration and adsorption to cellulose acetate filters.

5.1.3 Marker Virus Concentration Using Ammonium Sulfate

To test the efficiency of concentrating virus by ammonium sulfate precipitation, a 33 ml concentrate sample of marker virus was treated by adding 9 grams of ammonium sulfate and 1 ml of a 1/10 dilution of normal rabbit sera (to provide a carrier protein during precipitation). The mixture was stirred for 30 minutes at room temperature. The precipitate formed was collected by centrifugation and resuspended in 3 ml of distilled water thus giving a 10-fold reduction in volume. The 3 ml concentrate was then tested by RIA and the ratios observed compared with a positive control consisting of the same amount of marker virus found in the 30 ml concentrate added to 3 ml. The positive control gave a ratio of 0.19 while the 3 ml concentrate gave a ratio of 0.17. These results indicated greater than 95% recovery. Although this system works well, the amount of ammonium sulfate required to precipitate the virus is large and, therefore, due to weight consideration, this technique was eliminated from consideration.

5.1.4 Marker Virus Concentration Using Polyethylene Glycol

Polyethylene glycol (PEG) has been used in many instances to precipitate and concentrate viruses. Generally, most viruses will precipitate within 1 to 24 hours in the presence of 2 to 6% w/v PEG. To test the possibility of using PEG to precipitate marker virus, an experiment was set up using 30 ml concentrate samples containing 1 ml of a 1/10 dilution of normal rabbit sera and 0.5 M NaCl. PEG wa tested at concentrations of 2, 4, and 6% (w/v), with one set kept at room temperature for 1 hour and another kept at 4°C for 1 hour. At the end of this period, no precipitate was observed in any test, even after centrifugation, and RIA testing confirmed no precipitated virus. The test was repeated with samples incubated at room temperature and 4°C for 24 hours. Once again no precipitate was observed even after centrifugation. This observation was also confirmed by RIA indicating that no marker virus

had precipitated. Since this procedure presents the same drawbacks as the ammonium sulfate technique, it was eliminated from consideration.

5.1.5 Marker Virus Concentration Using Ultrafiltration

Using Millipore Pellicon filters, which have an exclusion limit of one million (all molecules less than one million molecular weight pass through), a 30 ml concentrate sample was filtered under vacuum at a flow rate of approximately 0.5 ml per minute. The filter was then backwashed with 3 ml of buffer. An RIA test was run on the filtrate, the backwashed material and a positive control. The results of the RIA test revealed no detectable marker virus present in either the filtrate or backwashed buffer indicating the marker virus had remained on the filter. Further tests with high pH buffers failed to release the marker virus. Therefore, due to the filtration time and difficulty in releasing the marker virus, no further testing using this technique was performed and it was eliminated from consideration.

5.1.6 Marker Virus Concentration Using Virus Adsorption

Development of a concentration system based on virus adsorption to cellulose acetate membranes in the presence of low pH and trivalent salts was adapted to marker virus concentration from the work of Wallis, Henderson and Melnick. The system uses cellulose acetate membranes (Millipore Corp.) with a porosity of 0.45μ and a diameter of 25 mm. The viruses are adsorbed to these membranes in the presence of 0.5 mM aluminum chloride ($AlCl_3$) and a pH of 3.5. The viruses can then be desorbed from the membranes in the presence of 0.05 M glycine buffer at a pH of 11.5.

To test the recovery of virus using this technique, a 0.1 ml amount of a given concentration of marker virus ($\approx 10^{11}$ PFU) was added to 30 ml of a 0.5 mM $AlCl_3$ solution at pH 3.5. This was then passed through a sterile Millipore filter. To elute the virus, 3 ml lots of pH 11.5 glycine buffer were passed through the filter and collected. To test for virus recovery, an RIA test was performed on the virus eluants and on the control containing the same amount of marker virus in 3 ml of glycine buffer as was in the 30 ml concentrate. These results can be seen in Table XV, Part A. The results showed that all the virus was eluted in the first 3 ml glycine wash with essentially 100% virus recovery, as indicated by having the same RIA ratio as the positive control. The remaining two washes showed little or no detectable marker virus.

TABLE XV- A. RESULTS OF VIRUS ELUTION FROM MILLIPORE FILTERS WITH SUBSEQUENT 3 ML WASHES TESTED BY RIA

B. RESULTS OF VIRUS ELUTION FROM MILLIPORE FILTERS WITH SUBSEQUENT 1 ML WASHES TESTED BY RIA

A	Ratio*
First 3 ml elution	0.34
Second 3 ml elution	0.99
Third 3 ml elution	1.01
Positive control	0.34

B	Ratio*
First 1 ml elution	0.56
Second 1 ml elution	0.37
Third 1 ml elution	0.58
Final 3 ml elution	1.05
Positive control	0.34

*Ratio of CPM of sample divided by CPM of negative control. (The lower the ratio, the more concentrated the marker virus.)

To determine if less than 3 ml of glycine buffer could be used to elute the virus, an experiment was set up using the same conditions as the previous experiment with the exception that three 1 ml washes, and then a final 3 ml wash with glycine buffer were used and collected separately, with the results determined by RIA. The results are shown in Table XV, Part B. As shown, marker virus was detected in all three 1 ml washes with the majority occurring in the second 1 ml wash. The final 3 ml wash showed no detectable marker virus. Based on these results, it was apparent that a minimum 3 ml wash was necessary for total virus recovery.

5.1.7 Selection of Concentration System

Two concentration approaches were tested based on the experiments done. The first system linked the Bio-Fiber concentrator, (as described in Section 5.1.1.3) which concentrated 400 ml of sample to 30 ml, with virus adsorption concentration (as described in Section 5.1.5), which concentrated the marker virus in 30 ml into 3 ml. The second approach eliminated the Bio-Fiber concentration and the entire sample was tested using the virus adsorption system. This approach concentrated the marker virus in 400 ml into 3 ml directly.

To determine the overall recovery of the marker virus using the first approach, 400 ml distilled water samples were concentrated to approximately 30 ml by the Bio-Fiber concentration system. The 30 ml concentrates were treated, adsorbed to filter and the marker virus concentrated into 3 ml of desorbing buffer. The concentrate was then tested by RIA to determine overall sensitivity.

Three 400 ml samples were concentrated and tested. They were designated A, B and C. The concentrated samples were tested by RIA with the appropriate controls. These results are shown below:

Sample	Ratio
A	0.23
B	0.22
C	0.24
Positive Control	0.25

With each sample, the ratio obtained was essentially the same as the ratio obtained with the positive control, indicating essentially 100% marker virus recovery using this approach.

To determine the overall recovery of marker virus using the second approach, marker virus was added to a 400 ml sample of distilled water containing 0.5 mM $AlCl_3$ at a pH of 3.5. This was then passed through a 25 mm sterile 0.45 Millipore cellulose acetate filter at a 20 ml/min flow rate. To elute the virus, a 3 ml volume of pH 11.5 glycine was passed through the filter and collected. To test for virus recovery, an RIA test was performed on the virus eluant and on a control, containing the same amount of marker virus in 3 ml of glycine buffer as was in the 400 ml sample. The results showed that the concentrate gave a ratio of 0.22 and the control gave a ratio of 0.23 when tested by RIA. The results indicated that this approach gave as good a recovery and concentration of marker virus as the first approach, with essentially 100% marker virus recovery.

The second approach, which was to treat the entire 400 ml by filter adsorption, was selected for further study, based on its speed of concentration (approximately 20 minutes to obtain the 3 ml concentrate) and ease of automation when compared to the Bio-Fiber approach.

5.1.8 Further Testing of the Selected Concentration System

Several experiments were performed to further test the virus adsorption concentration system. These experiments were as follows: effect of low pH without $AlCl_3$ on marker virus adsorption, effect of pretreated filters, effect of reusing filters, effect of processed water (A+B) and effect of substituting Cat-Floc (a quaternary ammonium compound) for $AlCl_3$.

5.1.8.1 Effect of Low pH Without $AlCl_3$

An experiment was set up to determine if lowering the pH to 3.5 without the addition of $AlCl_3$ would cause the virus to adsorb to the filter. In this way, without the addition of $AlCl_3$, the acid in the water could be neutralized and thus eliminate the need for returning the water sample for repurifying. In this experiment, marker virus was added to two different samples. The first sample was 30 ml of distilled water at pH 5.6. The second sample was 30 ml of distilled water adjusted to pH 3.5. Both samples were passed through

Millipore filters and the virus desorbed with 3 ml of pH 11.5 glycine buffer. The virus concentrates were then tested by RIA and the ratios compared to a positive control containing the same amount of virus in 3 ml of glycine buffer as was added to the initial 30 ml samples. The result showed that the distilled water sample virus without pH adjustment gave a ratio of 0.93, indicating that a small amount of marker virus had been retained on the filter (less than 5%). The distilled water adjusted to pH 3.5 resulted in a larger amount of virus still being adsorbed to the filter following the glycine buffer wash, giving a ratio of 0.27. This was a substantial adsorption of virus since the positive control gave a ratio of 0.19. The results of this experiment indicated that lowering the pH to 3.5 would result in a virus adsorption onto the filter of approximately 70-80%, thus showing that pH is a major factor in the adsorption of the marker virus to millipore filters.

5.1.8.2 Effect of Pretreating Filters

Another approach was tried to eliminate the need to add $AlCl_3$ and this was to pretreat the filters by passing through the filter an $AlCl_3$ solution and then passing through a sample of marker virus suspended in distilled water adjusted to pH 3.5.

A 30 ml solution of 0.5 mM $AlCl_3$ at pH 3.5 was passed through a Millipore filter. This was followed by 30 ml of distilled water adjusted to pH 3.5 and containing marker virus. The virus was then desorbed with pH 11.5 glycine buffer and the concentrate assayed by RIA, along with the appropriate controls. The ratios of the positive control and the concentrate when compared were the same, indicating that essentially 100% of the virus was recovered.

5.1.8.3 Effect of Drying Pretreated Filters

To determine the effect of drying on pretreated filters, two types of filters were prepared. The first pair (Pair A) was prepared by soaking 25 mm 0.45μ Millipore filters in a solution of 0.5 mM $AlCl_3$ at pH 3.5 for 1 hour. The second pair (Pair B) of filters was prepared by passing 30 ml of 0.5 mM $AlCl_3$ at pH 3.5 through the filters. Both pairs of filters were then allowed to dry overnight at 37°C.

After overnight drying, both sets of filters were tested for their ability to recover marker virus. Pair A and Pair B filters were tested using

400 ml of distilled water, at pH 3.5, to which marker virus had been added. Identical 400 ml samples were passed through each filter. Adsorbed virus was then desorbed using 3 ml of pH 11.5 glycine buffer and the concentrate tested by radioimmunoassay (RIA). The results of this experiment are shown below:

Filter Set	*Ratio
A-1	0.26
A-2	0.28
B-1	0.20
B-2	0.26
Positive control	0.19
Negative control	1.00

*Counts per minute (CPM) of sample/counts per minute of negative control.

The ratios obtained from the RIA testing were compared with a positive control which contained the same amount of marker virus added to 3 ml of glycine buffer as was added to the 400 ml sample. As shown, filter Pair B gave overall better recoveries than filter Pair A. This indicates that pretreating filters by passing a solution of pH 3.5 $AlCl_3$ through them, rather than just soaking, is possibly a better pretreatment procedure. Although both filter sets A and B did not give 100% virus recovery, as indicated by the higher ratios when compared to the positive control, drying of the filters did not seem to interfere too greatly with the recovery of marker virus.

5.1.8.4 Effect of Reusing Filters

To reduce the number of filters required for the adsorption step, the following experiment was set up to determine if it would be possible to reuse filters. Five 30 ml samples, containing a similar amount of marker virus, were treated with an appropriate amount of $AlCl_3$ and acid. Each sample was passed through the same Millipore filter with subsequent desorption with pH 11.5 glycine buffer. After each desorption, a 5 ml volume of 0.5 mM $AlCl_3$ at pH 3.5

was passed through the filter to regenerate it. The concentrates were then tested by RIA with the appropriate controls. The samples were designated A - E in order of their passage through the filter and compared according to their ratios obtained by RIA. These results are shown below:

Sample	Ratio*
A	0.18
B	0.15
C	0.26
D	0.14
E	0.17
Positive control	0.16

*Ratio of CPM of sample/CPM of negative control (the lower the ratio the more concentrated the marker virus).

There was no significant drop in the ability of the same filter to adsorb and release virus after five cycles. This experiment indicated with high probability that a filter could be reused.

5.1.8.5 Effect of Processed Water

A sample of processed shower water (A) was received from the NASA Project Monitor in the first part of May, 1975. The processed water was used to study its effect on the virus adsorption concentration procedure. The processed water was slightly opaque and after vigorous shaking gave a foam head of from 1/2 to 1" which would persist for approximately 1/2 hour. This created some concern since it was felt that excessive detergent might interfere with adsorption of virus to the filter. Since the volume of sample was small, 30 ml rather than 400 ml aliquots were used for testing.

To test the effect of the processed water on the concentration of virus by the adsorption technique, various levels of marker virus were added to 30 ml aliquots of processed water. Samples were adjusted to 0.5 mM $AlCl_3$ and adjusted with acid to pH 3.5, then passed through a cellulose acetate Millipore filter. Virus was then desorbed with 3 ml of pH 11.5 glycine buffer.

Results were assayed by RIA and compared to positive controls containing the same amount of marker virus in 3 ml of glycine buffer.

Concentrations of virus tested were 0.1 ml of 1/25, 1/50, and 1/100 dilutions of a marker virus concentrate added to 30 ml aliquots of processed water. The results of this experiment are shown below:

Test	Ratio
1/25	0.89
1/25 Positive Control	0.24
1/50	0.94
1/50 Positive Control	0.36
1/100	1.00
1/100 Positive Control	0.57
Negative Control	1.00

These results are shown in Figure 20. It is apparent that very poor recovery of virus was obtained, indicating a definite interference by the processed water.

Changes in pH and $AlCl_3$ concentration were investigated in an attempt to improve the concentration and recovery of virus in processed water.

5.1.8.5.1 Effect of pH

An experiment was set up to determine if lowering the pH of the samples would improve recovery. Results revealed that if a processed water sample containing 0.5 mM $AlCl_3$ was adjusted to pH 3.0 a better overall recovery was obtained. An experiment was run in which varying concentrations of marker virus were added to 30 ml aliquots of pH 3.0 processed water containing 0.5 mM $AlCl_3$. The same experiment conditions were used as were used in Section 5.1.8.5 with the exception of the lower pH. The results of the concentrate samples tested by RIA are shown below:

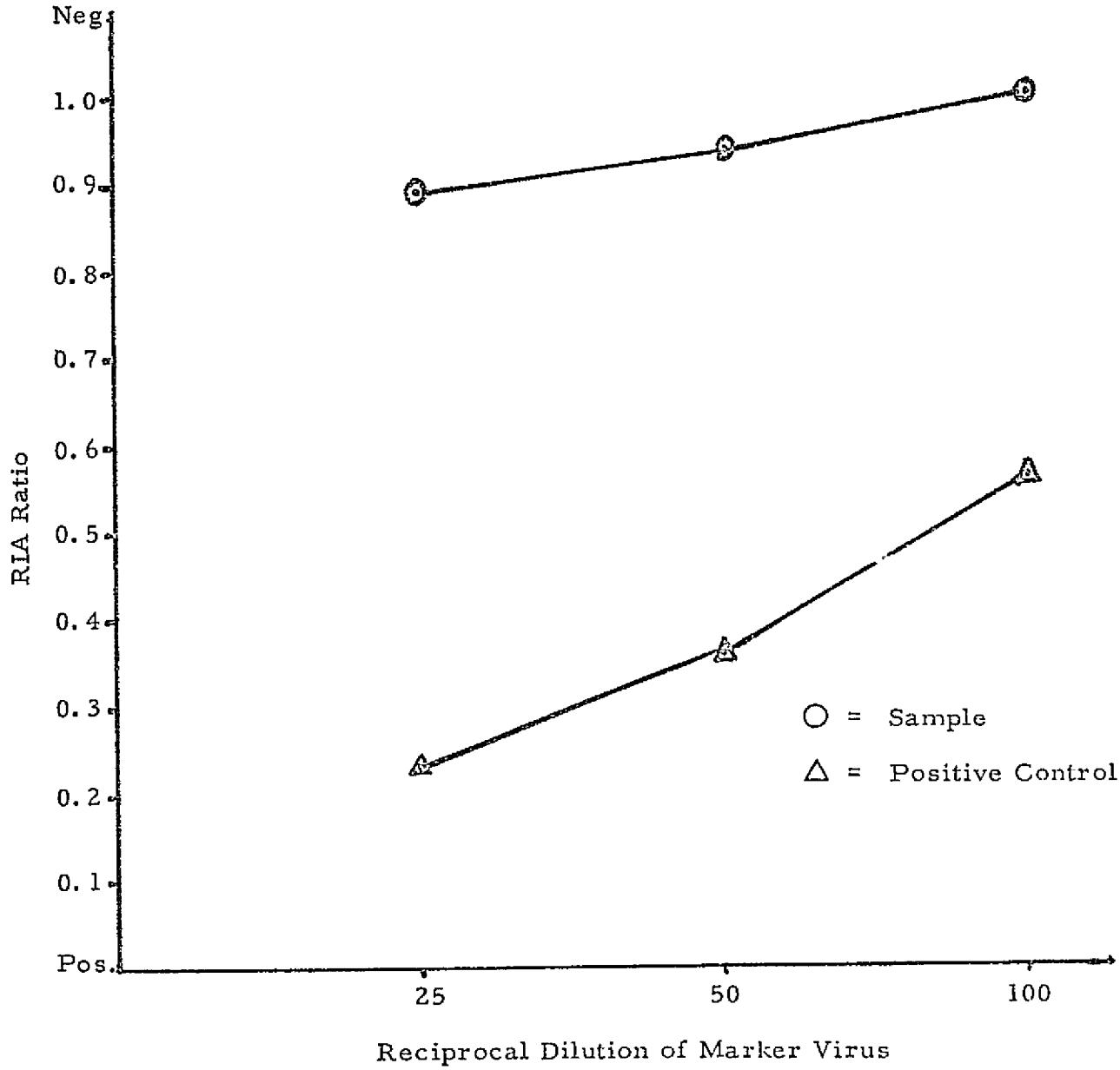

Fig. 20. Recovery of Virus Using Filter Adsorption with 0.5 mM $AlCl_3$ at pH 3.5 in Processed Water with Varying Concentrations of Marker Virus

Test	Ratio*
1/25	0.46
1/25 Positive Control	0.21
1/50	0.93
1/50 Positive Control	0.35
1/100	0.88
1/100 Positive Control	0.55
Negative Control	1.00

*CPM of sample/CPM of negative control.

These results, plotted in Figure 21, show that there was an improvement in recovery by lowering of the pH from 3.5 to 3.0. There was still, however, poor recovery when comparing results to the positive control.

5.1.8.5.2 Effect of Increased $AlCl_3$

It was felt that perhaps the effects of $AlCl_3$ were in some way being overcome by the presence of the detergent in the processed shower water. Based on this, it was decided that an increase in the concentration of $AlCl_3$ might improve recovery of marker virus.

An experiment was set up that duplicated the experiments in Section 5.1.8.5 and 5.1.8.5.1. The 30 ml aliquots of processed shower water were treated so as to contain 5 mM $AlCl_3$ (10 times the previous concentration) and adjusted to a pH of 3.0. To these treated water samples, the various dilutions of marker virus were added and the concentration procedure run. The results of this experiment are shown below:

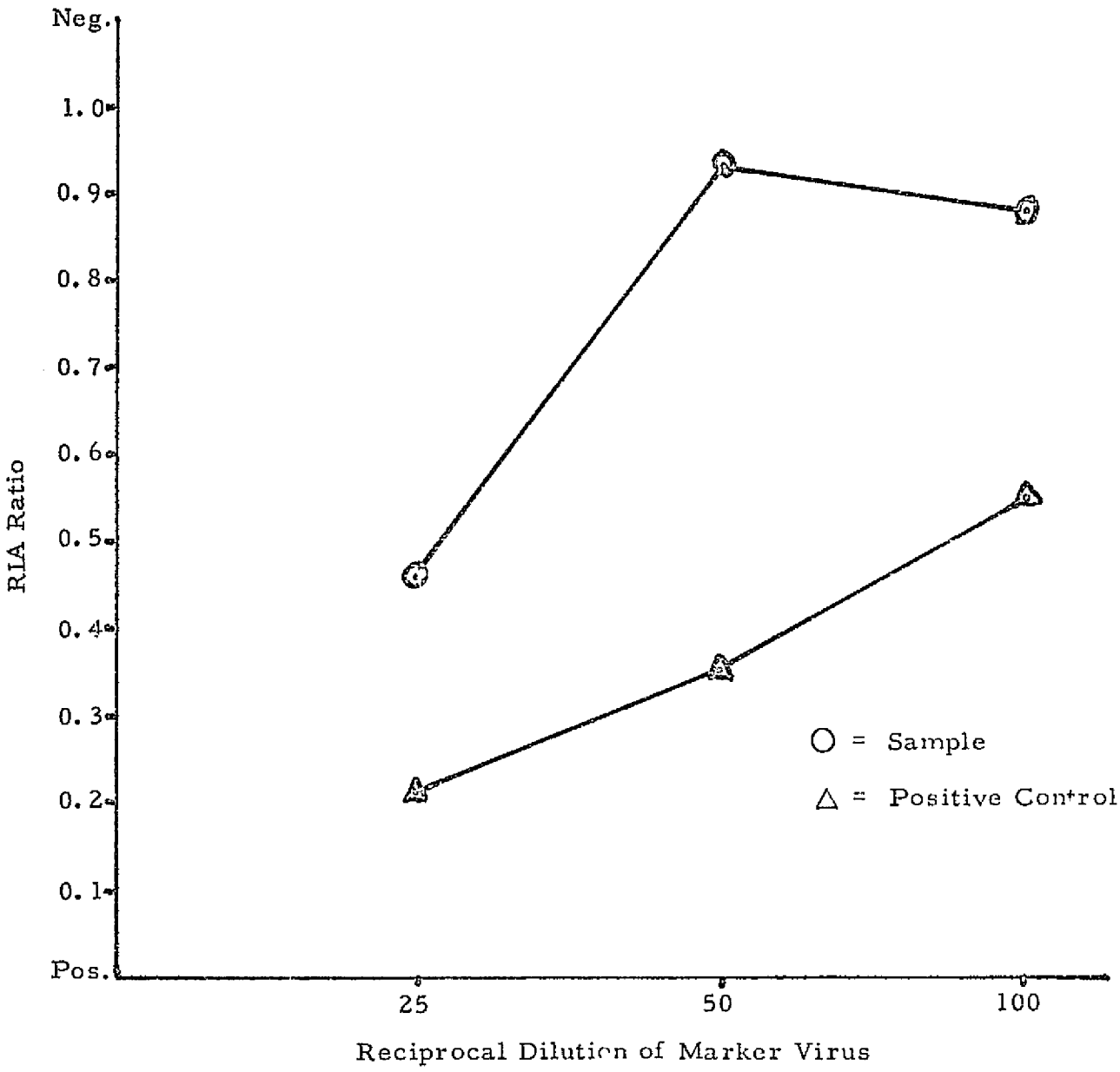

Fig. 21. Recovery of Virus Using Filter Adsorption with 0.5 mM $AlCl_3$ at pH 3.0 in Processed Water with Varying Concentrations of Marker Virus

Test	Ratio
1/25	0.26
1/25 Positive Control	0.22
1/50	0.43
1/50 Positive Control	0.38
1/100	0.63
1/100 Positive Control	0.59
Negative Control	1.00

These results are shown in Figure 22. Excellent results were obtained by using 5 mM $AlCl_3$ at pH 3.0. Recoveries at each dilution of marker virus tested ranged between 80 to 90% when compared to their positive control.

