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COLLABORATIVE NATIONAL STUDY USING MOLECULAR TECHNIQUES TO DETECT HEPATITIS A VIRUS AND VIRULENCE FACTOR GENES IN E. COLI: HAWAII MATCHING STUDY

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WATER RESOURCES RESEARCH CENTER
UNIVERSITY OF HAWAI'I AT MANOA
Honolulu, Hawai'i 96822

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Department of Wastewater Management
City and County of Honolulu
650 South King Street
Honolulu, Hawaii 96813
and
National Water Research Institute
10500 Ellis Avenue
P.O. Box 20865
Fountain Valley, CA 92728

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Principal Investigator: Roger S. Fujioka
Water Resources Research Center
University of Hawaii
2540 Dole Street
Honolulu, Hawaii 96822

CHAPTER ONE: PROJECT DESCRIPTION

I. Development of Collaborative Study

Effluents from wastewater, agricultural, animal, and industrial facilities are commonly discharged into environmental waters (streams, lakes, estuaries, harbors, ocean). Since these discharges are a potential source for a variety of water-borne pathogens, there is a public health risk if recreational waters are contaminated by these discharges. To address this public health risk, USEPA has developed recreational water quality standards based on culturable concentrations of fecal indicator bacteria (fecal coliform, *E. coli*, enterococci). Although the use of these microbial water quality standards have been useful, these standards are not always reliable because the fecal bacterial indicators die off much more rapidly in environmental waters, especially marine waters, than many fecal borne pathogens (human enteric viruses, enteric protozoans). As a result, the absence of fecal bacteria in environmental waters may not accurately predict the absence of sewage-borne pathogens.

In Hawaii, the USEPA recreational water quality standards have been shown to be inappropriate because the same fecal indicators used to establish water quality standards are multiplying in the soil environment of Hawaii and is the source of the naturally high concentrations of these fecal bacteria in all streams, storm drains and land run-off. In Hawaii, these naturally occurring, environmental sources of fecal bacteria are not indicative of sewage contamination because the sources of these bacteria are not from sewage or any other effluent discharge. Moreover, many sewage-borne pathogens (human enteric viruses, enteric protozoans) cannot multiply under environmental conditions. As a result, in Hawaii, the presence of fecal bacteria in environmental waters may not accurately predict the presence of sewage-borne pathogens.

Based on the limitation of using fecal bacteria indicators, there is a strong recognition that environmental waters should be monitored for the presence of pathogens. However, all attempts to monitor environmental waters for sewage-borne pathogens using traditional, culturable methods have been determined to be unfeasible. Thus, there is a need to develop newer methods to assess the sanitary quality of environmental waters based on detection of sewage-borne pathogens. Recent developments of molecular genetic methods indicate that these newer methods can overcome many of the limitations of culturable methods. Recognizing the potential of these new molecular genetic methods, Dr. Carol Palmer of the County Sanitation Districts of Orange County (CSDOC) , Dr. Mark Sobsey of the University of North Carolina and Dr. Roger Fujioka of the University of Hawaii collaborated in writing a joint proposal to the National Water Research Institute (NWRI). The goal of this joint study was to apply newer molecular genetic methods to assess coastal water quality in the United States by conducting comparable studies at coastal sites representing the east coast (North Carolina), the west coast (California) and the tropical pacific island coasts (Hawaii). NWRI approved funding for this two-year collaborative study in June of 1992. One of the requirements of NWRI funding was to secure matching services and funds from the grantees county or state organization. In Hawaii, the Water Resources

Research Center of the University of Hawaii was the agency to conduct the research. The agency most likely to benefit from this study and the agency which agreed to provide matching services and funds was the Department of Wastewater Management, City and County of Honolulu (CCH).

It should be noted that the two-year, collaborative study funded by NWRI was closely managed by the NWRI Research Board which required timely written and oral presentation reports by project personnel to evaluate the effectiveness of each funded project. Another requirement was the timely submission of the final report to NWRI upon completion of the joint collaborative study contract date of December 31, 1994. To meet this requirement, principal investigators from California, North Carolina and Hawaii submitted their final project report entitled, "Collaborative National Study Using Molecular Techniques to Detect Hepatitis A Virus and Virulence Factor Genes in E. coli" (NWRI Grant HR-92-06) to NWRI on February 28, 1995. Copies of this final report were sent to all cooperative agencies including the Department of Wastewater Management, City and County of Honolulu. This final report summarized all of the comparative data obtained at the three coastal areas of the United States. The summarized conclusion of this report is included as Appendix A.