5.1.8.5.3 Confirmation Testing

To confirm the effectiveness of this modification, the same testing approach was taken with a different processed water sample (designated processed shower water B). Visual observations of this sample revealed heavy contamination in the form of white flocculent material. The processed water was centrifuged since it was felt that this material might interfere with infiltration. After centrifugation, however, flocculent material remained in suspension making filtration difficult.

To evaluate the effect of this processed shower water on the concentration system, three conditions were tested. A sample of processed water was tested by adjusting the pH to 3.5 and adding $AlCl_3$ to give a concentration of 0.5 mM. This duplicated the original concentration parameters (Section 5.1.6). Another sample of processed water was tested by lowering the pH to 3.0 and adding $AlCl_3$ to yield a concentration of 0.5 mM. A final sample of processed water was tested by adjusting the pH to 3.0 and adding $AlCl_3$ to yield a final concentration of 5.0 mM. This duplicated the revised concentration procedure (Section 5.1.8.5.2). In each condition 30 ml of processed shower water B was used and passed through 0.45μ cellulose acetate filters. The adsorbed virus was subsequently desorbed using 3 ml of pH 11.5 glycine buffer

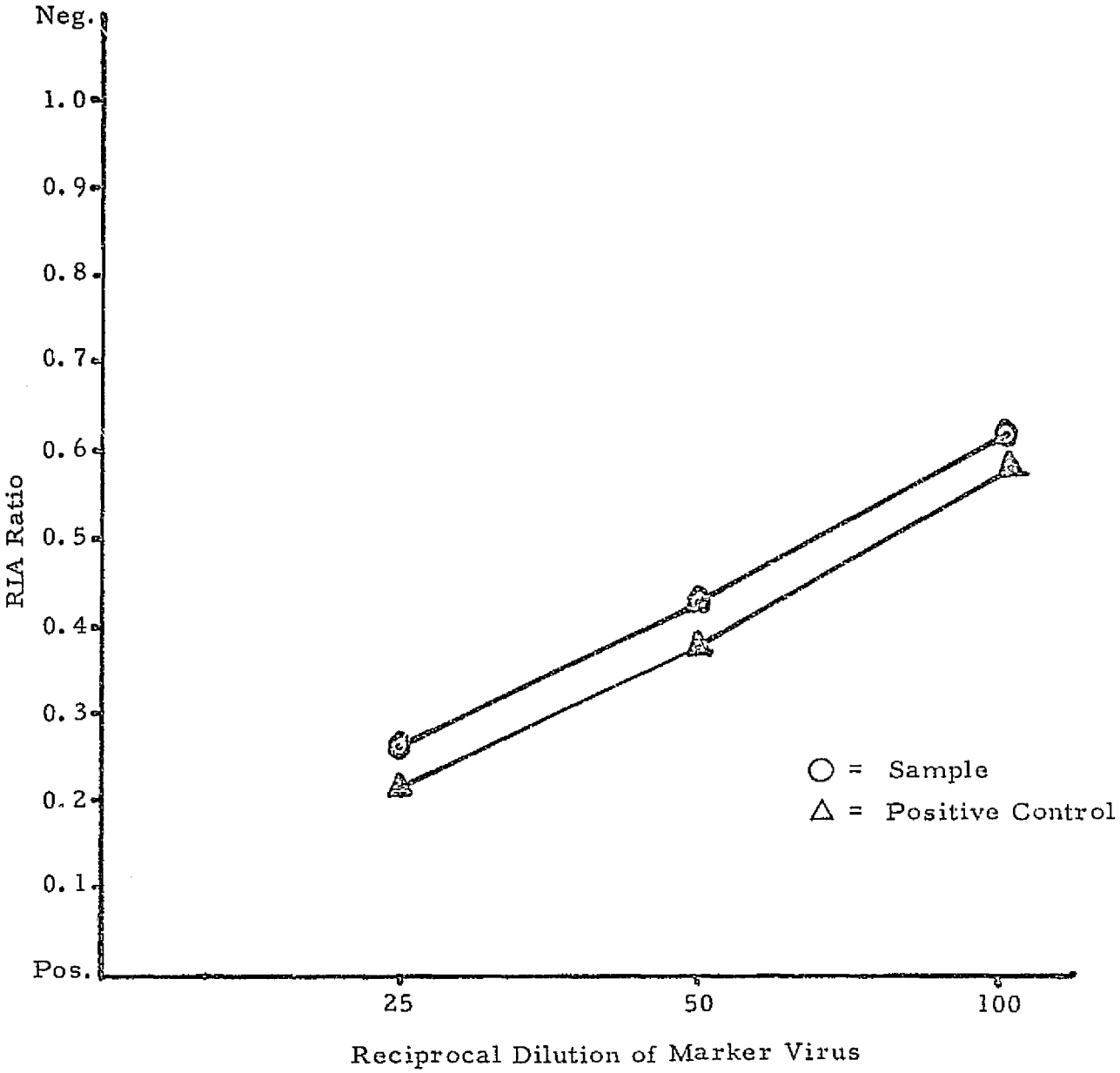

Fig. 22. Recovery of Virus Using Filter Adsorption with 5 mM $AlCl_3$ at pH 3.0 in Processed Water with Varying Concentrations of Marker Virus

at 0.05 M concentration. The concentrates were then tested by radioimmunoassay (RIA) utilizing appropriate controls. The results of this experiment are shown in Table XVI. The best recovery was obtained when the sample was adjusted to 3.0 with the $AlCl_3$ concentration at 5.0 mM. The results were similar to those obtained with processed shower water A. Based on these results this modification was included in all further testing.

5.1.8.5.4 Effect of Cat-Floc Replacing $AlCl_3$

An alternate virus concentration system was investigated based on the use of a cationic resin. The concept was reported in a government article (ASME publication 74-E NAS-6) entitled "Reverse Osmosis and Future Army Water Supply," by R. O. Schmitt. The article reports on reverse osmosis as a means of purifying water in the field for use as a potable water source. As a means of testing this system for its ability to pass virus, bacteriophage F_2 was used as a marker virus system in the same manner as envisioned on the contract. The paper reports that high percentages of bacteriophage F_2 were removed prior to water purification, by the use of a prefiltration system which included the addition of a cationic polymer.

A more detailed description of the prefiltration arrangement was found in Government report AD A005-557 entitled, "Removal of F_2 Virus from River Water by Army Water Purification Units". The report describes the prefilter as consisting of coal and sand. They reported that testing revealed this system was ineffective in removing the negatively charged bacteriophage F_2 particle until the prefiltration media was combined with a positively charged polymer (Calgon's Cat-Floc, a polyquaternary ammonium compound). Although effective virus removal was obtained with this system, reclamation of the virus from the prefilter was not described. Method of viral recovery and the volume of liquid required are important considerations in determining the suitability of the concentration system.

Cat-Floc was tested at 6.2 mg/liter (1 X) and 62.0 mg/liter (10 X). As a control, the established concentration system based on $AlCl_3$ was also run. Testing involved the use of 30 ml aliquots of processed water B to which marker virus had been added. The samples were adjusted to pH 2.5-3.0 and either Cat-Floc or $AlCl_3$ added. The samples were then passed through filters

TABLE XVI. EFFECTS OF CHANGES IN pH AND $AlCl_3$ CONCENTRATION OF RECOVERY OF F_2 VIRUS BY FILTER ADSORPTION

Sample Treatment		RIA RATIO*
pH	$AlCl_3$ Conc.	
3.5	0.5 mM	0.32
3.0	0.5 mM	0.19
3.0	5.0 mM	0.17
positive control		0.16
negative control		1.00

* = Count per minute of sample/count per minute of negative control

and the adsorbed virus desorbed with 3 ml of pH 11.5 glycine buffer. The virus concentrates were then tested and compared to an appropriate control representing 100% virus recovery. The results of this experiment showed the established $AlCl_3$ adsorption system effectively concentrated and recovered all of the added marker virus. Cat-Floc, on the other hand, when tested at 6.2 mg/liter (1 X) or 62.0 mg/liter (10 X) was ineffective in replacing $AlCl_3$.

5.1.9 Conclusions

A marker virus concentration system based on virus adsorption to cellulose acetate filters was selected for use in the viral monitoring system based on its speed of concentration and ease of operation when compared with the Bio-Fiber approach. Under this system, $AlCl_3$ and acid is added to 400 ml of sample to yield a pH between 2.5 and 3.0 and an $AlCl_3$ concentration of 5.0 mM. The treated 400 ml sample is then passed through a 25 mm, 0.45 μ, sterile cellulose acetate filter at a flow rate of approximately 20 ml/min. The adsorbed virus is subsequently desorbed with 3 ml of 0.05 M, pH 11.5 glycine buffer. With this system, greater than 95% marker virus recovery is obtained with a 100-fold concentration.

5.2 MARKER VIRUS DETECTION

The passive immune agglutination (PIA) test was proposed as the most logical immunological detection system for use under the stringent requirements of long term space travel. Therefore, the majority of effort was spent in developing and modifying this detection system. Several other detection systems were investigated during this project and will be discussed briefly. These detection methods involved fluorescent antibody (FA), radioimmunoassay (RIA) and enzyme labeled antibody (ELA).

5.2.1 Fluorescent Antibody

The fluorescent antibody technique involved the use of a fluorescent material bound to specific antibody (see Figure 9). The tagged antibodies were incubated with specific antigen resulting in an antibody-antigen complex. Excess non-complexed tagged antibody was removed by filtration or washing. Complexed antibody could then be detected either visually or photometrically by the intensity of light emitted from the complexes under exposure to ultra-violet irradiation.

The reagents used in this investigation consisted of fluorescein isothiocyanate-tagged antibody as well as 1.1μ polystyrene beads to which anti-marker antibody had been covalently bound. Purified anti-marker virus IgG isolated from Group I rabbit #2 was tagged using the dialysis method of Cherry et al (1971), with fluorescein isothiocyanate. The beads to which antibody was bound were used in both the fluorescent antibody test investigation and also in the RIA and ELA investigations. Benzene residues of the polystyrene bead surface were first nitrated and then reduced to the amino form yielding aminopolystyrene. The IgG molecules then were attached to the beads via diazonium linkage previously formed with nitrous acid. Unreacted diazo groups were filled by reaction with β-napthol, which imparted a salmon color to the beads. These antibody-coated beads then acted as a solid support which facilitated the separation, centrifugation, of bound and unbound elements in the reaction mixture.

To evaluate the FA detection system, the following tests were run. In the first experiment several dilutions of marker virus were filtered through a Pellicon Millipore filter, which had an exclusion molecular weight of one million. In this way the marker virus with a molecular weight of three million was retained on the filter. A sample of fluorescent labeled anti-marker virus antibody was then incubated on the filter. The non-bound, labeled antibody, with a molecular weight of approximately 200,000, was then washed through the filter and the fluorescence resulting from the bound antibody was read visually.

The second experiment involved the use of antibody-coated beads. In this test, various dilutions of marker virus were incubated with the antibody-coated beads. After incubation, free non-bound marker virus was removed and the beads collected by filtration. A sample of fluorescent labeled anti-marker virus antibody was then incubated with the beads. After an incubation period, the excess unbound labeled antibody was removed by filtration and the fluorescence on the beads, due to the bound labeled antibody, was read visually. Results from both experiments were hard to interpret due to a high degree of nonspecific adsorption of labeled antibody resulting in a similar degree of fluorescence from all the dilutions of marker virus tested. This nonspecific

adsorption seems to be a common problem with some fluorescent antibody tests. Based on the results of these tests, no further testing was performed using the fluorescent antibody approach.

5.2.2 Radioimmunoassay (RIA)

The development of a competitive RIA test involved the use of 1.1μ polystyrene beads to which specific anti-marker virus antibody had been bound (as described in Section 5.2.1). A second reagent consisted of purified marker virus labeled with radioactive iodine-125. To label the marker virus with ^{125}I, the chloramine-T method of Hunter and Greenwood was used.

Using purified IgG and marker virus, a competitive RIA test was developed (see Figure 11). The test was based on a competition between labeled and unlabeled marker virus for specific antibody sites on the beads. As the amount of unlabeled marker virus increased, the number of available antibody sites decreased, therefore resulting in less uptake of labeled marker virus. Varying dilutions of unlabeled marker virus were incubated with antibody coated beads. After 2 hours at 37°C, a sample of labeled marker virus was added and the mixture incubated for an additional hour. The beads were then washed, utilizing centrifugation, and radioactivity on the beads counted. Table XVII and Figure 23 show the result of an experiment in which various dilutions of unlabeled marker virus were tested. From this experiment, it was determined that under these test conditions a sample of purified marker virus containing 1×10^{13} PFU/ml with a protein content of 240 μg/ml, when diluted 1/25,600 gave a positive response with a ratio of 0.81 when compared with the control uptake containing no unlabeled marker virus. The results of this test indicated that under these conditions, a sample of marker virus containing 4×10^{8} PFU/ml or approximately 3 ng/ml could be detected by RIA as a definite positive.

5.2.3 Enzyme Labeled Antibody (ELA)

Using purified anti-marker virus IgG and marker virus, an ELA test was developed (see Figure 10). In this test, as with the RIA and FA, 1.1μ polystyrene beads, to which anti-marker virus IgG had been covalently bound, were used. Also used in the test was specific antibody to which the enzyme horseradish peroxidase had been bound using the method of Nakane and Kawaoi.

TABLE XVII. RESULTS OF RIA TESTING WITH VARIOUS DILUTIONS OF MARKER VIRUS

Reciprocal of Marker Virus Dilution*	CPM (Avg. of Two Tests)	Ratio Sample CPM/Control CPM
400	1,795	0.04
1,600	5,785	0.14
3,200	10,310	0.25
6,400	20,435	0.49
12,800	26,775	0.64
25,600	34,050	0.81
51,200	39,600	0.95
**Negative	41,805	---

*Initial concentration of 1×10^{13} PFU/ml.

**Contained no marker virus.

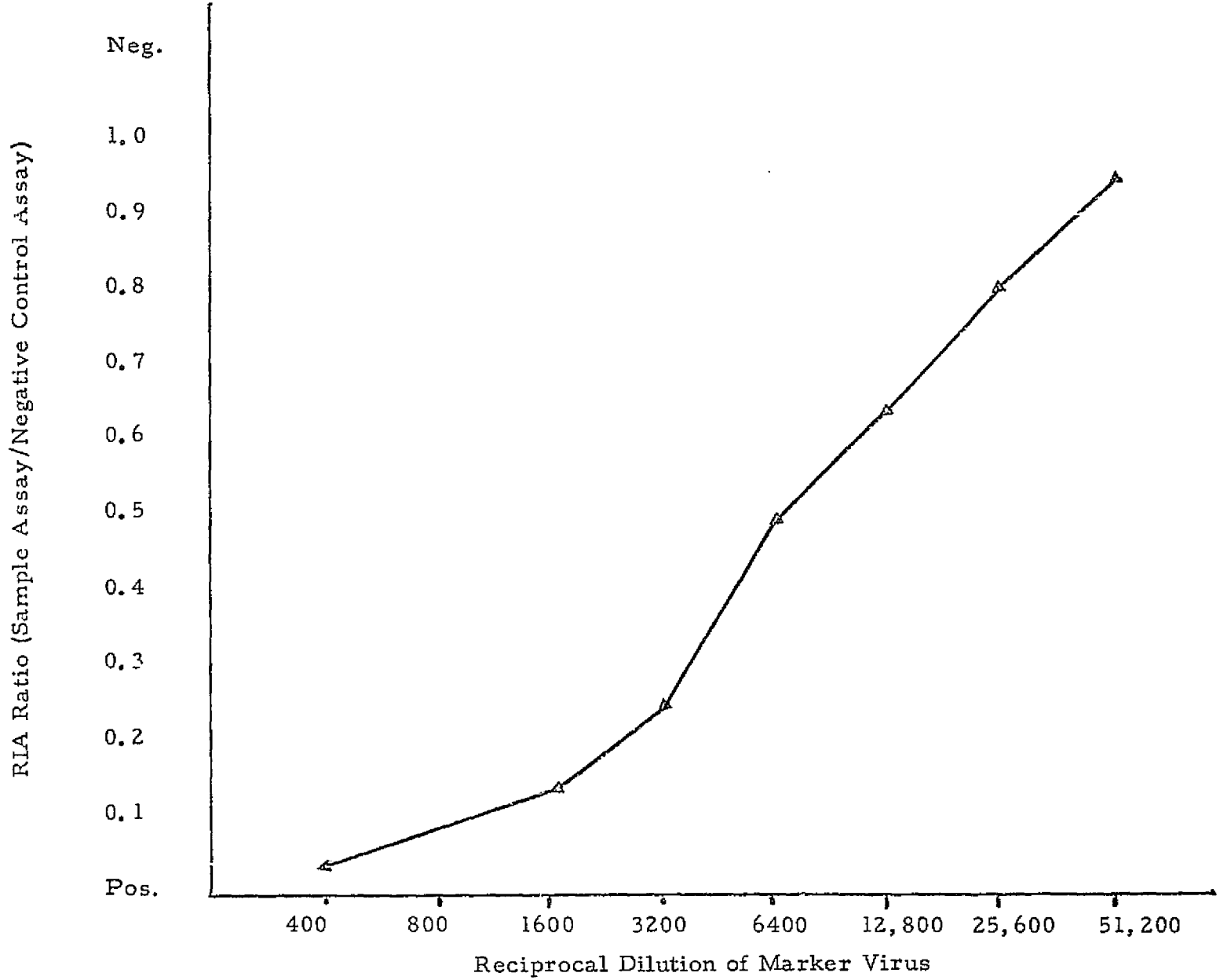

Figure 23. Response of a Competitive RIA for Marker Virus as a Function of Virus Dilution

The test was based on a three-step incubation. In the first step antibody-coated beads were incubated for 2 hours at 37°C with samples containing various dilutions of marker virus. Marker virus was thereby bound to the beads. Following the removal of unbound marker virus, an incubation was performed for 1 hour at 37°C in which enzyme-labeled antibody (conjugate) was added. Following this incubation, excess enzyme-labeled antibody was removed by centrifugation and the beads incubated with the dye and substrate for which the enzyme was specific. In this case, the substrate was hydrogen peroxide and the dye was ABTS (2, 2^1-Azino-diethylbenzthiaoline-sulfonic acid). The decomposition of hydrogen peroxide by horseradish peroxide, which was directly related to the amount of labeled antibody on the beads, with ABTS as hydrogen donor, was determined by measuring the color development after 1 hour at 37°C. The result of this experiment is shown in Table XVIII and in Figure 24. Marker virus dilutions were compared to negative test samples containing no marker virus. This detection system was able to "see" a 204,800 dilution of marker virus concentrate representing $\approx 5 \times 10^7$ PFU/ml. This represented an approximate 8-fold detection sensitivity increase over the competitive RIA test system (see Section 5.2.2).

5.2.4 Direct Passive Immune Agglutination (PIA)

The direct passive immune agglutination test uses 1.1 μm polystyrene beads to which specific antibody has been adsorbed (see Figure 8). These beads differed from those used in the FA, RIA and ELA in that the antibody was adsorbed rather than covalently bound. When these antibody coated beads are incubated with an antigen (marker virus), which is specific for that antibody, cross-linking between beads occurs resulting in clumping which is observed as a change in light transmission read visually or photometrically. This system can be used to detect either antigen or antibody.

To prepare the polystyrene beads for use in this test, a given concentration of 1.1 μ polystyrene beads was mixed with purified anti-F_2 IgG diluted to a concentration of 3-5 mg/ml. Generally about 60% of the available protein is absorbed onto the beads. After the absorption was complete, the beads were centrifuged and resuspended in 0.1 m glycine, 1% NaCl plus 0.2% gelatin, pH 8.2, to a 1/25 dilution of the stock bead suspension.

TABLE XVIII. RESULTS OF ELA TESTING WITH VARIOUS DILUTIONS OF MARKER VIRUS

Reciprocal of Marker Virus Dilution *	Optical Density 425 mm
12,800	1.200
25,600	0.630
51,200	0.410
102,400	0.155
204,800	0.075
** Negative	0.000

* Initial concentration 1 x 10^{13} PFU/ml

** Contained no marker virus

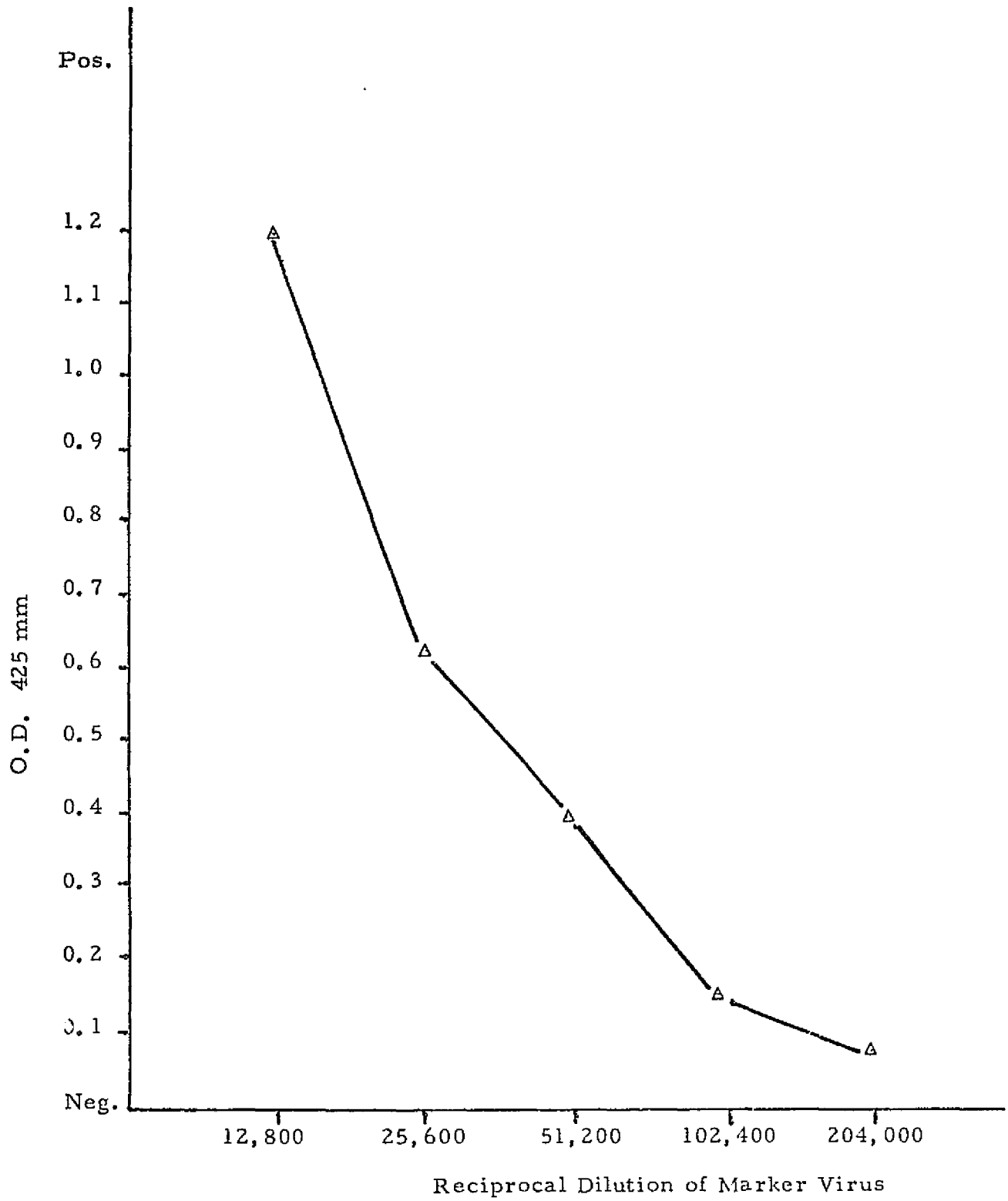

Figure 24. Response of a Non-Competitive ELA for Marker Virus as a Function of Virus Dilution

The sensitivity of the PIA is affected by the reaction concentration of the beads. Therefore, an initial step in the development of a PIA test system was to react the beads at various dilutions with specific antigen (marker virus) to determine the proper working dilution of beads.

An experiment was set up to determine the optimum dilution of beads by diluting the stock preparation so that the reaction mixture, when mixed with dilutions of marker virus, falls in the proper range. The dilution mixtures were incubated overnight at 45°C and 37°C. Each sample was then diluted up to give a final bead concentration of 1/1000 of stock using 0.1 M glycine, 1% NaCl pH 8.2. The degree of agglutination occurring was read spectrophotometrically at 475 nm as percent transmittance (% T). The results of this experiment can be seen in Table XIX. Each sample run was compared to a control containing unclumped beads with results expressed as a ratio of sample to control. As seen in Table XIX, two trends are evident as the bead dilution increases. First, as the bead dilution increased, the detection sensitivity increased. Second, as the dilution of beads increased, the prozone effect becomes evident. Prozone shows up when the amount of antigen (marker virus) overwhelms the amount of antibody present. When this occurs, all available antibody sites are complexed with antigen, therefore eliminating the possibility of cross-linking of bound antigen with unbound antibodies on adjacent beads which would result in beads clumping. This results in a false negative test. However, as the amount of antigen decreases, more antibody sites become available for secondary binding, and clumping of beads occurs, resulting in positive tests. Of the two temperatures tested, 45°C showed the best sensitivity. Utilizing these results, dilutions of beads selected for further testing were made based on maximum sensitivity while also showing a good range of positive tests over a number of dilutions. Bead dilutions of 1/200 and 1/400 were selected for further testing with tests being performed at 45°C. Using these conditions and a 1/3200 dilution of marker virus, tests were performed with incubation for 2, 4, 5, 6 and 7 hours. As shown in Table XXI, the 1/200 bead dilutions gave a better overall sensitivity when compared to the 1/400 dilution. Based on this information, a 1/200 bead dilution was picked as the optimum for testing.

TABLE XIX. RESULTS OF OVERNIGHT PIA

Bead Dilution	37°C Assay Results* on Marker Virus Dilutions of: 200	800	3,200	12,800
50	43	100	100	100
100	65	65	100	100
200	97	62	78	100
300	99	89	64	100
400	98	92	74	95
500	100	100	76	96
600	100	100	84	89

Bead Dilution	45°C Assay Results* on Marker Virus Dilutions of: 200	800	3,200	12,800
50	36	81	80	96
100	49	58	100	98
200	91	54	65	97
300	98	73	52	98
400	97	87	56	81
500	100	96	68	85
600	96	93	68	82

*Results expressed as a ratio of sample to control.

TABLE XX EFFECT OF INCUBATION TIME ON SENSITIVITY OF PIA UTILIZING A 1/3200 DILUTION OF MARKER VIRUS WITH A 1/200 AND 1/400 BEAD DILUTION

Incubation Time (hours)	Assay Result* for Bead Dilution of: 1/200	1/400
2	94	100
4	90	98
5	81	86
6	75	91
7	74	85

*Results expressed as a ratio of sample to control.

5.2.4.1 Effect of Salt and pH on Direct PIA

Utilizing 1/200 dilution of beads at 45°C the effects of pH and salt concentration were investigated. An experiment was set up to test the effect of various salt concentrations and pH on the sensitivity of the direct PIA test. Using 0.1 M glycine buffer at pH 8.2, the salt concentration was tested at 0.5%, 1.0% and 2.0%. The test was performed at 45°C for 6 hours with a bead concentration of 1/200 and various dilutions of marker virus. The results showed no significant increase or decrease in sensitivity.

The next experiment tested the effects of pH. The same test conditions were used with a 1% salt concentration being employed, with pH 7.0, 7.6, 8.2, 9.0, and 9.5 being tested. The results showed that over this range of pH's there was no significant increase or decrease in sensitivity.

It should be noted that, with each new condition tested, appropriate controls were run to eliminate the problem of false-positive tests occurring due to nonspecific agglutination. Based on the previous results, the original testing buffer consisting of 0.1 M glycine + 1% NaCl at pH 8.2 was kept as the buffer of choice.

5.2.4.2 Comparison with Detection Method

Using six purified marker virus batches, each batch was compared and rated based on its protein content, plaque assay, complement fixation titer, PIA ratios and RIA ratios. When tested by PIA, a 1/1600 dilution of each batch was used and, when tested by RIA, a 1/3200 dilution of each batch was used. As shown in Table XXI, there was good correlation between the various assay techniques and the amount of protein and plaque-forming units present. Marker virus preparation, Batch D, which had the highest protein content and PFU/ml, also gave the strongest response by RIA and PIA and gave the highest CF titer. This similar trend in reaction by diverse methods of detection indicates that the PIA test system was working and was consistent.

5.2.5 Indirect Passive Immune Agglutination

In order to eliminate the problem of prozone formation, the indirect PIA test was developed. The indirect PIA is a test based on bead agglutination. It differs from the direct PIA in that antigen (marker virus) instead of antibody is bound to the beads. The test works as follows: A sample

TABLE XXI. COMPARISON OF VARIOUS MARKER VIRUS DETECTION TECHNIQUES

Marker Virus Batch	Assay Results for Detection Techniques				
	Protein ug/ml	Plaque PFU/ml	RIA*	PIA**	C.F. Titer
A	138 (4)***	5.2×10^{12} (4)	0.61 (4)	83 (4)	1280
B	225 (3)	9.2×10^{12} (3)	0.37 (2)	82 (3)	1280
C	246 (2)	1.4×10^{13} (2)	0.39 (3)	78 (2)	1280
D	375 (1)	2.0×10^{13} (1)	0.25 (1)	52 (1)	5120
E	117 (5)	8.7×10^{12} (5)	0.66 (6)	87 (5)	1280
F	105 (6)	7.2×10^{12} (6)	0.65 (5)	91 (6)	1280

*Tested at 1/3200 dilution of marker virus(results shown as ratio of CPM sample/CPM control).

**Tested at 1/1600 dilution of marker virus (results expressed as a ratio of sample to control).

***Numbers in parenthesis indicate order of reaction strength.

of marker virus is incubated with a predetermined concentration of antibody. After an incubation period, latex beads are added to which antigen has been bound. If free antibody is present, clumping of beads occurs. If, however, the antibody has been neutralized by the marker virus, no clumping occurs. Therefore, in the indirect PIA test, clumping of the beads is a negative test indicating that there was either no marker virus or marker virus was present in an amount that could not neutralize all the antibody added. If, on the other hand, clumping does not occur, this would indicate that enough marker virus was present to neutralize the antibody added and thus prevent clumping when the antigen coated beads were added. No clumping would then indicate a positive test.

5.2.5.1 Test Development

Antigen (marker virus) coated beads were prepared in the same manner as the antibody coated beads used in the direct PIA, using batch D antigen. Utilizing these beads, an experiment was set up to determine the minimum amount of antibody necessary to cause good detectable clumping (the less antibody used, the more sensitive the test). The amount of antibody (purified anti-marker virus antibody IgG 14-15-19) required is dependent on the bead concentration; therefore, a block titration was set up in which dilutions of antibody were tested against dilutions of marker virus coated beads. Based on the results shown in Table XXII, a bead dilution of 1/400 was selected for testing with an antibody dilution of 1/1600.

5.2.5.2 Comparison with Direct PIA

A comparison was made between the direct and indirect PIA test. Both tests required 5 hours and incubations were performed at 45°C. The direct PIA was performed using antibody-coated beads at a 1/200 dilution incubated with various dilutions of marker virus for 5 hours. The indirect test was performed using a 1/1600 dilution of antibody incubated with an equal volume of various dilutions of marker virus for one hour at 45°C. After this incubation period, marker virus coated beads were added at a concentration of 1/400 and incubated for an additional 4 hours. The results of this experiment

TABLE XXII. RESULTS OF OVERNIGHT ANTIBODY BLOCK TITRATION

	Assay Results* for Anti-marker Virus Dilutions of:						
Bead Dilution	100	200	400	800	1600	3200	6400
50	63*	62	62	72	96	96	91
100	100	99	84	96	100	100	100
[illegible]	80	80	75	83	78	94	100
300	65	69	62	62	51	66	95
400	58	67	65	68	57	59	89
500	55	64	62	61	61	58	96

*Results expressed as a ratio of sample to control.

are shown in Table XXIII. The results from the direct PIA showed a characteristic trend with the lower dilutions of marker virus giving weak readings due to prozone. As the dilutions of marker virus increased, the reading became stronger and then weaker again as the limits of detection were reached. It is this prozone effect that is eliminated when an indirect PIA is run. No agglutination of beads was seen until a marker virus dilution of 12,800 was reached. At this dilution, there was not enough marker virus present in the sample to neutralize the antibody present and agglutination occurred. Although the prozone effect is eliminated by the indirect PIA, the sensitivity is the same as the direct PIA, with both tests able to detect a 1/6400 dilution of marker virus. Because of this advantage, the indirect PIA was further investigated as the method of detection. These investigations included experiments to determine the sensitivity of the indirect PIA using a visual readout, which would simplify automation, as opposed to an instrumental photometer readout.

5.2.5.3 Visual Detection of Indirect PIA

To investigate the possibility of using a visual readout for detecting clumping, beads were observed under darkfield illumination at 480X magnification. The degree of clumping observed was then determined by comparison with negative controls.

An experiment was set up using the optimum conditions established for the indirect PIA. These conditions included testing at a bead concentration of 1/400 and an antibody concentration of 1/1600. An overnight incubation at 45°C was set up with positive samples, containing marker virus coated beads and antibody, and with negative samples containing just antigen-coated beads. Four tubes of each were run. After the incubation period, samples from each tube were removed, placed on slides, covered with coverslips and observed. The results showed that with the negative samples a majority of beads occurred singly or in twos with occasional clumps ranging from 3-5 beads. The positive sample showed a few beads occurring singly and in groups of 3-5 with large clumps of 12-15 beads occurring frequently. The results of this experiment are shown in Table XXIV.

TABLE XXIII. COMPARISON OF DIRECT (D) PIA WITH INDIRECT (I) PIA

		Assay Results for Marker Virus Dilutions of:						
		200	400	800	1600	3200	6400	12,800
D.	PIA	96	89	68	55	58	90	98
I.	PIA*	100	100	100	100	100	100	92

*Negative control ratio = 89

TABLE XXIV COMPARISON OF VISUAL AND PHOTOMETRIC READOUT OF BEAD AGGLUTINATION

Negative Samples	Observation	Photometric Reading*
1	Majority of beads single and double some in clumps of 3 - 5 beads.	100
2		100
3		100
4		100
Positive Samples		
1	Some single and double beads, numerous clumps of 3 - 5 beads frequent clumps of 12 - 15 beads.	82
2		73
3		72
4		66

*Results expressed as a ratio of sample to control.

An experiment was set up to determine if visual readings could be made to determine positive or negative tests after 5 hours incubation at 45°C. In this experiment, 1/1600 and 1/800 dilutions of antibody were tested using an optimum bead dilution of 1/400. Five positives containing antibody and five negatives containing no antibody were set up for each antibody dilution and incubated for 5 hours at 45°C. The samples were then read visually and spectrophotometrically. The results of this experiment are shown in Table XXV. From the results of this experiment it appeared that spectrophotometric readings in the low 80's were required for any visual readings to be made. Based on this experiment, a blind study using a 1/800 antibody was made. Ten tubes containing positive and negative samples were randomly arranged and read visually and spectrophotometrically after a five-hour incubation. A test showing agglutination when compared to a control was given a "+" and a negative a "-". The results of this experiment can be seen in Table XXVI. As shown, all positive agglutinations detected spectrophotometrically were also observed visually.

It should be noted, however, that a careful examination of each slide is needed to be made before any decision could be made. In many cases negative test slides had areas which appeared positive with positive test slides having areas appearing negative. A decision on whether a slide was positive or negative depended on the number of fields appearing positive or the number of fields appearing negative. In some cases the decision was not clear cut.

It was decided to test the system using an antibody concentration of 1/400 under the same conditions. Testing revealed that, even when using this increase in antibody concentration, no increase in visual bead agglutination was observed. Spectrophotometric readings also showed no real increases in agglutination.

To determine the sensitivity of the indirect PIA using a 1/800 antibody dilution, an experiment was set up testing various dilutions of marker virus. The test involved a half-hour preincubation step with marker virus samples and antibody and then a five-hour incubation with marker virus-coated beads. The samples were then read visually and spectrophotometrically. The results of this experiment are shown in Table XXVII.

TABLE XXV. RESULTS OF A FIVE-HOUR TEST OF THE INDIRECT PIA READ VISUALLY AND PHOTOMETRICALLY

1/1600 Antibody	Observations	Photometric Readings*
1	No definite difference in bead clumping when compared to negative.	89
2		88
3		92
4		93
5		91
1/800 Antibody		
1	A more detectable difference, with bead clumps of 8-10 when compared to negative.	82
2		84
3		83
4		84
5		75

*Results expressed as a ratio of sample to control.

TABLE XXVI. RESULTS OF BLIND STUDY OF INDIRECT PIA READ VISUALLY AND PHOTOMETRICALLY

Sample	Visual Reading	Spectrophotometric Reading
1	-	100
2*	+	84
3*	+	82
4	-	100
5*	+	85
6	-	100
7	-	100
8*	+	83
9*	+	84
10	-	100

\- No overall bead agglutination when compared to control

\+ Overall bead agglutination when compared to control

* 1:800 antibody dilution

TABLE XXVII. SENSITIVITY OF INDIRECT PIA USING 1/800 ANTIBODY DILUTION READ VISUALLY AND SPECTROPHOTOMETRICALLY

	Assay Results	
Marker Virus	Visual Reading	Spectrophotometric Reading*
200	-	100
400	-	100
800	-	100
1600	-	100
3200	-	99
6400	+	90
12,800	+	79
25,600	+	76
51,200	+	79

*Results expressed as a ratio of sample to control.

\- No overall bead agglutination when compared to control

\+ Overall bead agglutination when compared to control.

There was correlation between the visual readings and the spectrophotometric readings. The overall sensitivity of the test using a 1/800 antibody, necessary for any visualization of clumping, is lower than when using a 1/1600 antibody dilution. In this test, with a 1/800 antibody dilution, we could detect a 1/3200 dilution of marker virus, whereas when we use a 1/1600 dilution of antibody, a 1/6400 dilution of marker could be detected. Both tests employed the same marker virus preparation

5.2.6 Modified Indirect PIA

Using the present optimized indirect PIA, a 0.7 ml volume of sample is first incubated with an equal volume of diluted antibody. Following this, 0.2 ml of antigen-coated beads are added giving a bead dilution of 1/400. After an appropriate incubation time, 2.4 ml of glycine buffered saline (GBS) is added to bring the dilution of beads to 1/1000 for reading photometrically.

An attempt was made to simplify the number of steps required under the present system by seeing if it would be possible to incubate the sample at the same bead concentration as is used for reading. In this way the final addition of GBS would be eliminated. To determine which bead dilution would give the best agglutination an experiment was set up in which bead dilutions ranging from 1/400 to 1/1000 were tested. The test was run as follows: To 1.5 ml of GBS, 1.5 ml of a 1/1600 antibody dilution was added. This was followed by the addition of 0.2 ml of an appropriate dilution of marker virus coated beads giving the desired final bead dilutions. The mixture was then incubated at 45°C for 5 hours and the agglutination read against an appropriate control. Of the bead dilutions tested, only a dilution of 1/900 gave significant agglutination. Based on these results this bead dilution was selected for further testing.

5.2.6.1 Comparison of Detection Methods

To determine the overall sensitivity of this modified indirect PIA, when compared to the established indirect PIA, a side-by-side study was made. The experiment compared the modified indirect PIA, in which a single bead dilution of 1/900 was used requiring no additional dilution to read the tests, with the established indirect PIA which used an initial 1/400 bead dilution that was diluted before reading to a final 1/1000 bead dilution.

To determine maximum sensitivity, a 1/1600 and 1/3200 antibody dilution was used for pre-incubation of the sample before addition of beads. Both test systems involved a 1/2-hour pre-incubation with antibody and then a five-hour incubation with marker virus coated beads at 37°C. There is a similarity of sensitivity between the two techniques. At a 1/1600 antibody dilution, both systems can detect a 1/800 dilution of marker virus; at a 1/3200 antibody dilution a greater sensitivity is obtained; with the indirect PIA able to detect a 1/1600 dilution of marker virus while the modified indirect is able to detect up to a 1/3200 dilution of this particular marker virus preparation. The modified indirect PIA appeared to have the same sensitivity as the established indirect PIA.

5.2.6.2 Simplification of Test System

The modified indirect PIA test has several advantages. The major advantage is the elimination of the need to dilute the sample prior to reading. This approach also creates another advantage in that the same sample can be used as the reference control. This can be accomplished by taking a reading at the beginning of the incubation period with beads and establishing this as the zero point. Another reading is then taken after the five-hour incubation period to determine if any agglutination has taken place. This would then eliminate the need for the simultaneous use of a reference control.

To test the feasibility of using the same sample as the reference control, a modified indirect PIA test was set up using cuvettes as the reaction tubes. The test involved incubating 1.5 ml volumes of serial dilutions of marker virus with equal volumes of a 1/1600 dilution of purified antibody. This mixture was incubated for 1/2 hour at 45°C. Following this incubation period, 0.2 ml of marker virus coated latex beads was added, yielding a final bead dilution of 1/900. At this point, one tube was used to set a Bausch and Lomb spectronic 20 single beam spectrophotometer to read 70% light transmittance. All other test samples were then read and their base line % transmittance readings recorded. The test samples were then incubated for 5 hours at 45°C. During this time the spectrophotometer stayed on with the dial settings unaltered. A bead control, containing no antigen or antibody, was also run with the test to correct any drift in the spectrophotometer. Following the five-hour incubation period, test samples were re-read and their % transmittance recorded. Assay

results were assessed in terms of the change in transmittance between the samples prior to incubation and following incubation. The results of this test are shown in Table XXVIII. The ΔT values are plotted and shown in Figure 25. Marker virus dilutions up to 1/1600 were all positive as indicated by no agglutination of beads. A 1/3200 dilution of marker virus showed only slight agglutination indicating a strong positive. A 1/6400 dilution showed moderate agglutination indicating a moderate positive with a 1/12,000 dilution of marker virus showing almost maximum agglutination, thus indicating a weak positive. There is a wide difference in readings between a positive and negative test which allows for reading in the dynamic range over three serial dilutions of virus. This modified indirect PIA appeared to work well and lent itself well to automation.