II. Goals and Delay in Completing The Matching Study to City & County of Honolulu

As mentioned earlier, the CCH agreed to provide yearly contracts to WRRC to match the two years of funding by NWRI. The specific goals of the contract with the CCH are as follows: a) Develop and apply molecular genetic methods to assess the quality of environmental waters in Hawaii. b) Train personnel at the City and County of Honolulu Sand Island Water Quality Laboratory in the use of molecular methods. c) Assist in setting-up and establishing a functioning molecular microbiology laboratory at the CCH Water Quality Laboratory. d) Make recommendations to the CCH on future use of molecular methods.

Although the CCH contract with WRRC was planned as a matching study to the NWRI grant, two factors contributed to the delay in completing the matching study for the CCH. First, the CCH contract started approximately one year after the starting date of the NWRI study. Second, a major objective of the CCH contract with WRRC was the training of the laboratory personnel at the CCH Sand Island Laboratory in the use of molecular methods and the establishment of that capability at the CCH laboratory. Completion of this last objective was delayed because of personnel changes at the CCH laboratory. In summary, the first CCH laboratory technician trained by WRRC left the services of the CCH. After a delay, the second CCH laboratory technician trained by WRRC took a series of leaves from work and was then re-assigned to other duties upon return to duty status. This was followed by an approximate one year period, when the CCH laboratory was understaffed and did not have anyone available for WRRC to train. It was not until Spring of 1997 that CCH was able to provide laboratory personnel to be trained in molecular methods. As a result, the CCH contract with WRRC underwent a series of no-cost extensions through June of 1997.

III. Training CCH Laboratory Personnel

Training CCH laboratory personnel in the use of molecular methods, assisting in furnishing that laboratory with all the necessary equipment and reagents and establishing that laboratory's capability to independently analyze water using molecular methods were major objectives of the study funded by CCH.

In Hawaii, Bruce Roll, a Ph. D. candidate at the University of Hawaii working under the direction of Roger Fujioka was selected as the research microbiologist to conduct the experiments in Hawaii and to train the CCH laboratory personnel. Since this was a collaborative study, Bruce traveled to CSDOC and to the University of North Carolina to learn the methods used in these two cooperative laboratories and to learn about water quality problems in these two states. Bruce provided numerous individual training sessions for the laboratory personnel in the CCH laboratory. However, Bruce was also involved in the following two formal training workshops developed specifically to train CCH personnel and to ensure that all methods used in Hawaii were similar to the methods used at the CSDOC laboratory:

1. August 31-September 15, 1992, University of Hawaii: Genetic Analysis: DNA Methods Workshop. Dr. Carol Palmer and Dr. Yu Li Tsai of CSDOC traveled to Hawaii to conduct this training workshop to ensure that the same molecular methods used in CSDOC were being used in Hawaii. Another purpose of this trip was to enable CSDOC scientist to learn about the water problems in Hawaii and to compare the environmental conditions in Hawaii with those in Southern California. Laboratory personnel from the University of Hawaii, the City and County of Honolulu and the State Department of Health received training during this workshop which covered the basic principles of molecular genetic methods, the reagents required, the molecular reactions and the means to measure the products of the molecular reactions. This workshop was comprehensive and covered molecular methods using gene hybridization and gene amplification technology. The excellent handouts used in this workshop are included as Appendix B.

2. August 1-7, 1993. University of Hawaii: Environmental Virus Detection Workshop. Dr. Carol Palmer, Dr. Yu Li Tsai and Fred Bonilla of CSDOC traveled to Hawaii for two purposes. First to review and compare the first year's data collected by the three collaborating laboratories and to discuss plans to complete the project. Second, to conduct a training workshop specifically on the use of Membrex Vortex Flow Filtration Concentrator and the RT-PCR technique which is required to measure for human enteric viruses and Hepatitis A virus whose genes are only RNA rather than the typical DNA genes. The same set of people who were trained in the first workshop was trained in this second workshop. The handouts of for this second workshop are included as Appendix C.