5.2.6.3 Quantitation of the Modified Indirect PIA

The concern over quantitation of a positive response was raised by the Contract Monitor and an approach was formulated which would possibly quantitate a positive response. The approach involved monitoring the test continually after the addition of the beads. If the test is negative, the beads will start to agglutinate with the agglutination increasing as time progresses. If the test is strongly positive, the beads will not agglutinate, which should become apparent at some time during the monitoring. At a certain time during the test there should occur a detectable difference between a positive and a negative test. At this point an additional concentration of antibody is added to samples that have not reached a predetermined level of agglutination. A new zero time is established and the monitoring continued. With this approach it was felt that the free marker virus and the marker virus bound to the beads would compete for the added antibody. The speed of the subsequent bead agglutination would then be inversely related to the concentration of free marker virus. In other words, the more free marker virus present, the more antibody it would bind leaving less antibody available to agglutinate beads, therefore, increasing the time before bead agglutination could be detected.

5.2.6.3.1 Determination of Agglutination Curve

An agglutination curve was plotted to establish a typical negative response and to determine when additional antibody should be added. This was accomplished by running the modified indirect PIA with a negative sample

TABLE XXVIII. RESULTS OF MODIFIED INDIRECT PIA TEST USING SERIAL DILUTION OF MARKER VIRUS

Incubation Time (hours)	Assay Results* for Marker Virus Dilutions of: 200	400	800	1600	3200	6400	12,800	25,600	51,200	102,400	Control**
0	67.0	67.0	66.0	68.0	67.5	67.5	67.0	68.0	67.0	70.0	64.5
5	67.0	66.5	66.0	68.5	71.0	82.5	91.5	97.0	96.0	99.5	65.5
ΔT	0	0	0	0.5	3.5	15.0	24.5	29.0	29.0	29.5	1.5

*%T at 475 nm.

**Control beads sample containing no antibody or antigen.

containing no marker virus. Spectrophotometric monitoring of the test was performed following the addition of marker virus-coated beads to the preincubation antibody-sample mixture. Readings were recorded every one-half hour for five hours. A bead control was also run containing beads but no antibody to monitor any nonspecific agglutination. Figure 26 shows that there is a linear agglutination response of the negative sample with bead agglutination starting from time "0" with no apparent lag period. The bead control showed a change in transmittance after 5 hours of only "2" indicating little nonspecific bead agglutination.

5.2.6.3.2 Effect of Additional Antibody

Based on the negative test agglutination curve, it was determined that during the test a two-hour reading would be taken after the addition of beads. The difference in readings between the initial and two-hour reading would be calculated and all samples with change in transmittance readings less than 10% would then have additional antibody added. Those samples with a change in transmittance of greater than 10% would complete the five-hour incubation without additional antibody added. Three different concentrations of antibody were selected for testing: 1/400, 1/800, and 1/1200. In each case the antibody dilution represented the final antibody concentration of the sample mixture taking into account that a 1/3200 dilution of antibody was already present. The results of the experiments with each antibody dilution are shown in Figures 27, 28 and 29. With each experiment the modified indirect PIA was employed to test serial dilutions of marker virus. Each graph shows the readings after two hours and five hours. The dotted line represents the cut-off value. All samples with test readings falling below the line received additional antibody and all samples with test readings falling above received no additional antibody. The results of testing with 1/400 dilution of antibody are shown in Figure 27. The results of testing with 1/800 is shown in Figure 28 and the results of testing with 1/1200 antibody can be seen in Figure 29.

The results of testing with 1/400 antibody (Figure 27) gave the exact reverse of the reactions anticipated. With this dilution of antibody the higher concentrations of marker virus gave a more rapid and complete agglutination, as the marker virus concentration was diluted out, the agglutination became less rapid and less complete. This was the opposite of what was

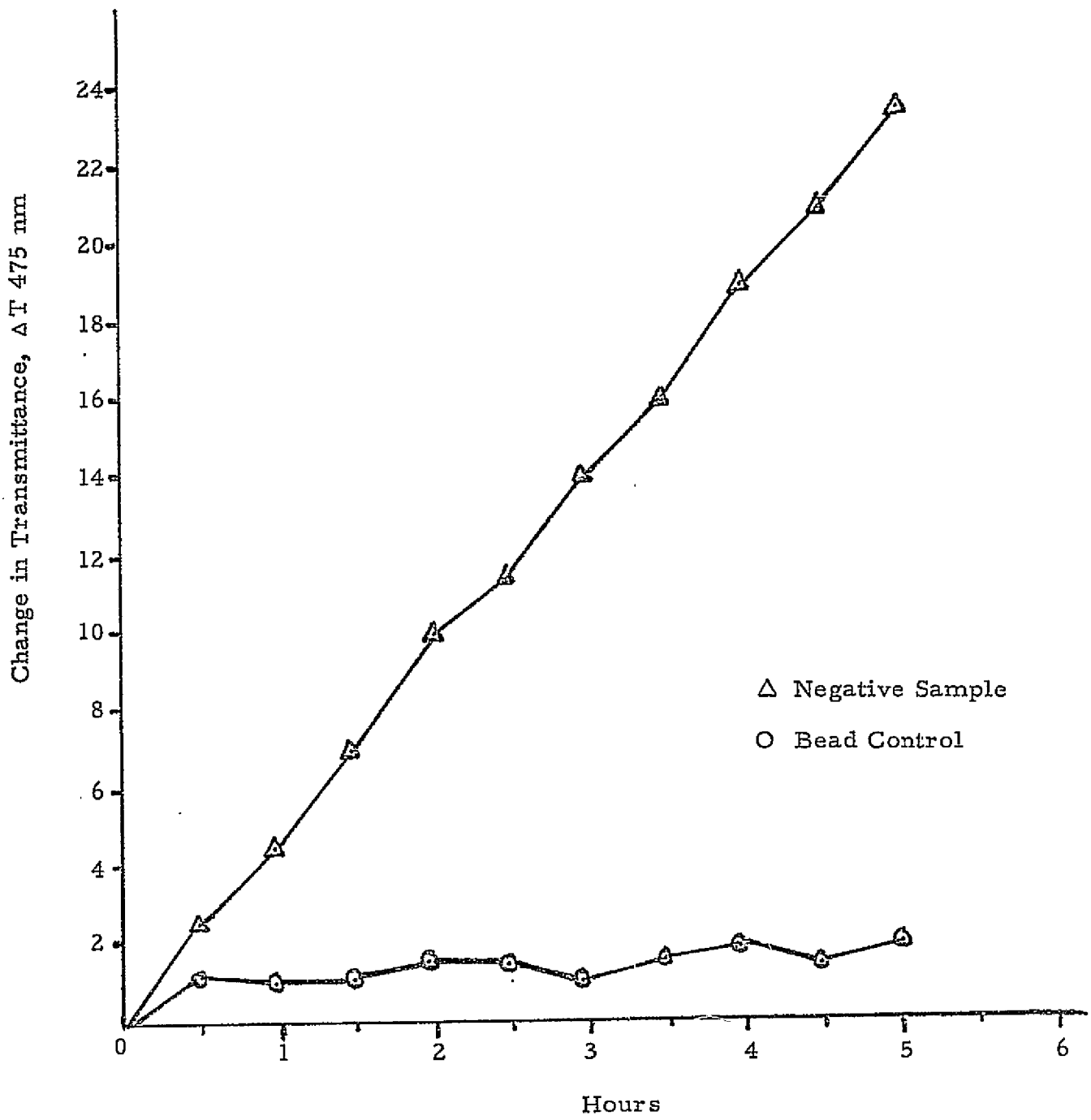

Figure 26. Agglutination Response Curve for Negative Sample and Bead Control Using Modified Indirect PIA

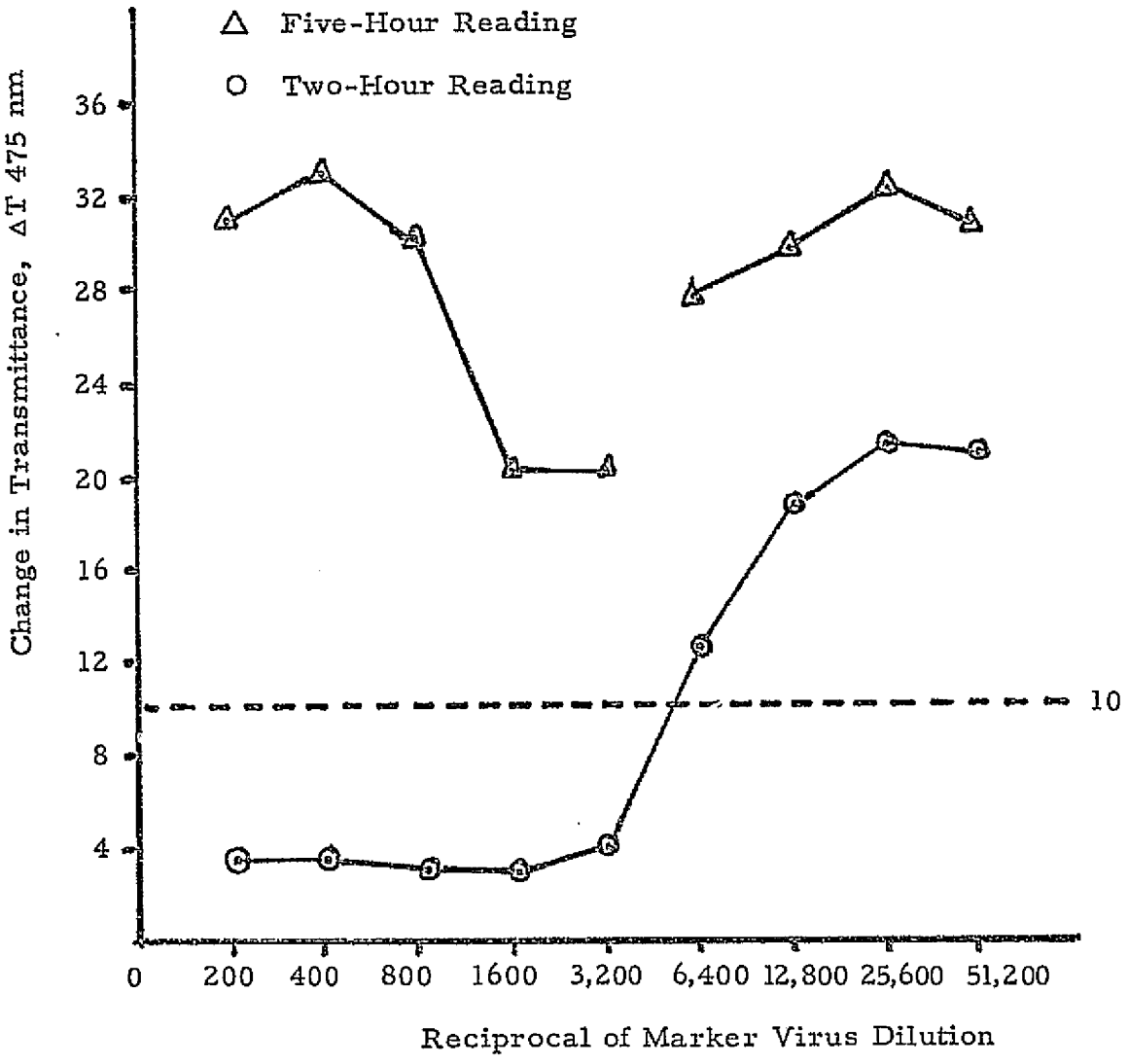

Figure 27. Effect on Modified Indirect PIA by the Addition of Antibody (Final 1/400) After Two Hours to All Samples with a Change in Transmittance Less Than 10

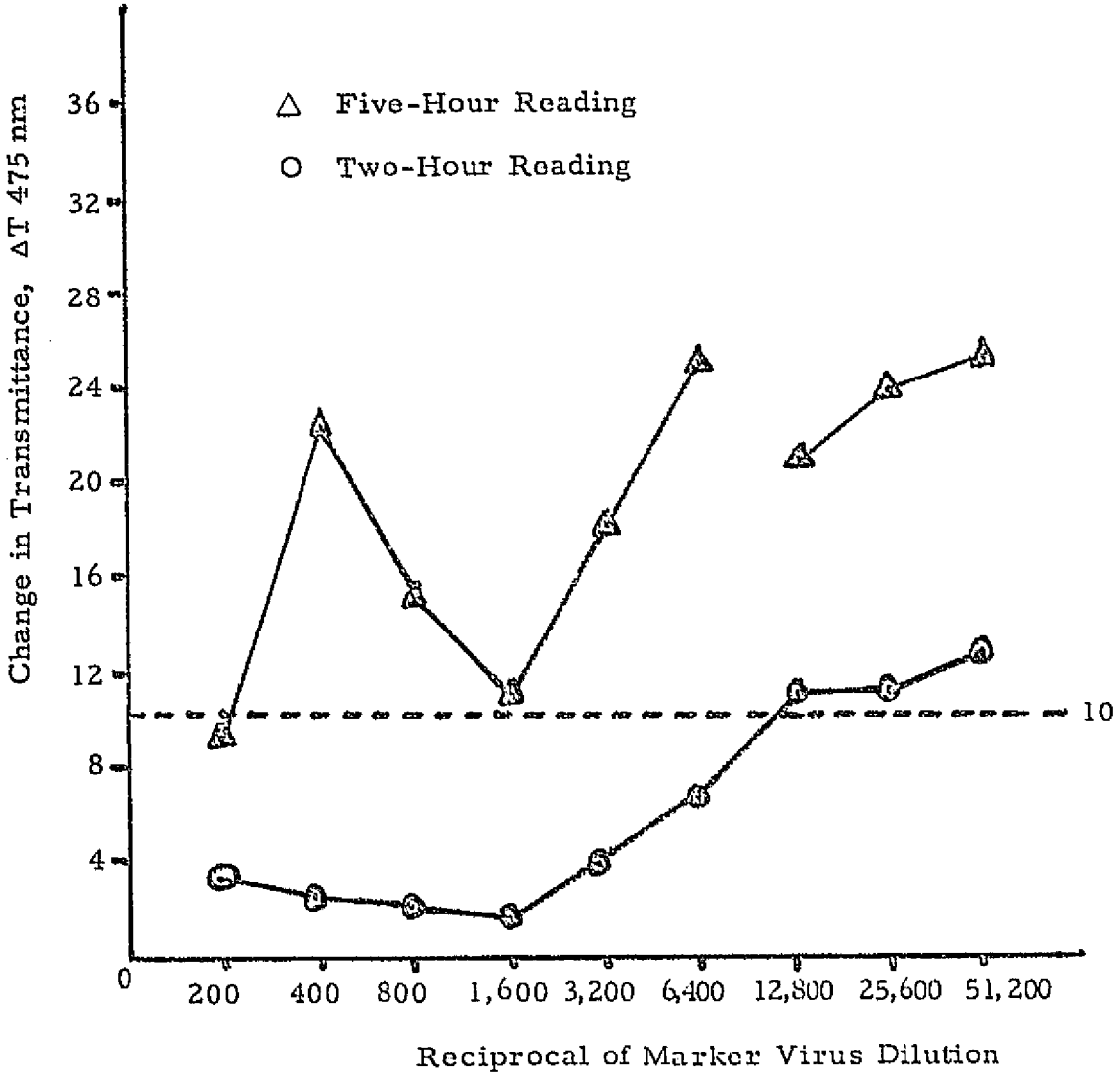

Figure 28. Effect on Modified Indirect PIA by the Addition of Antibody (Final 1/800) After Two Hours to All Samples with a Change in Transmittance Less Than 10

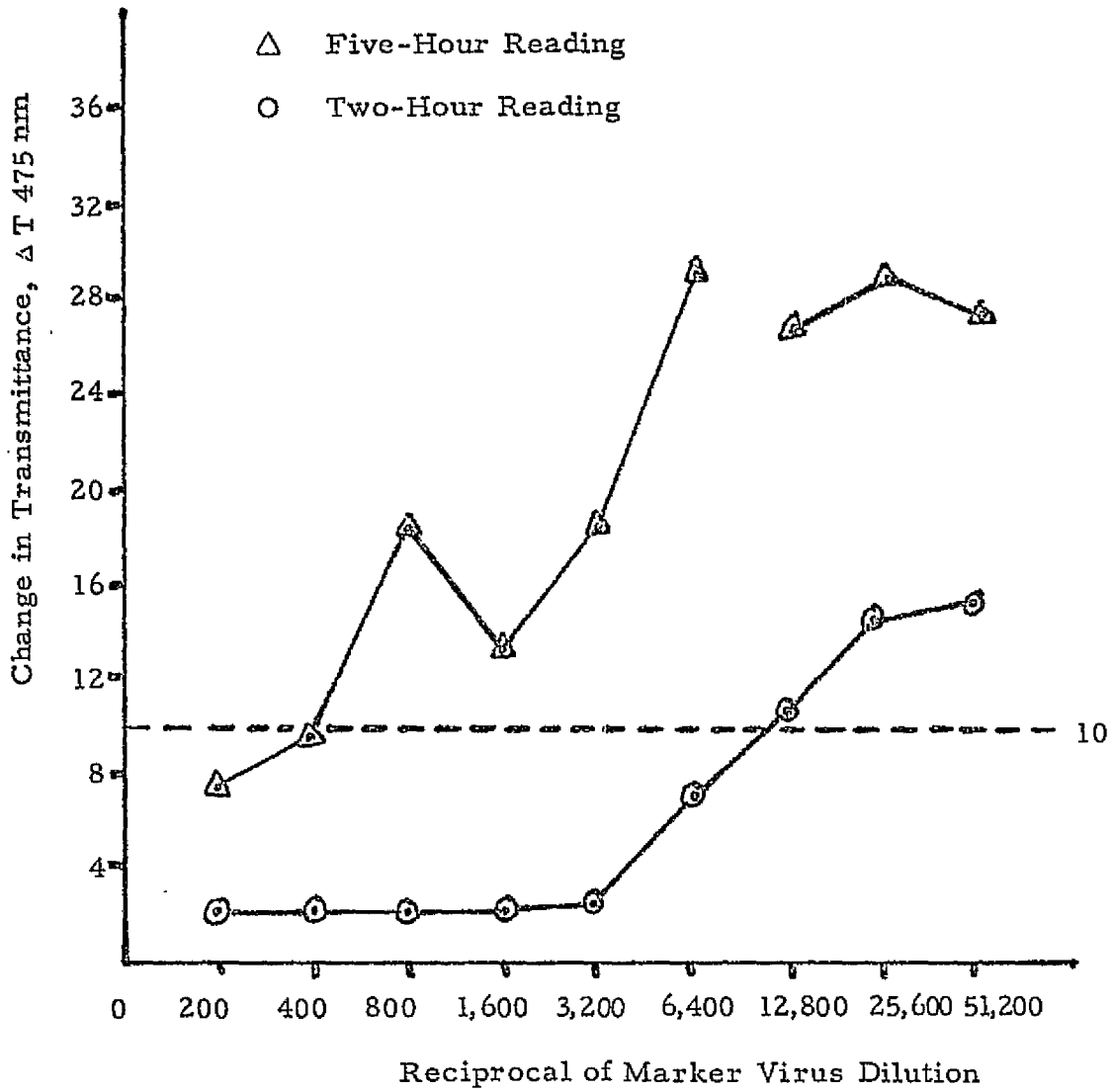

Figure 29. Effect on Modified Indirect PIA by the Addition of Antibody (Final 1/1200) After Two Hours to all Samples with a Change in Transmittance Less Than 10

expected. It was felt, as described earlier, the more free virus present the slower and less intense the agglutination would be, and as the virus was diluted out, the more rapid and complete the bead agglutination would be.

When 1/800 and 1/1200 antibody (Figures 28, 29) dilutions were tested, response patterns as anticipated were seen. With both dilutions, however, peaks of unexpected rapid agglutination were seen. These occurred at 1/400 marker virus dilution with 1/800 antibody and at 1/800 marker virus dilution with 1/1200 antibody.

5.2.6.3.3 Conclusions

The approach of quantitation by adding additional antibody during the bead incubation of the modified indirect PIA does not appear to be feasible due to the occurrence of unquantitatable agglutination. The agglutination is probably the result of a secondary reaction in which free antibody-coated marker virus coming in contact with marker virus coated beads results in bridges between beads and thus agglutination.

5.3 TEST REQUIREMENT FOR AUTOMATION

The detection system as developed involved the use of 1.5 ml of sample mixed with 1.5 ml of antibody with 0.2 ml of beads added after the initial incubation step. The final mixture then contained anti-marker virus antibody at 1/3200 and marker virus-coated beads at a 1/900 dilution.

It was necessary to change the volumes of the components of the test to accommodate requirements for automation. These requirements involved using the entire volume from the concentration step and changing the volume of antibody and beads that were added. In accordance with the engineering concepts developed, the volumes of the reagents were changed so that to the 3 ml of concentrate sample, 1.0 ml of antibody and 1.0 ml of beads were added. This resulted in a total volume of 5.0 ml with the antibody at a final dilution of 1/3200 and the beads at a final dilution of 1/900. A volume of 1.0 ml was selected for use since this amount can be accurately pumped using a peristaltic system.

With this reagent volume change, additional buffering capacity was added to the antibody and bead reagents. This involved suspending antibody and beads in 1.0 M glycine buffer + 1% NaCl at pH 8.0. This increased buffer strength would insure that the pH of the sample solution would be in an optimum range for bead agglutination. Testing revealed that when compared with the old conditions, this volume and buffer modification had no effect on the sensitivity of the test.

6.0 PROPOSED VIRAL MONITORING SYSTEM

The proposed viral monitoring system, as developed, consists of two main components, the concentration step and the detection step. The concentration step is designed to concentrate marker virus from a 400 ml sample into 3 ml. This system is based on virus adsorption to a cellulose acetate filter in the presence of $AlCl_3$ and low pH with subsequent desorption from the filter at high pH. As developed for automation, the 400 ml processed water sample is pumped through a filter (25 mm) at 20 ml per minute. Before passing through the filter, an $AlCl_3$ buffer concentrate is added at 0.5 ml per minute which lowers the pH to 2.5-3.0 and adds 5 mM $AlCl_3$. Following the initial 400 ml of treated sample, an additional 10 ml of untreated sample is pumped through the filter to remove residual $AlCl_3$ which has been shown to interfere with the PIA detection system. To remove the adsorbed virus 3 ml of 0.05 M glycine buffer, pH 11.5, is pumped through the filter at 30 ml per minute. The first ml is pumped through and collected, with the next two ml collected after a one minute delay. This delay was incorporated since it appeared to increase the overall recovery of marker virus. To the 3 ml marker virus concentrate, 1.0 ml of a predetermined dilution of anti-marker virus antibody is added and the mixture incubated for 1/2 hour at 45°C. Following this, 1.0 ml of a predetermined dilution of 1.1 μ latex beads coated with marker virus is added and at this point a spectrophotometric reading is made to determine the light transmission of the suspension. This mixture is then incubated for 5 hours at 45°C. Following this incubation period the light transmission of the solution is checked again and compared to the original reading. If agglutination of beads occurs, as a result of free antibody interacting with the marker virus coated beads, an increase in light transmission will result. In this case the test is considered negative for marker virus in the concentrate. If no agglutination, or only partial agglutination, of the beads occurs, indicating complete or partial inactivation of antibody by the presence of marker virus, the test is considered positive.

7.0 EFFECT OF AgBr AND IODINE

The use of AgBr at 70 ppb Ag or iodine at 5 ppm is being considered as a germicide to be added to the reclaimed water. It was, therefore, necessary to test the effects of these ions on the total viral monitoring system and on the viability of the marker virus.

7.1 EFFECT OF AgBr AND IODINE ON THE VIRAL MONITORING SYSTEM

The total viral monitoring system, involving the concentration and detection step, was tested for its ability to concentrate and detect virus in the presence of AgBr (at 70 ppb Ag) or iodine (at 5 ppm) in deionized, tap, and processed (B). The iodine suspensions were prepared by adding 5 ppm sublimed iodine w/v to the appropriate water samples.

Sample volumes of 400 ml were used when testing with deionized or tap water and volumes of 30 ml were used when testing processed water (B). With each condition tested, marker virus was added to the prepared samples and then $AlCl_3$-buffer added at a ratio of 1 part buffer to 40 parts sample. The samples were then passed through cellulose acetate filters and the virus desorbed with 3 ml of pH 11.5 glycine buffer. Detection of marker virus was made using the proposed modified indirect PIA. With each type of water sample tested two controls were run. The first control (Control #1) involved concentrating and detecting marker virus in the particular type of water tested in the absence of added ions. This would insure proper operation of the concentration step. The second control (Control #2) represents a 100% virus recovery and was prepared by adding the same concentration of marker virus to 3 ml of glycine buffer as was added to the starting 400 ml samples. This control acted to monitor the concentrating efficiency of the system under various conditions.

The results of these experiments are shown in Table XXIX. Testing the total viral monitoring system using deionized, tap, and processed water revealed that the AgBr had significant interference with marker virus recovery in deionized, tap, and processed water. The presence of iodine interfered significantly in tests with the deionized and tap water, but did not interfere in the processed water.

TABLE XXIX EFFECTS OF AgBr (70 ppb Ag) AND IODINE (5 ppm), IN DEIONIZED, TAP, AND PROCESSED WATER ON THE VIRAL MONITORING SYSTEM

Suspension Medium	Percent Apparent Recovery of Marker Virus in Concentrate
Deionized Water (Control #1)	100
100% Virus Recovery (Control #2)	100
Deionized Water + AgBr	< 25.0
Deionized Water + Iodine	<12.5
Tap Water (Control #1)	100
100% Virus Recovery (Control #2)	100
Tap Water + AgBr	<25.0
Tap Water + Iodine	<12.5
Processed Water (Control #1)	100
100% Virus Recovery (Control #2)	100
Processed Water + AgBr	<12.5
Processed Water + Iodine	100

7.2 EFFECT OF AgBr AND IODINE ON MARKER VIRUS ANTIGENICITY

The effect on the antigenicity of marker virus in the presence of dosed deionized or tap water treated with AgBr or iodine was studied. Experiments were performed using the different types of treated water at natural pH and at a pH of 2.8 (a condition found in the concentration step).

The experimental conditions involved suspending marker virus in the different types of treated water and incubating for 1/2 hour at room temperature. Samples were serially diluted and tested using the PIA detection system. With each condition tested a control was run in which marker virus was suspended in a particular type of water in the absence of added ions. In addition to this, each test also included an antibody and a bead control which were both run in the presence of AgBr or iodine. The results of these experiments showed that it was possible to detect (by PIA) 100% of the added marker virus in AgBr treated deionized and tap water. On the other hand, marker virus could not be detected in iodine treated deionized water, but could be detected completely in iodine treated tap water.

When the pH of the deionized and tap water was brought down to pH 2.7-2.8 (as is done in the concentration step), marker virus could still be detected completely in the deionized and tap water treated with AgBr. However, with both the iodine treated deionized and tap water, marker virus could not be detected.

Control testing demonstrated that both AgBr and iodide ions, at the levels used above, did not interfere with the agglutination of marker virus-coated beads incubated with anti-marker virus antibody.

7.3 EFFECT OF AgBr and IODINE ON VIABILITY

The effect on marker virus viability of AgBr (70 ppb Ag) and iodine (5 ppm) in deionized and tap water was studied. Marker virus was suspended in various types of treated water at a level of approximately 10^7 plaque forming units (pfu) per milliliter. Samples were held at room temperature and tested for viable marker virus, using the plaque assay method, after 1 hour and then after 1 day. A control was run in which the viability of marker virus was tested in deionized and tap water containing no added ions. The results of this experiment can be seen in Table XXX

TABLE XXX EFFECT OF AgBr (70 ppb Ag) AND IODINE (5 ppm), IN DEIONIZED AND TAP WATER, ON THE INFECTIVITY OF MARKER VIRUS

Incubation Medium	Viral Titer (PFU/ml) Following Incubation for:	
	1 Hour	1 Day
Deionized Water	2.79×10^7	1.82×10^6
Deionized Water + AgBr	1.98×10^7	2.72×10^6
Deionized Water + Iodine	0*	0
Tap Water	8.4×10^6	0
Tap Water + AgBr	6.2×10^6	0
Tap Water + Iodine	0	0

*Not detectable by plaque assay.

iodine in deionized and tap water completely inactivated the virus after one hour. On the other hand, when compared with the controls, little or no inactivation of marker virus was observed with AgBr treated deionized or tap water.

It is interesting to note that tap water was effective in inactivating marker virus after one day. This probably reflects the degree of chlorination found in city water. In comparison only, a one-log drop in titer is seen with deionized water.

7.4 CONCLUSION

Both AgBr at 70 ppb Ag and iodine at 5 ppm interfere in some way with the viral monitoring system. It was concluded that AgBr probably interferes with the concentration step since the virus could be detected in AgBr treated, deionized, and tap water under all test conditions. Iodine, on the other hand, probably interferes with the antigenicity of the virus. It is apparent that this interference is overcome by the presence of other substances found in tap and processed water since marker virus could be detected under these conditions. In pH 2.7-2.8 tap water, however, the iodine appears to be active and interferes with marker virus antigenicity.

AgBr had little or no effect on virus infectivity under the conditions tested; while iodine, in deionized and tap water, totally inactivated the virus after one hour.

8.0 ANALYSIS OF SYSTEM

Testing of the developed viral monitoring system was performed to establish the sensitivity and repeatability of the system. In addition, tests were performed to establish process accuracy and reliability. The required volume of expendable reagents was determined based on the design parameters established on the current program.

8.1 DETERMINATION OF SENSITIVITY

The sensitivity of the proposed modified indirect passive immune agglutination detection system (as described in section 5.3) was determined by testing serial dilutions of purified marker virus. Each level of virus was tested in triplicate following established testing protocols. The testing protocol involved diluting the marker virus in pH 11.5 glycine buffer and assaying 3 ml of each dilution. To these 3 ml samples, 1 ml of a predetermined anti-marker virus antibody was added and the sample incubated at 45°C for 30 minutes. To this mixture 1.0 ml of marker virus-coated beads was added and the sample incubated for 5 hours. The degree of agglutination was then determined by the difference in light transmittance between the initial and final 5-hour readings.

The results of the triplicate sensitivity runs are shown in Table XXXI and graphed in Figure 30. The degree of agglutination, as shown by an increase in light transmittance, was expressed as a percentage of the increase in light transmittance recorded for the negative (antibody) control.

As shown by the range of the triplicate runs, good reproducibility was observed with each dilution of virus tested, with a maximum range of 1.5 seen at any one dilution. Plotting the results indicated that a stright line response could be seen between a virus concentration of 5.0×10^8 PFU/ml to 3.8×10^9 PFU/ml. Under these conditions, all virus concentrates greater than 3.8×10^9 PFU/ml would show no agglutination and all concentrates less than 5.0×10^8 PFU/ml showing maximum agglutination. Based on these results, the indirect PIA test as developed can significantly detect a marker virus concentration in the range of 1.0×10^9 PFU/ml. Any virus concentration greater than this value would result in no bead agglutination and could not be quantitated.

TABLE XXXI SENSITIVITY OF THE MODIFIED INDIRECT PIA TEST RUN IN TRIPLICATE

REPLICATE TEST #	ASSAY RESULTS* FOR VARIOUS DILUTIONS OF MARKER VIRUS VIRAL TITER (LOG PFU/ml)				
	9.875	9.579	9.278	8.973	8.698
1	9	13	39	83	100
2	9	11	43	78	96
3	11	15	43	76	100
Mean	9.4	15.3	42.0	79.0	98.6
Range	2.0	4.0	4.0	7.0	4.0

* Results expressed as the ratio $\frac{\Delta T \text{ of sample}}{\Delta T \text{ of negative}} \times 100\%$ with ΔT representing the difference in % transmittance between the initial and final reading.

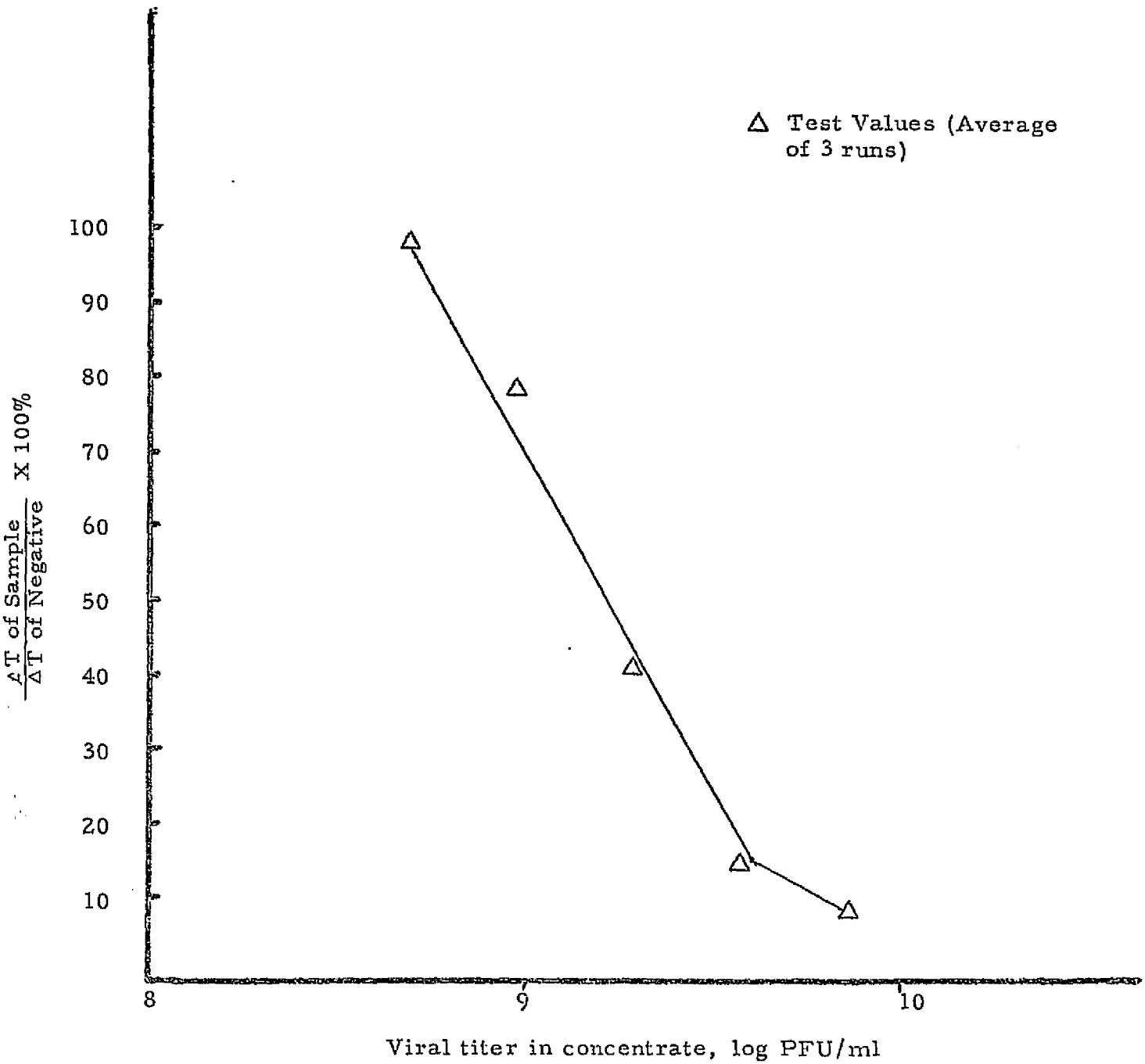

Figure 30. Sensitivity of the Modified Indirect PIA Test

8.2 SYSTEM REPRODUCIBILITY

A determination of repeatability of the entire concentration-detection system was obtained from repetitive testing of a single concentration of marker virus. The same concentration of marker virus was added to each of ten 400 ml deionized water samples. These samples were then tested by the developed monitoring system. To determine virus recovery, the 3 ml concentrates obtained were diluted out and tested using the developed detection system. Consideration was also paid to repetitive testing of negative samples as well as nonspecific agglutination bead controls, as shown in Table XXXII. There was good correlation between the 10 repetitive, negative runs with a calculated mean ΔT of 16.5, a range of only 3.0 and a standard deviation of 1.04. The repetitive bead controls showed very little nonspecific agglutination and good correlation with a calculated mean of 2.45 a range of 2.0 and a standard deviation of 0.72.

To test the reproducibility of recovery (assuming 100% repeatability), the concentration system concentrate samples were diluted so that when tested the detection response would occur in the dynamic range of the dose response curve. With the amount of virus tested, this necessitated a 1/16 dilution of the concentrates. As shown in Table XXXIII, the mean result for the diluted viral concentrates fell above the response of the 100% virus recovery control. The ten replicate runs showed a range of 16 with a standard deviation of 5.06. When compared with the control, a range of virus recovery from 100% to 73% with a mean recovery of 86% was obtained. Percent recoveries were calculated using the dose response curve found in Figure 30.

8.3 SYSTEM ACCURACY

Accuracy of the proposed viral monitoring system was determined by comparing the proposed modified indirect passive immune agglutination detection system with other detection methods. These methods included viable virus assay, radioimmunoassay and protein determinations. Selected samples of varying marker virus concentrates were tested by the above technique. Accuracy of the proposed detection system was then determined by comparing the level of response obtained from the other detection methods.

TABLE XXXII RESULTS OF TEN REPETITIVE NEGATIVE SAMPLES AND BEAD CONTROL BY THE INDIRECT PIA

Replicate Sample No.	ASSAY RESULTS* Negative Sample**	Bead Control***
1	18.0	2.0
2	16.0	2.0
3	16.5	2.0
4	18.5	3.5
5	15.0	2.5
6	16.0	2.0
7	16.0	2.0
8	16.5	2.0
9	17.0	2.5
10	16.0	4.0
Mean	16.5	2.45
Standard deviation	1.04	0.72
Range	3.0	2.0

* = Results expressed as the difference in % transmittance between the initial and final five-hour reading.

** = Negative Sample consists of 3 mls of buffer not containing F_2 plus 1.0 ml of anti-F_2 antibody plus 1.0 ml of F_2-coated beads.

*** = Bead Control consists of 3 mls of buffer not containing F_2 plus 1.0 ml of buffer not containing antibody plus 1.0 ml of F_2-coated beads.

TABLE XXXIII REPETITIVE TESTING OF POSITIVE SAMPLE CONCENTRATIONS AS ASSAYED USING THE MODIFIED INDIRECT PIA

Test Identification	Assay Results*
Replicate Sample 1	62
2	64
3	74
4	72
5	72
6	69
7	70
8	74
9	63
10	78
Positive Control (100% Recovery of Marker Virus)	63
Negative Control (0% Recovery of Marker Virus)	100
Mean for replicate assays	70
Standard deviation for replicate assay	5.06
Range for replicate assays	16.0

* Results expressed as the ratio of $\frac{\Delta T \text{ of sample}}{\Delta T \text{ of negative}} \times 100\%$ with ΔT representing the difference in % transmittance between the initial and final reading.

Ten different lots of marker virus concentrate were tested. In the case of the indirect PIA and the RIA the concentrates were diluted 1/3200 to bring the concentration of the virus into the dynamic range of the test. The RIA test was performed as described in Section 5.2.2 and the indirect PIA test performed as described in Section 5.3. The plaque assay and protein determination were made on the marker virus concentrates directly. The plaque assay was performed using the agar layer technique and the proteins were determined using the Lowry method. The results of this experiment are shown in Table XXXIV. The various virus concentrates were listed according to their strength of reaction by the indirect PIA (the lower the assay result, the higher the virus concentration for that dilution).

With the exception of virus concentrates D and R a good correlation was seen between the response of the PIA detection method and the RIA and protein determination response. There was a definite trend toward less protein content and higher RIA ratios (indicating less virus present) as the PIA response decreased. When comparing the PIA reactions for each concentrate with the plaque assay, not as strong a correlation could be drawn. However, with the exception of concentrate lots K, L, M, and D. There was a correlation between decreasing PIA response and decreasing plaque forming units per milliliter. The overall correlation of reactions using different detection systems indicates good accuracy.

8.4 SYSTEM RELIABILITY

A major component of the viral monitoring system is the concentration step. This system is based on virus adsorption to and subsequent desorption from cellulose acetate filters. It was thought that these filters could be reused instead of being replaced after each run. It was, therefore, necessary to test the reliability of reusing these filters. To accomplish this the filters were subjected to repetitive adsorption, desorption, and regeneration cycles (as described in Section 6.0), with the addition of a known cencentration of marker virus every third cycle. This approach was continued until the filter no longer functioned properly, either by clogging or no longer retaining virus.

TABLE XXXIV EVALUATION OF VIRAL CONTENT IN VARIOUS LOTS OF F_2 VIRUS CONCENTRATES USING FOUR ASSAY METHODOLOGIES

	ASSAY RESULT / ASSAY METHODOLOGY			
Lot	Indirect* PIA	Protein (ug/ml)	RIA**	Viral Titer (PFU/ml)
K	13	833	0.27	8.0×10^{12}
L	13	803	0.28	1.2×10^{13}
M	25	681	0.32	1.0×10^{13}
D	33	1024	0.24	2.0×10^{13}
R	40	821	0.28	2.3×10^{13}
O	53	660	0.35	1.4×10^{13}
pool #2	68	538	0.43	1.0×10^{13}
A	75	439	0.48	5.2×10^{12}
Q	93	335	0.69	5.0×10^{12}
P	93	210	0.91	4.9×10^{12}

* Ratio: (ΔT of sample/ ΔT of negative control) x 100

** Ratio: cpm of sample/cpm of negative control

ΔT = difference in % transmittance between initial and final reading.

cpm = counts per minute

PFU = plaque forming units

Reliability of the detection system was also investigated by performing a shelf life study of the two reagents (anti-marker virus antibody and marker virus-coated beads) used in the indirect PIA. Using a single batch of each reagent diluted to working strength, the level of response of a negative and positive test was monitored over a period of weeks.

Another important consideration was the effect that peristaltic pumping would have on the reagents used in the detection system. An experiment was run comparing the reactivity of pumped and non-pumped reagents.

The result of the filter studies are shown in Table XXXV. The marker virus concentrates obtained on every third run were diluted out so that the response would fall in the dynamic range of the test. A total of 27 runs were performed with a total of nine live runs. The results showed there was a trend towards less virus recovery, the more cycles the filter had gone through. Cyclic runs were made using deionized water and filter clogging did not become apparent until the 15th cycle. Additional cycles performed after the 15th run had to be performed at slower sample and elution buffer pumping rates. Following the 27th run, the filter became completely clogged and could no longer be used. Even though the filter had clogged after the 27th run, virus was still being adsorbed and recovered.

The results of the shelf life study are shown in Table XXXVI. Both the antibody and beads were diluted to working strength and held at $4^{\circ}C$. Sodium azide was added to a final concentration of 0.02% as a preservative. To test the shelf life of the beads, samples of the reagents were taken each week for 8 weeks and used in a positive test (sample with marker virus) and a negative test (sample without marker virus). The testing procedure used is described in Section 5.3. As shown in Table XXXVI, there was a slight increase in the agglutination of the positive control after the 8th week, indicating an increase in nonspecific agglutination. The negative control showed no change in agglutinating strength after the 8th week, indicating no loss of antibody strength or bead reactivity. It will be necessary to perform a more extended shelf life study of these reagents since a 6-month supply schedule is proposed.

TABLE XXXV EFFECT OF REUSING FILTERS ON CONCENTRATION AND RECOVERY OF MARKER VIRUS AS ASSAYED USING THE MODIFIED INDIRECT PIA

Regeneration Cycle No.	Assay Results*
3	41
6	47
9	57
12	55
15	48
18	59
21	63
24	59
27	72
100% Recovery Control	48

*Results expressed as the ratio of $\frac{\Delta T \text{ of sample}}{\Delta T \text{ of negative}} \times 100\%$ with ΔT representing the difference in % transmittance between the control and final reading.

TABLE XXXVI SHELF LIFE STUDY OF ANTI-MARKER VIRUS ANTIBODY AND MARKER VIRUS COATED BEADS DILUTED TO WORKING STRENGTH AND HELD AT 4°C AND ASSAYED USING THE MODIFIED INDIRECT PIA

Period of Storage (Weeks)	Modified Indirect PIA Results for Stored Reagents Applied to a Test Sample	
	Positive Sample*	Negative Sample*
0	1.5	17.5
4	2.5	16.5
8	4.5	17.5

*Difference in % transmittance between the initial and final reading

8.5 SYSTEM SPECIFICITY

To insure the bead agglutination observed in the indirect PIA test was due to the specific interaction of the marker virus (antigen) and anti-marker virus (antibody) reagents, experiments were set up using a different antibody and different antigens. In the case of the antibody, anti-adenovirus hexon-specific rabbit antibody was used and in the base of antigens, adenohexons, rubella antigen and influenza were used. For each experiment, the conditions of the finalized indirect PIA (Section 5.3) were applied, with either the marker virus (antigen) or anti-marker virus (antibody) being replaced by the nonspecific reagent. In each case, the nonspecific antibody or antigens were adjusted to the same protein level as the specific reagents.

In the first experiment, a series of dilutions of marker virus were tested over the dynamic range of the indirect PIA (those dilutions that gave no agglutination to maximum agglutination) using anti-adenohexon antibody instead of specific anti-marker virus antibody. Included in the test was a sample that contained just anti-adenohexon antibody and marker virus coated beads. Results showed that the anti-adenohexon antibody did not agglutinate the beads in any of the serial dilutions of marker virus tested and did not agglutinate the beads when tested in the absence of marker virus. This indicated that the marker virus coated beads in the indirect PIA were agglutinated due to a specific interaction of antibody against the marker virus, not as a result of nonspecific interactions of rabbit IgG with the coated beads.

When testing the effect of nonspecific antigens, a similar experiment was run. In this case, however, the dilution of marker virus was replaced by dilution of the various antigens. All other conditions were the same as those in the indirect PIA with specific anti-marker virus antibody and marker virus coated beads being used. Included with each antigen tested was a sample which contained just antigen and marker virus coated beads. The results of the experiment with adenohexons, rubella, and influenza antigens showed that at no dilution tested did these antigens significantly interfere with the agglutination reaction between the marker virus coated beads and specific anti-marker virus antibody, and that when incubated with the marker virus coated beads alone, these antigens did not cause any nonspecific agglutination of the beads.

An experiment was designed which tested the effects of peristaltic pumping on the marker virus-coated beads and anti-marker virus antibody. The result of the experiment with the pumped reagent when compared with non-pumped reagent showed no difference in either anti-marker virus antibody strength or marker virus-coated bead reactivity.

8.6 VOLUME OF EXPENDABLES

The viral monitoring system, as developed, involves introduction of marker virus into the reclamation system, a marker virus concentration step and a detection step (see Section 6.0). Each step requires specific reagents for operation. Concentrated marker virus is required to challenge the system. The concentration step involves the use of a filter, $AlCl_3$-buffer concentrate and 0.05 M glycine buffer, pH 11.5. The detection step involves the use of marker virus coated beads and anti-marker virus antibody. Calculations of the volume of reagents required were made based on a 6-month resupply schedule and the performance of two tests a day. These calculations are shown on Table XXXVII.

TABLE XXXVII VOLUME OF EXPENDABLES FOR THE OPERATION OF THE VIRAL MONITORING SYSTEM*

		Total
I.	Challenge of the Water Reclamation System	
	- Marker Virus (10 ml per test**)	7200 ml
II.	Marker Virus Concentration	
	- Filters (Nine test per filter)	80 count
	- $AlCl_3$ - buffer (10 ml per test)	7200 ml
	- 0.05 M glycine buffer, pH 11.5 (3 ml per test)	2160 ml
III.	Marker Virus Detection	
	- Anti-marker virus antibody (1.0 ml per test)	720 ml
	- Marker virus coated beads (1.0 ml per test)	720 ml

* Based on 2 tests per day with a 360 day resupply schedule

** At a concentration of 2×10^{14} PFU/ml when using the finalized indirect PIA detection system

9.0 END-TO-END TESTING OF THE SYSTEM

End-to-end testing of the proposed viral monitoring system was performed as described in Section 6.0. Testing involved ten (10) different quantities of marker virus added to 400 ml samples of deionized water. The concentration range of marker virus included a maximum which represented a total breakdown of the reclamation system and a minimum equivalent to the limit of detection. The lowest concentration of marker virus to be tested was determined by the sensitivity determinations found in Section 8.1.

9.1 LEVELS OF MARKER VIRUS TESTED

Based on sensitivity data found in Section 8.1, the PIA system developed here has the capacity to detect a marker virus concentration of $\approx 1 \times 10^9$ PFU per milliliter of test sample. When linked with the concentration system which has the ability to concentrate a sample 100-fold, the total viral monitoring system has the capacity to, in effect, detect $\approx 1 \times 10^7$ PFU per milliliter.

An ability to detect up to a 99.99% drop in seed marker virus concentration passed in the purified water was established by NASA as a requirement for the system. When applying this requirement to the VCD (vapor compression distillation) water purification system, the following calculations were performed. A 1 ml sample of marker virus introduced into the closed urine loop of the VCD will be diluted by a factor $\approx 2 \times 10^4$, since the closed loop contains approximately 40 pounds of sample or 20 liters. If we introduce marker virus at 2×10^{15} PFU/ml we see an immediate dilution to 1×10^{11} PFU/ml. Therefore, we might assume that a total filure (i.e., 100% passage) of the system would introduce 1×10^{11} PFU/ml of marker virus into the reclaimed water with partial failure resulting in less marker virus getting through. If instead, only 0.01% of the marker virus passes through (i.e., 99.99% rejection), the concentration in a water sample will be 1×10^7 PFU/ml, and therefore just sufficient to meet the detection threshold of the PIA.

A starting marker virus concentration of 4×10^9 PFU/ml of sample was used due to a limited supply of marker virus at the time of testing. A range of marker virus finishing at 1.95×10^6 PFU/ml of sample was tested by marking serial dilutions of the initial starting marker virus concentration.

This concentration represented less than the concentration of marker virus to be expected in the case of total failure by the VCD system but which was adequate to produce a strong response in the PIA. Each dilution was tested by triplicate end-to-end runs using the proposed monitoring system (Section 6.0). Included with each dilution were three positive controls representing 100% marker virus recovery. With these controls, overall recovery at each dilution could be determined. With each dilution series, a negative and bead control was performed. The negative control contained marker virus coated beads and anti-marker virus antibody. This control was used to determine the degree of specific bead agglutination. The bead control contained only marker virus coated beads and was used to determine the degree of nonspecific agglutination. Test results were expressed such that a positive test would approach 0% and a negative would approach 100%. The results of this testing is shown in Table XXXVIII and graphed in Figure 31. The results listed represent an average calculated for each triplicate set. There was excellent correlation between the concentrate samples and their corresponding 100% recovery controls. The response curves are almost identical, indicating essentially 100% virus recovery from the marker virus concentration.

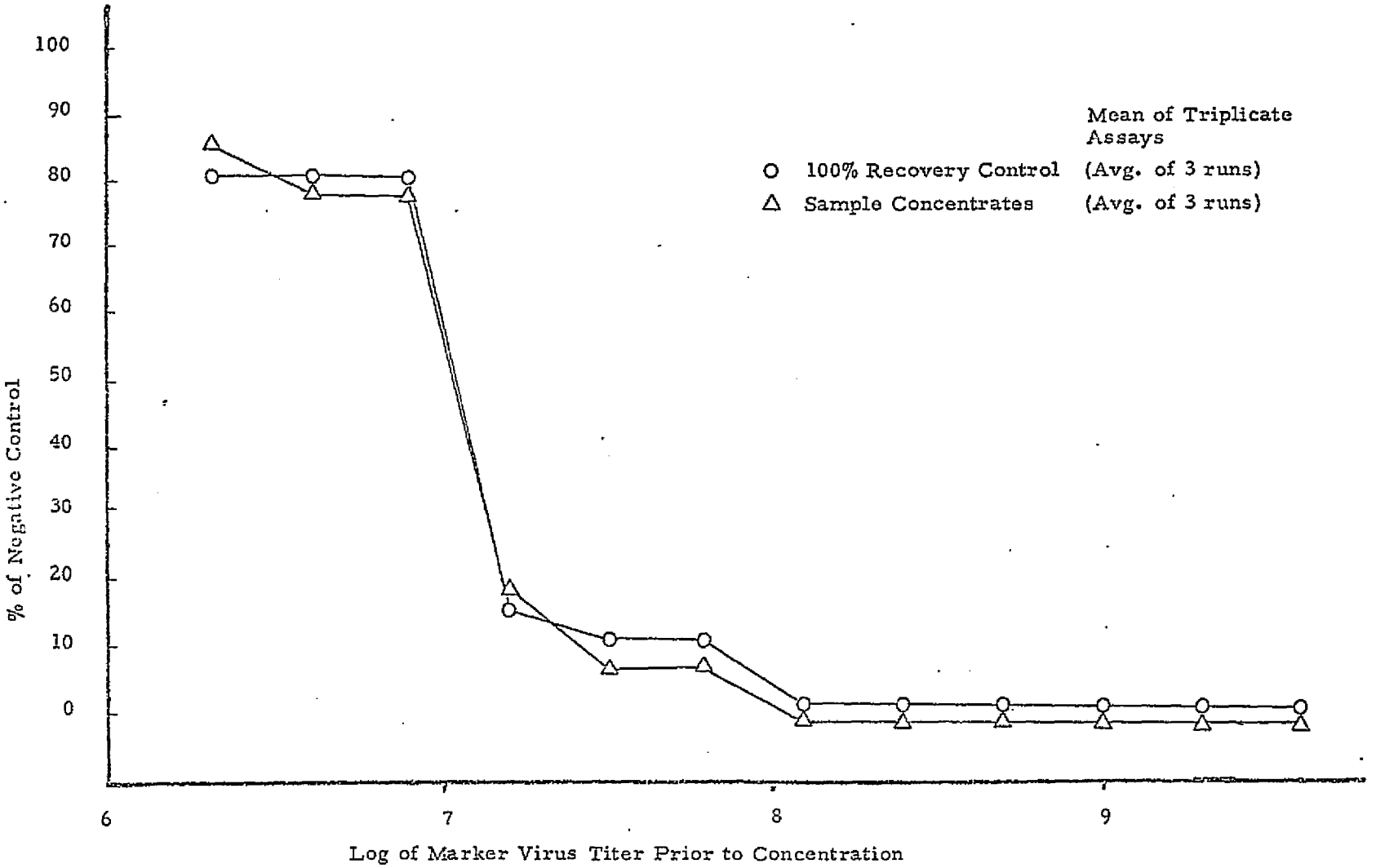

Figure 31 Results of End-to-End Testing of the Proposed Viral Monitoring System

TABLE XXXVIII RESULTS OF END-TO-END TESTING OF THE PROPOSED VIRAL MONITORING SYSTEM

Marker Virus in 400 ml Sample (PFU/ml)	Assay Results*	
	Concentrate	100% Recovery
4.00×10^9	0	0
2.00×10^9	0	0
1.00×10^9	0	0
5.00×10^8	0	0
2.50×10^8	0	0
1.25×10^8	0	0
6.25×10^7	8*	12
3.13×10^7	7	12
1.56×10^7	19	16
7.80×10^6	78	80
3.90×10^6	78	80
1.95×10^6	86	80

*Results expressed as the ratio $\frac{\Delta T \text{ of sample}}{\Delta T \text{ of negative}} \times 100\%$ when ΔT represents the difference between the initial and final % transmittance readings

10.0 TESTING OF THE WATER RECOVERY SYSTEMS

The development of a virus testing system for use in space application was based on the assumption that the water recovery systems are capable of rejecting or inactivating viruses when operating properly. To determine if the proposed recovery systems do reject marker virus, a rigorous on-ground testing program was proposed.

Testing was to be performed on two NASA-selected water recovery systems located at the NASA contractor's facilities. The first system employed reverse osmosis to recover shower water for recycling. The second system involved the use of a low pressure distillation system to recover potable water from urine.

To test this system, marker virus was introduced upstream and looked for in the reclaimed water. To detect the presence of marker virus in reclaimed water, plaque assay, PIA and ELA testing was performed.

10.1 REVERSE OSMOSIS WATER RECOVERY

The Reverse Osmosis (R.O.) Wash Water reclamation system being developed at Clemson University was challenged and tested for its ability to pass marker virus.

The R.O. system is shown diagramed in Figure 32. With this design a minimum of 20 liters of shower water is pumped through the system at an average of 75 liters per minute (lpm) under a pressure of approximately 1000 psi. Heat produced by the pump is regulated by a heat exchanger. This minimizes the probability of heat damage to the marker virus. Four membranes are linked into the system which, under good conditions, have the ability to produce approximately 10 ml/minute of reclaimed water when ganged together.

10.1.1 TESTING CONDITIONS

Two marker virus challenge runs of the R.O. system were performed. In the first run, distilled water was used as the starting material and in the second run a shower water concentrate was used. For each run, it was decided that 1.5×10^{14} PFU/ml would be introduced into the system. The volume of the system immediately diluted the marker virus to 5×10^{9} PFU/ml (assuming 30 liters in the system). Therefore, in a total breakdown

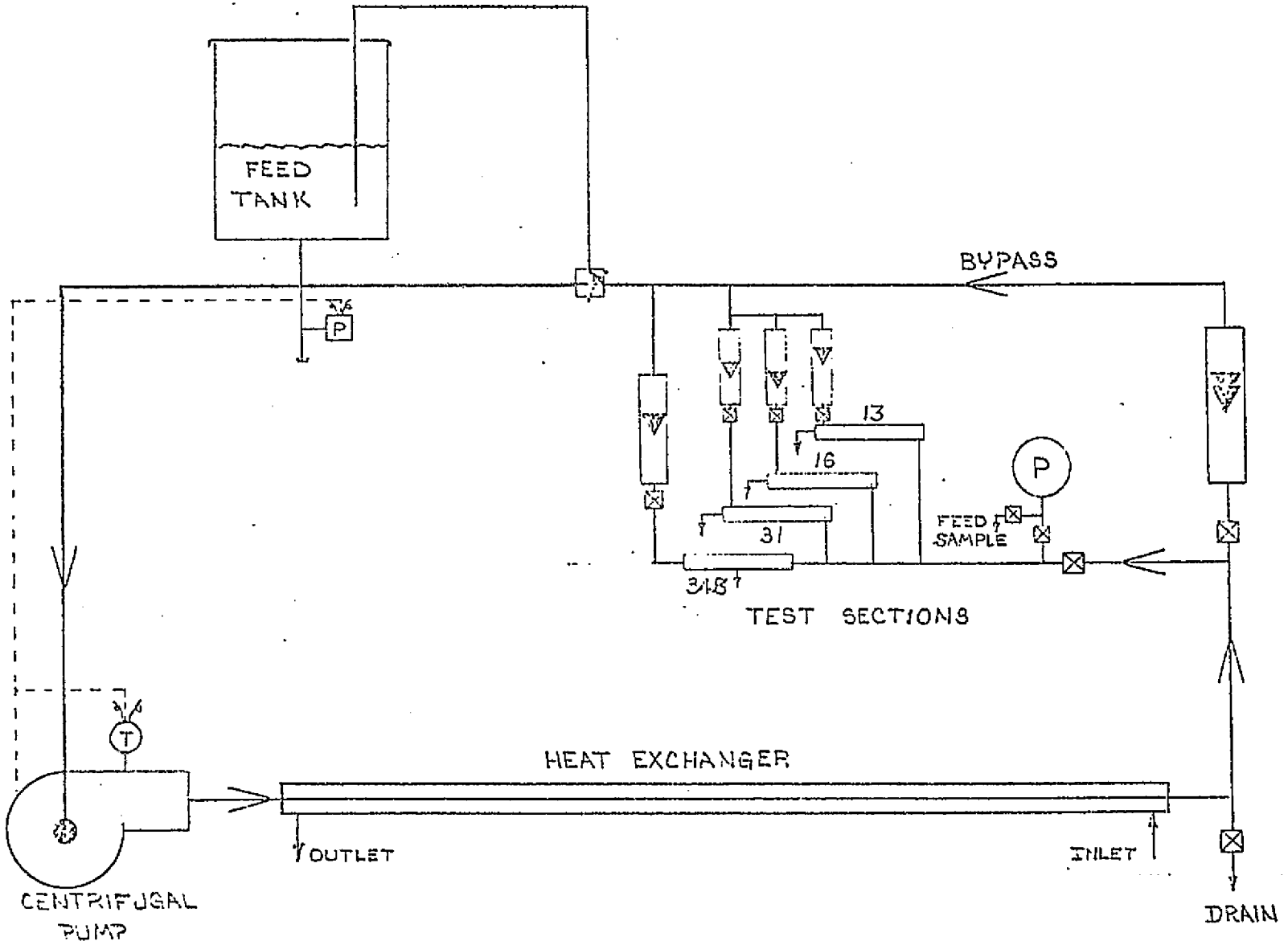

Figure 32. Reverse Osmosis System Schematic Diagram
(Courtesy of Clemson University)

of the system, one would expect to see 5×10^9 PFU/ml in the reclaimed water. At this input level of virus and utilizing the viral concentration system, we would be able to detect a 99.9% drop in marker virus concentration in the reclaimed water utilizing the PIA and a ≈99.9% drop in marker virus concentration in the water when testing by ELA.

During both test runs eight samples were collected. These samples are explaimed below:

Sample No. 1 - 1000 ml of unchallenged starting water collected from the input side

Sample No. 2 - 1000 ml of reclaimed water collected from the unchallenged system

Sample No. 3 - 1000 ml of challenged starting water collected from the input side

Sample No. 4 - 1000 ml of reclaimed water collected from the challenged system (pool of membranes 31 and 16)

Sample No. 5 - 1000 ml of reclaimed water collected from the challenged system (pool of membranes 13 and 34S)

Sample No. 6 - 1000 ml of reclaimed water collected from the challenged system (pool of membranes 31 and 34S)

Sample No. 7 - 1000 ml of reclaimed water collected from the challenged system (pool of membranes 16 and 13)

Sample No. 8 - 1000 ml collected from input side of system prior to shut-down.

The samples numbered 1 through 8 were collected in sequence with samples 4 and 5 being collected following the collection of samples 1, 2 and 3. To collect samples 6 and 7 the membranes were repooled. Sample 8 was collected last. All samples were stored in sterile bottles and held at 4°C prior to testing. With the distilled water run, samples 3 and 8 were tested by plaque assay, PIA and ELA, with only plaque assaying being performed with samples 3 and 8 from the shower water run. These sample results were used to determine the rate of inactivation of the virus while in the system. Samples 4, 5, 6 and 7 were tested by plaque assay, PIA and

ELA to determine if marker virus was present in the reclaimed water. Sample 2 was used as a negative control for each testing system. When testing by PIA or EIA, 400 ml samples were concentrated using the developed marker virus concentration system. The 3 ml concentrate was then tested. The results of the distilled water run are shown in Table XXXIX and the results of the shower water run are shown in Table XL. All results were expressed as the level of PFU/ml present as indicated by each detection system. It should be noted that the plaque assay method only detects infectious marker virus particles while both the PIA and ELA are capable of detecting inactivated (non-infectious), but still antigenic, marker virus. The following conditions were held for the distilled water run: temperature was 52°C, flow rate was 77 lpm, pressure was held at 1000 psi, there was an initial volume of 33.5 liters and a final volume of 29 liters. After the distilled water run, the system was heated to 88°C for 5 minutes to inactivate remaining virus. The shower water run consisted of a six-fold concentration of shower water which initially was a 1 to 5 dilution of SDS (sodium dodecal sulfate) in water previously used for showering. The following conditions were held for the shower water run: temperature was 52°C, flow rate was about 63 lpm, pressure was held at 1000 psi, there was an initial volume of 26.5 liters and a final volume of 17.5 liters.

Test results of sample 3 and 8 of the distilled water run, shown in Table XXXIX, indicated that while inside the system there was a sharp drop in infectivity of the marker virus (as shown by the plaque assay) with little or no effect on the antigenicity (as shown by the results of the PIA and ELA). Testing of the reclaimed water samples 4, 5, 6 and 7 revealed that significant levels of virus were passed in samples 5 and 6 with little or no detectable marker virus passed in samples 4 and 7. It was possible to determine by the pattern of pooling that membrane 34S was common to both samples and was suspect to passing ≈10% of the level of marker virus present in the system. The other membranes appeared to pass little or no detectable virus.

Test results of samples 3 and 8 of the shower water run, shown in Table XL, indicated that while inside the system there was a significant drop in infectivity and antigenicity of the added marker virus. Testing of reclaimed water samples 4, 5, 6 and 7 showed significant numbers of virus being passed in samples 5 and 6, as was found with the distilled water run.

TABLE XXXIX ASSAY OF RECLAIMED WATER PRODUCED BY THE REVERSE OSMOSIS SYSTEM: APPLICATION TO DISTILLED WATER SEEDED WITH MARKER VIRUS

Reclaimed Water Sample No.	Assay Results* (PFU/ml) Detection System		
	Plaque Assay	PIA	ELA
2	**	**	**
3	6.2×10^8	3.6×10^9	4.3×10^9
4	1.1×10^2	**	**
5	3.7×10^4	**	1.26×10^8
6	8.5×10^1	3.2×10^9	1.78×10^8
7	**	**	**
8	1.7×10^2	2.9×10^9	3.66×10^9

* Expressed in terms of viral titer

** No marker virus detected

TABLE XL ASSAY OF RECLAIMED WATER PRODUCED BY THE REVERSE OSMOSIS SYSTEM: APPLICATION TO SHOWER WATER SEEDED WITH MARKER VIRUS

	Assay Result* (PFU/ml)		
	Detection System		
Sample No.	Plaque Assay	PIA	ELA
2	**	***	**
3	8.8×10^8	"	3.23×10^9
4	1.6×10^2	"	**
5	5.0×10^4	"	**
6	1.0×10^4	**	**
7	1.2×10^2	***	**
8	7.2×10^6	"	**

* Expressed in terms of viral titer

** No marker virus detected

*** No results due to interference by samples.

The level was not enough, however, to be detected by ELA. It was noted that with the exception of sample 6S, the PIA detection system did not function correctly with the concentrate samples, in that bead agglutination was inhibited. The problem appeared to be the concentration of detergent along with the virus when concentrating using the filter adsorption technique. With the shower water run, as with the distilled water run, membrane 34S suspected as the cause of passed marker virus. The other membranes appeared to pass little or no detectable virus.

10.1.2 CONCLUSIONS

The R.O. System tested at Clemson University utilized four (4) membranes. Of the four membranes, only one membrane, 34S, appeared to pass significant detectable levels of marker virus (approximately 10% of that found inside the system). When testing reclaimed water produced from shower water, significant levels of detergent appeared to be present in the marker virus concentrate. The presence of this detergent interfered with bead agglutination in the PIA test.

11.0 ENGINEERING MODEL DESIGN

11.1 CONFIGURATION DEVELOPMENT

The engineering model design was conducted as a parallel effort to the laboratory development of the test system to detect viral contamination in a waste water reclamation system used in long term space flights. Engineering schematics and preliminary sketches of a workable system,based on the laboratory test protocol, were developed. Engineering recommendations were presented when the test requirements dictated subsystems difficult to automate. Several alternative instrument process arrangements and techniques were evaluated for optimum configurations. Alternative power sources were evaluated. Efforts were made to minimize disposables and waste material. At the completion of evaluation efforts, an instrument assembly drawing was completed to present a preliminary configuration of the instrument arrangements. Sufficient views and subassemblies were drawn to show the operation of the instrument and detection technique. A preliminary instrument control panel and electrical schematics were drawn. The following discussion presents the alternatives and design evaluation considered leading up to the present engineering design.

11.2 PRELIMINARY DESIGN

11.2.1 Sample Concentration

The initial task in the design of the virus detector was to develop a virus concentrator to reduce the sample size from 400 ml down to less than 3 ml. Initial work to concentrate the virus involved a two-step approach. The sample was first reduced from 400 ml down to approximately 30 ml by use of a hollow fibre concentrator. A schematic for performing this is shown in Figure 33. The hollow fiber filter concentrator system utilizes two pumps operated by a single reversible motor. Water from the reclamation system is allowed to enter the concentrator loop with one pump circulating the liquid at a rate of 100 ml/mm and maintaining a positive pressure of 10 psig. An additional pump maintains a negative pressure of -10 in. Hg on the hollow fibre and pumps liquid passing through the hollow fibre into a 400 ml zero-g container. Virus are retained in the positive pressure loop. When the 400 ml receiver is full, all valve positions are switched and the pumps are reversed to allow

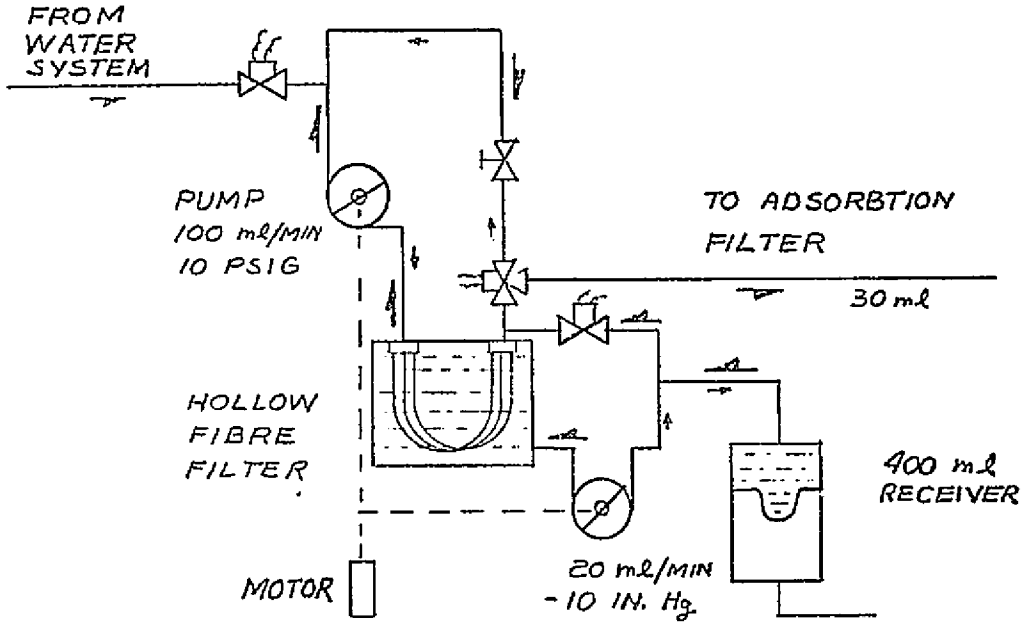

Figure 33 Filter Concentrator Virus Water Monitor

the liquid (30 ml) and concentrated virus in the positive press loop to flow to the adsorption filter system.

The second of the two part concentration step involved reducing the sample from 30 ml to 3 ml, was to be performed by filter adsorption utilizing $AlCl_3$. Further testing indicated the adsorbtion technique could be used to remove marker virus from the 400 ml water sample. The hollow fiber system was subsequently removed from the design, simplifying the system.

11.2.2 Pumping/Metering System

Two techniques for metering and pumping of liquids were considered and schematics for each are presented in Figures 34 and 35.

The flow schematic shown in Figure 34 presents a metering system utilizing pistons and as a sample collector and metering device. Water to be tested is collected in the 400 ml sample collection piston until the piston rod engages a valve control switch which reverses the water and $AlCl_3$ valve positions to allow flow of $AlCl_3$ to move the piston to a stop position. The valves are then switched to flow into the 400 ml catch tank with the virus being absorbed into the filter. The correct proportions of buffer/sample and beads are then metered into a similar piston device at the read/incubate location. Liquid in the 400 ml catch tank may be used to flush the read cylinder and the 400 ml sample collection piston. In addition, that liquid would backflush and reconstitute the filter for the next cycle. Regulated air was utilized as a power source to move the liquids.

A peristaltic pump metering system is shown in Figure 35. Metering and valving of liquids is performed by the action of peristaltic pumps sequenced to operate at the appropriate times. Reagent storage is handled by diaphragm or bellows type containers similar to the piston metering system without air pressure to drive the liquids.

The peristaltic pump metering system does not experience liquid leakage problems, however, metering of liquids is not as accurately controlled and some periodic maintenance is required to exchange pump tubing if the system is not operated periodically or if excessive useage takes place at high pressures.

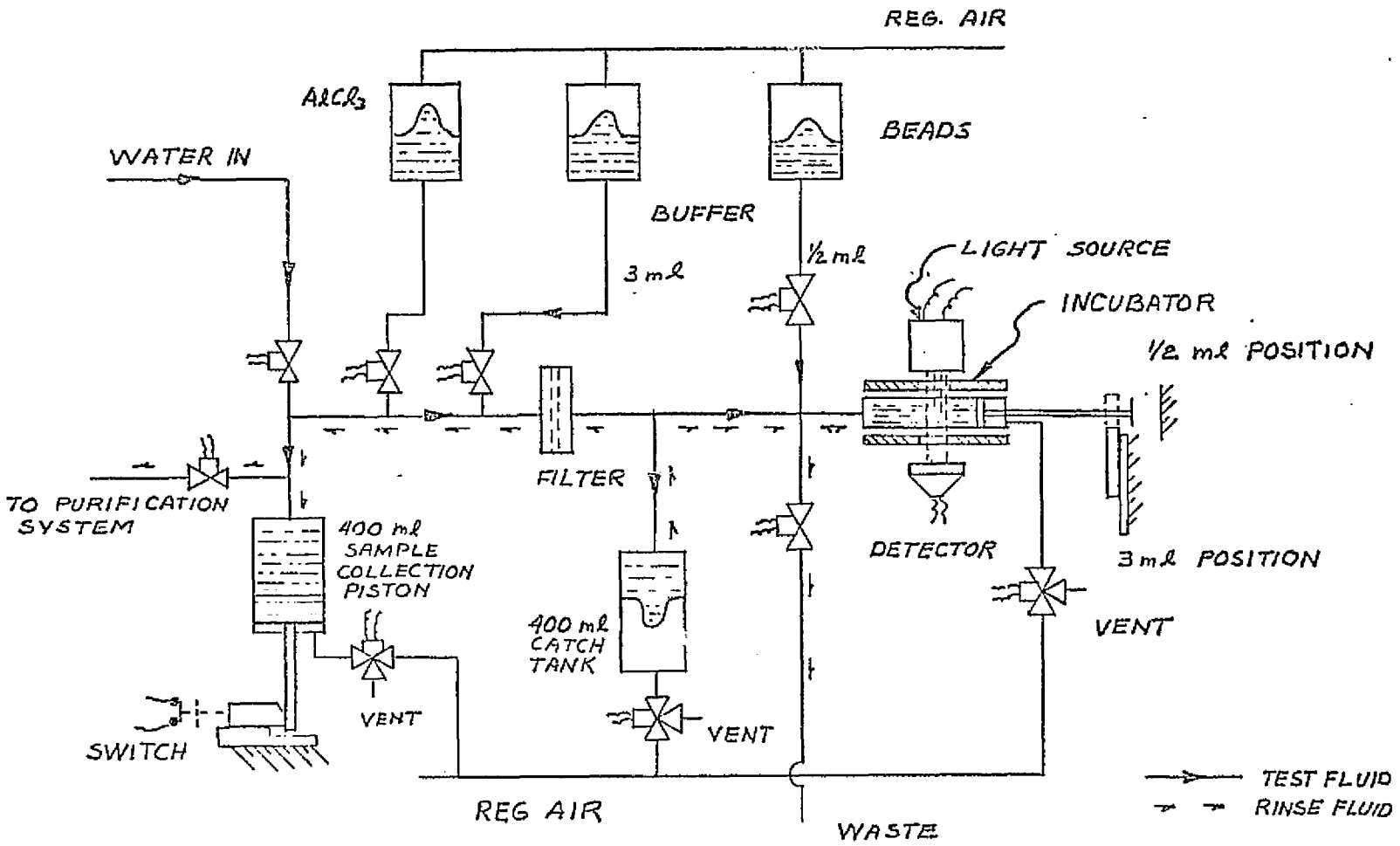

Figure 34 Piston Meter System Virus Water Monitor

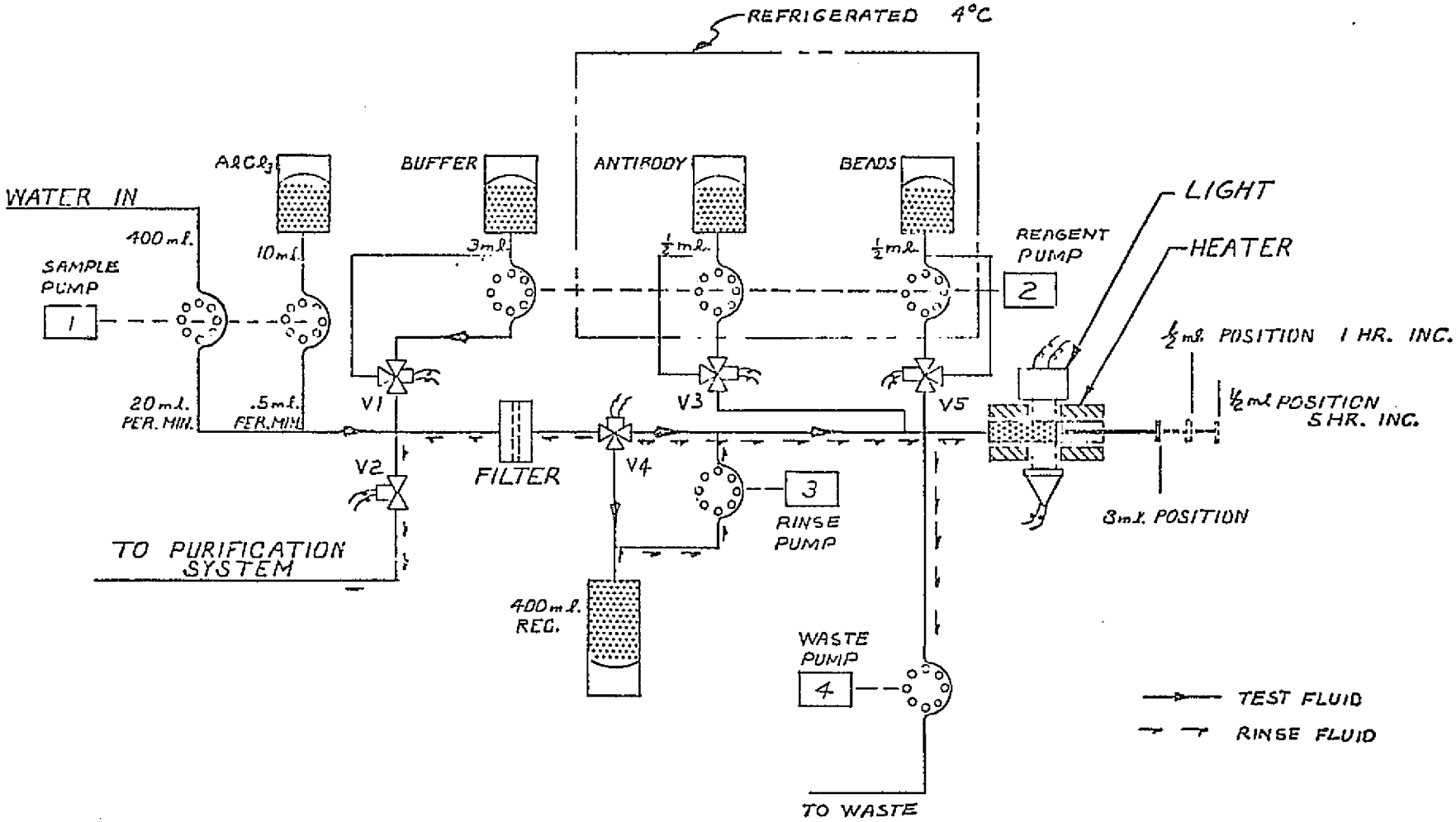

Figure 35 Fluid Flow Schematic Virus Water Monitor

After considerable debate, the peristaltic pump arrangement was chosen as most desirable for the system, primarily to eliminate the requirements for air pressure as a power source in addition to the electrical interface. The difficulty associated with excessive usage on the pump tubing is not considered applicable since the worst case requirement is 20 minutes operation per test. Difficulties associated with tubing collapse, due to insufficient useage, is not considered a problem since a test will likely be performed twice in 24 hours.

11.2.3 Detection System

The detection system utilizes a transmitted light photometer with the amount of light varying directly with the agglutination of particles. With a monodispersed 1 micron bead solution (non-agglutinated), each bead intercepts a portion of the light preventing it from reaching the detector. If the beads are agglutinated, the effective area blocking light is reduced and the amount of transmitted light increases.

The design of the detection system involved the selection of optimum light sources and detectors which could most reliably measure the transmittance of light. Several light sources such as mercury arc, carbon arc, zirconium arc, and tungsten filaments were considered for this application. Since the arc lamps require high voltage, somewhat bulky, power supplies for starting, the tungsten filament bulb presented the simplest installation for this application.

Photomultiplier tubes and photodiodes were compared for application as desirable detectors. Photodiodes do not require a high voltage power supply as the photomultiplier tube does. In addition, PMTs are much bulkier than photodiodes. Since photodiodes have less sensitivity than their counterparts, an analysis was performed to insure adequate light level. The analysis indicated a 10^3 average level light above the noise level of the photodiode utilizing a 6 watt tungsten filament bulb as a light source.

The test cell portion of the detector system must recieve and mix the sample, incubate the sample, and read the reacted liquids by transmitted light while in zero-g with a minimum of cross contamination (holdup volume) between samples. Three reaction cell configuration that were evaluated were

(1) conventional flow thru cell, (2) the glass Bellofram Diaphragm container, (3) and the glass syringe configuration. The flow thru cell is used extensively in earth-bound spectrophotometers but is undesirable for zero-g applications because mixing, incubation, and agglutination would have to be performed in a separate expandable container through which considerable wash liquid would be required to minimize cross talk. The glass diaphragm type expandable container allows space for adequate mixing and agglutination to take place, however, holdup volume is characteristic of diaphragm containers leaving them subject to cross-contamination. A glass syringe provides nearly zero holdup volume as compared to the Bellofram Diaphragm (10% holdup) and Bellows (30% holdup), however, liquid sealing and smooth friction-free operation must be contended with. Gas chromatograph equipment suppliers have developed a syringe piston made of Teflon with an internal O-ring behind a thin section of the piston periphery, which provides a permanent resilient seal. The Teflon/glass interface provides an inert self-lubricated seal while the internal O-ring gives the Teflon permanent resilience at the sealing area.

Figure 36 presents a preliminary design of the optical detector assembly. A 12-watt tungsten filament instrumentation lamp supplies the light and is mounted to an adjustable base to allow positioning of the focal point in the center of the test cell assembly. A convex positive lens gathers and focuses the light at the center of the test cell through to the sample detector face. An interference filter, located downstream from the lens, only passes wavelengths of light from 475-530 mu. A 50/50 beamsplitter splits off 1/2 the available light downstream of the filter for use at the reference detector. The purpose of the reference detector is to maintain a constant light level through a feedback circuit, controlling the lamp power supply. Heaters are located around the test cell to maintain 45^{o}C incubation temperature. The detector assembly is designed to allow the sample detector to be rotated about the axis of the test cell to permit detection by transmitted or scattered light. The lamp housing is designed to permit cooling by convection.

11.2.4 System Arrangement

The system arrangement, as presented in Figure 37, presents a proposed preliminary design of a fully functional laboratory prototype capable of operation in zero-g environment. The system is configured to functionally

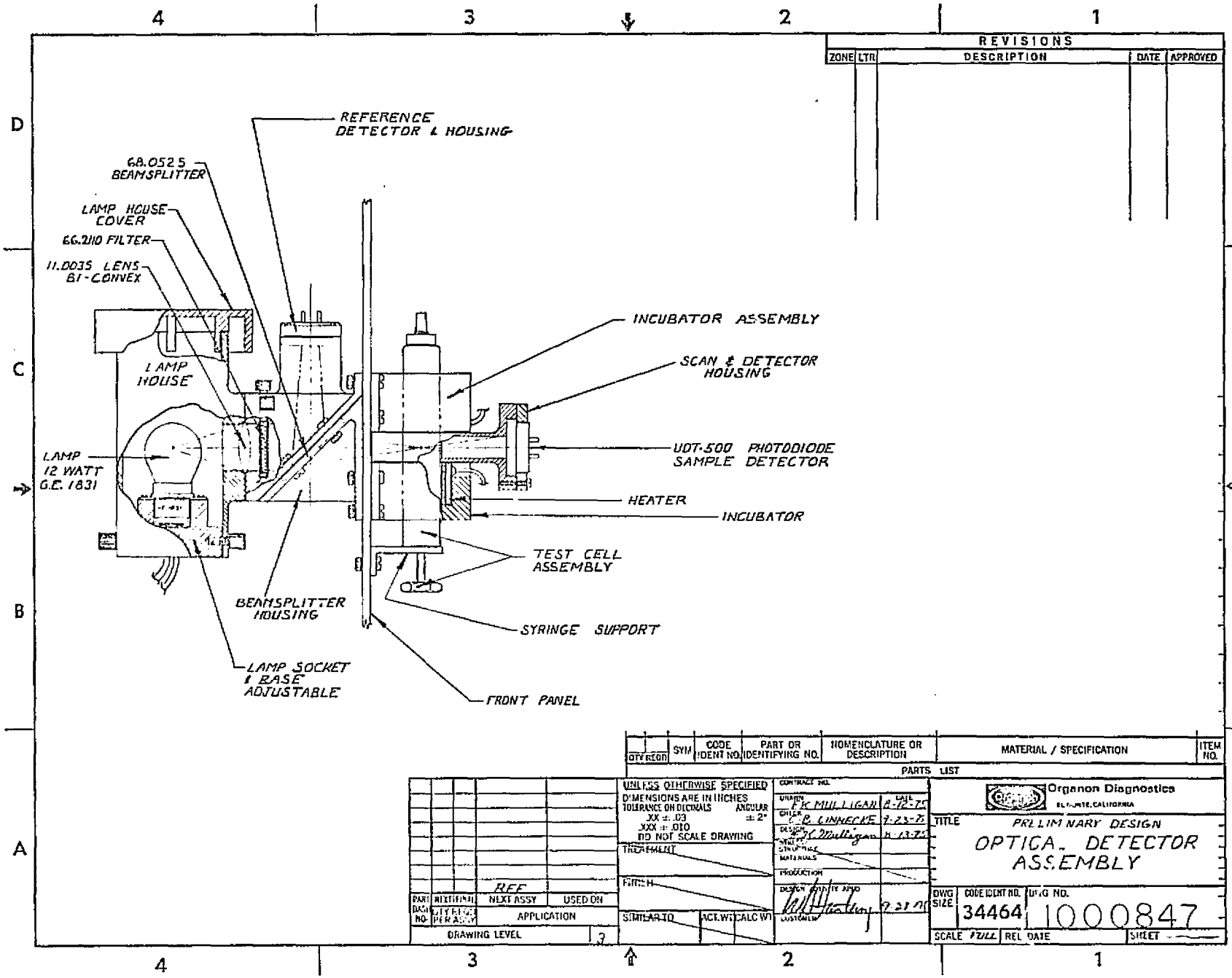

Figure 36 Optical Detector Assembly

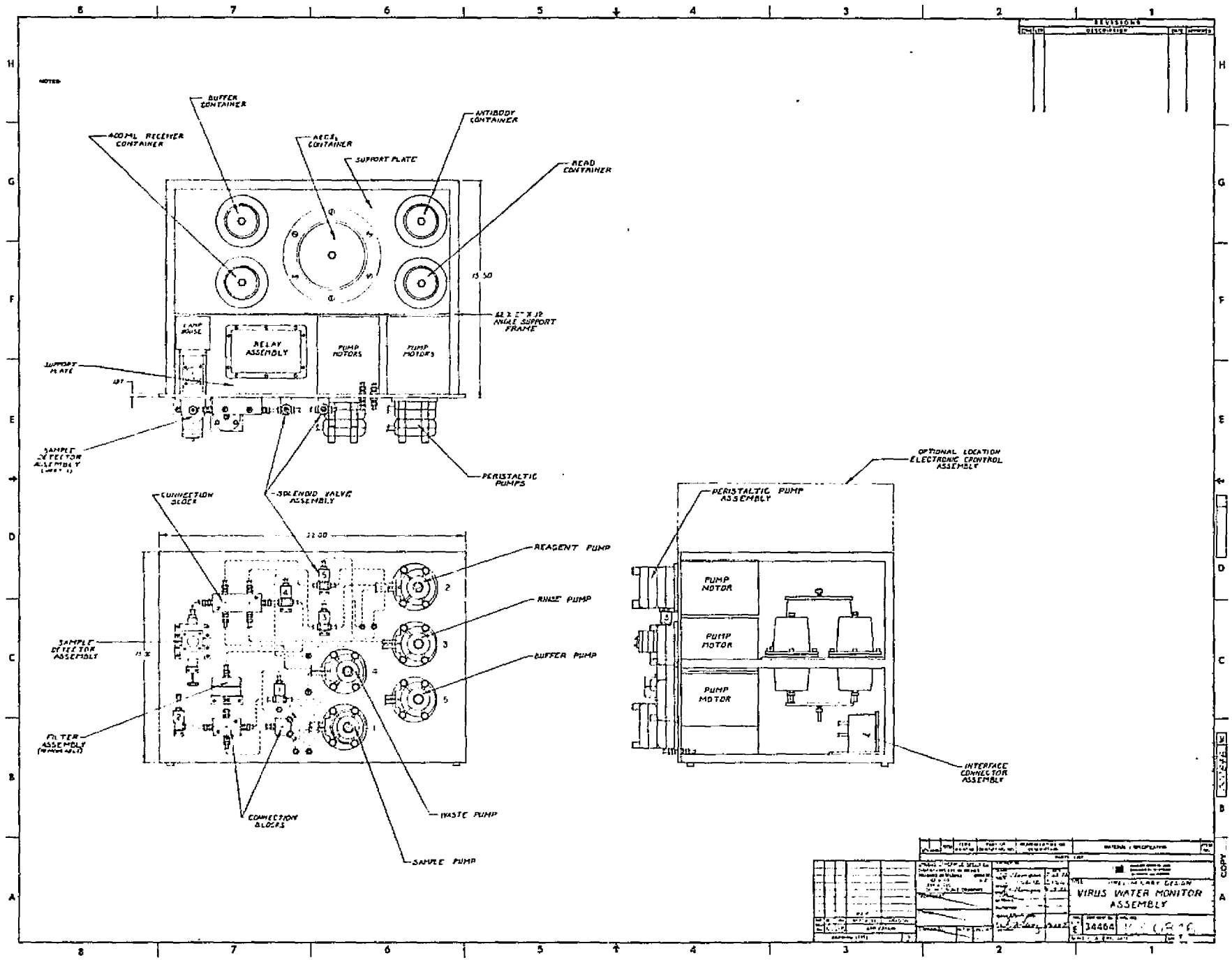

Figure 37 System Arrangement

operate as a flight prototype. It is packaged to present a reasonable estimate of the size and weight of a flight prototype, without sacrificing the accessibility required to all components, during laboratory evaluation. All pumps, solenoid valves, filters and the test cell of detector assembly, will be mounted to a panel totally accessible to the operator. The liquid storage containers, relays and pump motors, are located behind the panel in an open frame-work accessible from the back of the instrument. The electronic readout and control system is in a separate module which may be optionally placed on top or beside the instrument at the convenience of the operator. Mounts for containers and the absorption filter requiring maintenance or replacement will be designed for easy removal. The design of the instrument support structure lends itself to laboratory testing in inverted or on-end orientation for simulation of zero-g.

11.2.5 Readout Electronics and Electrical Control System

As previously mentioned, the electronic control assembly will be located in a separate module, Figure 38, to minimize electrical noise difficulties and permit the instrument operator the flexibility to re-orient the mechanical system without the readout. Instrument controls to automatically start, stop and flush the system are on the front panel. Manual controls will be located in a separate box to permit manual actuation of each function independently with light indicating operation of the function. Readout will consist of a digital panel meter (DPM) and positive and negative lights. The DPM will indicate % change in light transmission while the lights will display the results of the sample. A baseline signal taken at the beginning of the final incubation will be stored and then subtracted from the sample reading taken continually. The difference is compared to a preset threshold. Whereupon exceeding the threshold, the negative light will come on indicating no virus. If after 5 hours, the threshold level has not been exceeded, the positive light will indicate virus is present in the system. A series of lights will indicate which step of the test process is being performed. A digital clock displays elapsed time (minutes) into the incubation processes for operator convenience.

The instrument programming control circuit will consist of digital electronic timing, preset for the operation time of each function or functions to be operated. No limit switches are required for the operation of the instrument

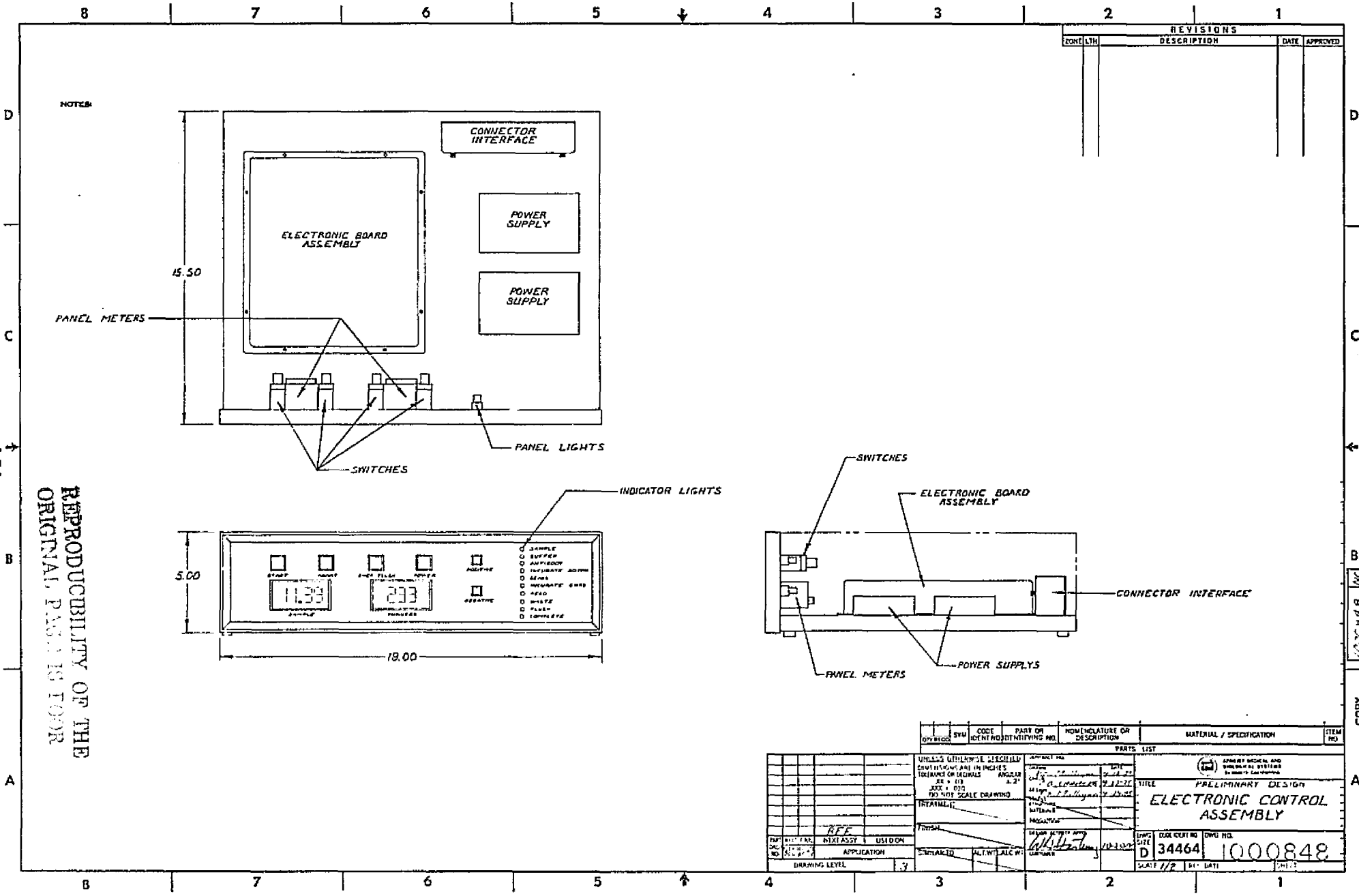

Figure 38 Electronic Control Assembly

but may be added if necessary. The start switch will initiate the sequence of operations. The timing control circuitry will step the system through all functions listed in Table XLI and will hold for restart by an operator. An abort switch will be provided to stop the system at any time. An emergency flush switch will cycle the instrument through the last four steps should the process be stopped partway through the program. The electronics for the programming control circuit and the detector circuit, Figures 39 and 40, can be mounted on wire wrap boards, providing a compact electronic package easily convertible to a spacecraft instrument.

TABLE XLI SEQUENCE OF OPERATIONS OF VIRUS WATER MONITOR

Step		
1.	Run Pump 1 with V4 open, collect sample	20 min.
2.	Run Pump 2 with V1 open, 3 ml buffer	1 min.
3.	Run Pump 2 with V3 open, add antibody	30 sec.
4.	Incubate	1/2 hour
5.	Run Pump 2 with V5 open, add beads	30 sec.
6.	Read and store baseline	--
7.	Incubate	5 hours
8.	Read and subtract baseline and compare to threshold	
9.	Turn on green light (negative) or red light (positive)	
10.	Run Pump 4, pump waste	30 sec.
11.	Run Pump 3, flush	12 sec.
12.	Run Pump 3 with V2 open, flush	20 min.
13.	Run Pump 4, waste	30 sec.
14.	Hold for reset by operator.	

NOTES:
1. Heater and light source are on during full operation of instrument.
2. Electronics requiring warm-up may need to be on.

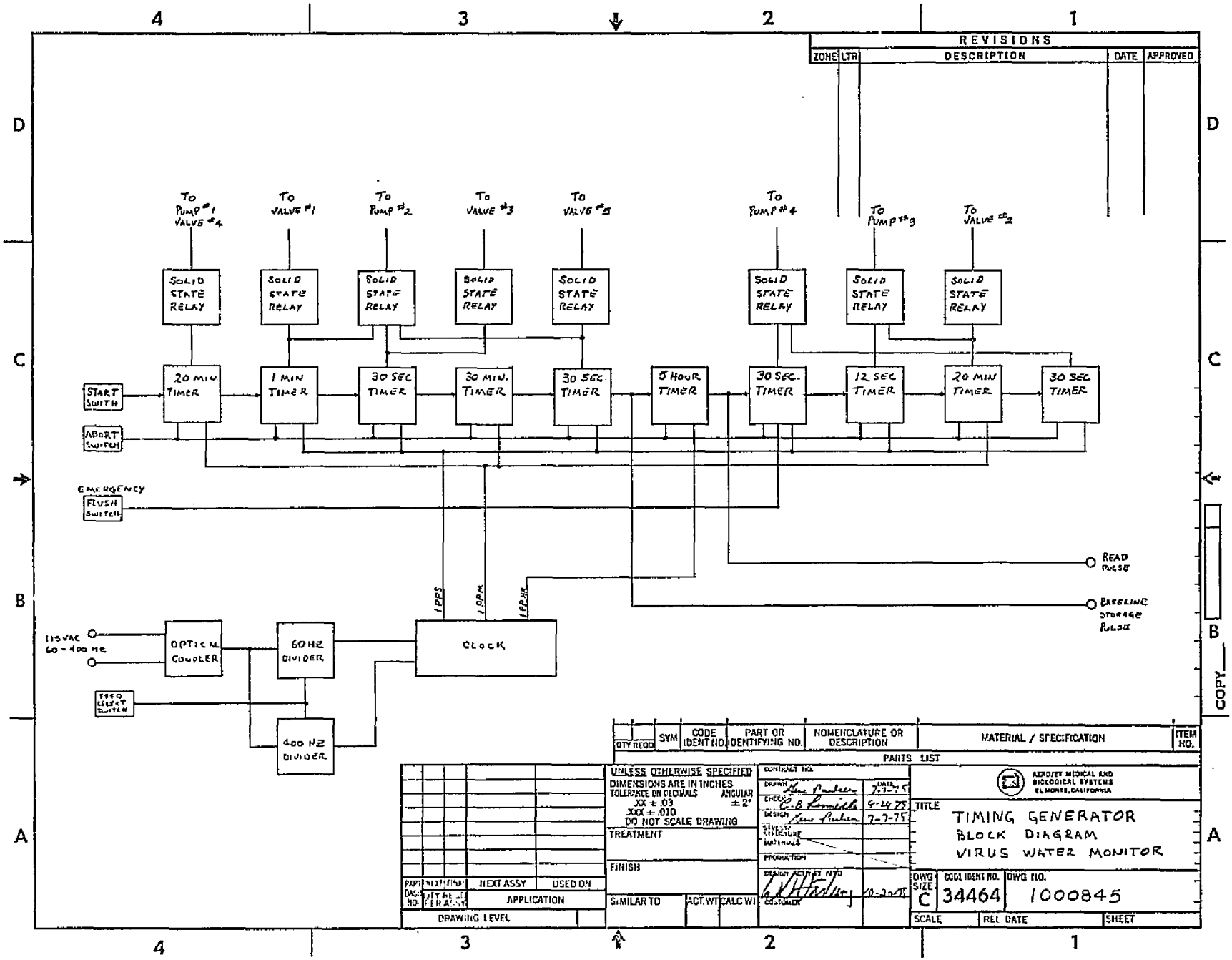

Figure 39 Programming Control Schematic

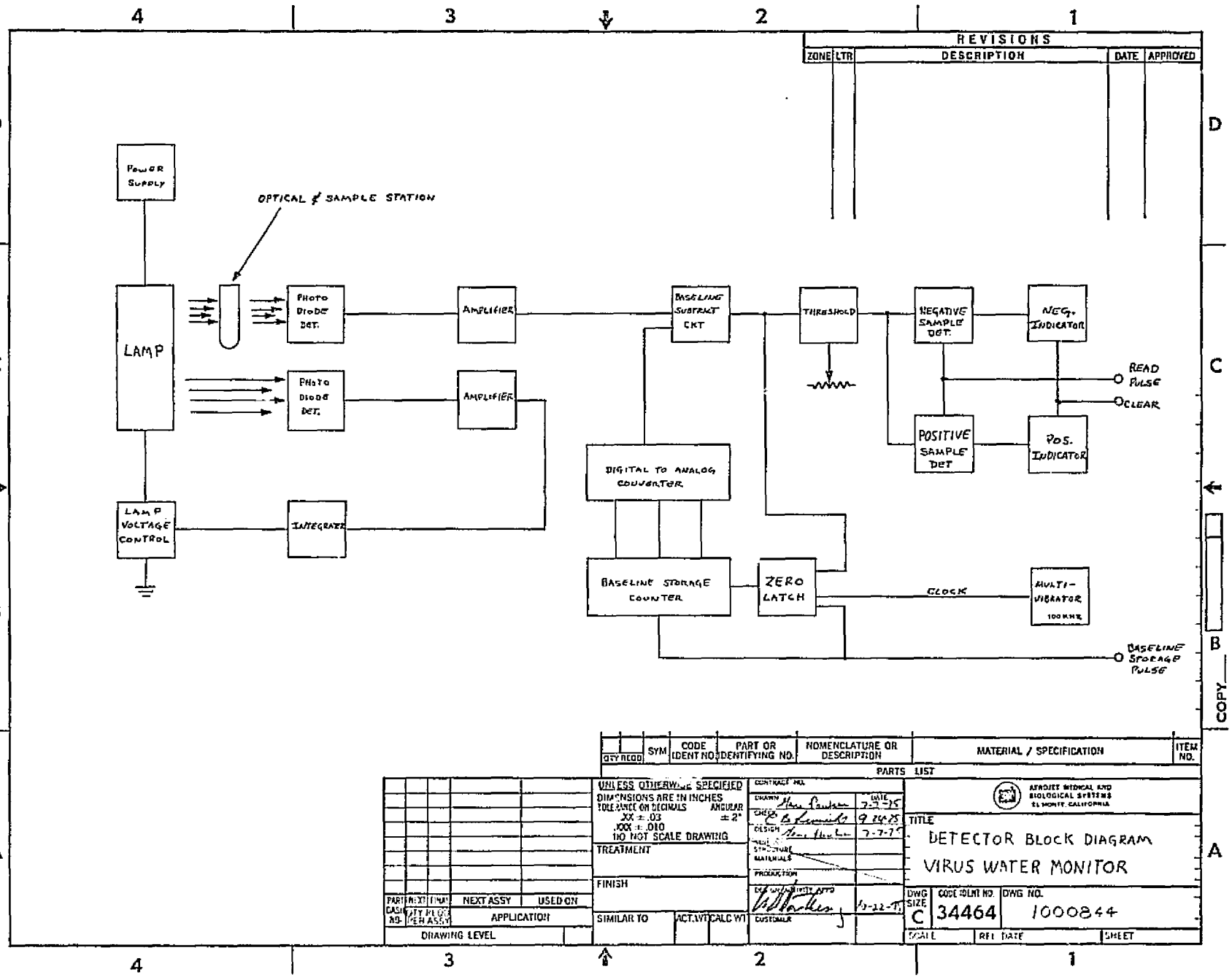

Figure 40 Detector Schematic

12.0 CONCLUSIONS AND RECOMMENDATIONS

A subsystem concept for the viral monitoring of reclaimed water has been developed. The system is based on the introduction of a marker virus upstream and monitoring for its presence downstream.

The development of the monitoring subsystem involved a concentration step and a detection step. The selected concentration step was based on marker virus adsorption and subsequent desorption from cellulose acetate filters. This system has been shown to work quickly ($\approx$ 20 min. to concentrate 400 ml into 3 ml) and efficiently ($\approx$ 100% virus recovery). The detection system was based on an indirect passive immune agglutination technique which, as developed, has the ability to detect marker virus at a concentration of 1×10^9 PFU/ml. The results of laboratory end-to-end testing have shown that, under the performance guidelines established for automation, the viral monitoring system has proven to work quickly and efficiently with the simplicity of the system lending itself well to automation.

When testing this subsystem with NASA-supplied reclaimed water samples obtained by Reverse Osmosis (RO), the nature of the sample did not interfere with concentrating the marker virus using filter adsorption, nor with the subsequent PIA. There was, however, interference in the PIA detection system with samples obtained from the Clemson University RO reclamation system. The problem appeared to be the concentration of detergent along with the marker virus when carrying out marker virus concentration using the filter adsorption technique. The detergent present in the concentrate, with the exception of one sample, apparently prevented the beads from agglutinating. The presence of detergent in the concentrate, however, did not appear to interfere with the marker virus concentration since marker virus, in the sample concentrate, was detected utilizing the ELA approach.

The requirement of the viral monitoring subsystem to detect up to a 99.99% drop in marker virus concentration (established by NASA), in the starting water, can be met by the present system. This would require an initial introduction of marker virus at a 10^{15} PFU level. Less initial

marker virus input could be used if a more sensitive detection system were employed. An enzyme-labeled antibody (ELA) system, investigated on this program, has proven to be 10-12 times more sensitive than the PIA detection system and does not appear to be subject to the interference observed with the bead agglutination test. With this detection system an initial input marker virus concentration at a 10^{14} PFU level could be used.

An engineering preliminary design has been performed as a parallel effort to the laboratory development of the marker virus test subsystem. Engineering schematics and drawings present a preliminary instrument design of a fully functional laboratory prototype capable of Zero-G operation. The instrument consists of reagent pump /metering system, reagent storage containers, a filter concentrator, an incubation/ detector system, and an electronic readout and control system.

The reagent pump /metering system utilizes peristaltic pumps sequenced at the appropriate times for the movement of reagents. Collapsible, flexible diaphragm-type containers are utilized for reagent storage. The detector/incubation system utilizes a syringe developed for gas chromatographs as a reaction and detector cell. The detection is by a transmitted light photometer with amount of light varying directly with the agglutination of particles. A heater blanket will maintain the reaction cell at the desired incubation temperature.

The instrument is packaged in two modules isolating the electronic control and readout from the reagent storage and pumping system. The instrument package has been designed to present a reasonable estimate of the size and weight of a flight prototype with the capability of laboratory testing in inverted or on-end orientation for simulation of Zero-G.

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