Bruce Roll successfully trained Lucy Abe, the first CCH laboratory personnel designated for this training. However, Lucy unexpectedly left the employment of the CCH. After a period of delay, Janet (Kim) Shin was designated as the new CCH laboratory personnel to be trained in molecular methods. Although good progress was being made in this training, Janet took a series of personal leaves and the training was not completed. Upon returning to full employment status,

Janet was assigned other duties at CCH and Bruce was not able to complete the training. This was followed by a period of about one year when no one from the CCH laboratory was available to be trained. Meanwhile, Bruce completed all the experiments planned in the NWRI contract and the final report for this contract was completed in February of 1995. Bruce earned his Ph.D. degree in May of 1995 and left Hawaii to take a position with the Portland, Maine Water District.

In 1996, Fred Bonilla who had worked in Dr. Palmer's laboratory at CSDOC and had traveled to Hawaii during the second workshop in 1993, matriculated at the University of Hawaii to obtain a masters degree in microbiology under the supervision of Dr. Roger Fujioka. Since, Fred was already trained in molecular methods at CSDOC and was completely familiar with the project in Hawaii, he was given the assignment to complete the training of CCH laboratory personnel.

In the Spring of 1997, the CCH was finally able to designate laboratory personnel to be trained by WRRC. As a result, WRRC re-initiated its program to train CCH personnel in molecular methods and to establish that capability at the CCH laboratory. This WRRC training activity included the development of a molecular method workshop entitled, "Ensuring the Quality and Safety of Hawaiian Water" and was held at the new Pearl City DOH laboratory from January 7-10, 1997. This workshop was co-sponsored by Centers for Disease Control and Prevention, Hawaii State Department of Health, Hawaii Water Environment Association, Hawaii American Water Works Association and the Water Resources Research Center. This workshop was a good introduction for the newly assigned CCH laboratory personnel who were designated to be trained in the molecular methods. Upon completion of this workshop, Fred Bonilla worked closely with the CCH laboratory to individually train laboratory personnel at the CCH and to establish the capability of this new technology at the CCH laboratory. These individual training sessions which took place at the University of Hawaii and then at the CCH laboratory continued through September of 1997, well beyond the project termination date of June 31, 1997. As a result of these training sessions, Allen Kainuma, Robert Chinn, Samuel Kon and Remi Gose have been trained in the use of molecular methods. Most significantly, the capability to use molecular genetic methods to detect for the presence of microorganisms in water and in sewage has now been established at the CCH Sand Island Laboratory.

CHAPTER TWO: MOLECULAR METHODS USED

I. Introduction

The methods described in this chapter are experimental methods which are not included in "Standard Methods for the Examination of Water and Wastewater" and therefore are not methods which are approved for routine monitoring of environmental waters. Moreover, it only represents the methods used in this study. There are many other methods and many variations of any method. However, the methods described are feasible, reliable and recommended when environmental waters are to be analyzed by molecular genetic methods.

II. Basis of Molecular Genetic Methods

Use of molecular genetic methods to measure for the presence of different types of microorganisms is now at the cutting edge of science. Molecular genetic methods have exploited the unique sequences of nucleic acid components (nucleotides) which make up the genome of every living cell and the specificity with which these nucleotide sequences hybridize to complementary sequences of nucleotides. Based on this biological principle, many molecular genetic methods such as DNA:DNA hybridization, DNA:RNA hybridization, DNA fingerprinting, DNA and RNA sequencing, and the more recent gene amplification method called polymerase chain reaction (PCR) have been developed. By analyzing for unique genes, every microorganism can potentially be identified by these molecular genetic methods. Since the polymerase chain reaction (PCR) method is the most sensitive and feasible molecular genetic method to analyze environmental waters for the presence of specific microorganisms, this was the gene probe method most extensively used in this study. However, there are several necessary steps before a water sample can be analyzed using molecular methods and each of these individual steps play an important role in how well the final molecular detection method performs. This chapter describes the choronological steps to analyze a water sample using a gene probe (PCR) method.

III. STEP ONE: Concentration of Viruses and Bacteria From Environmental Waters.

The first problem in the detection of microorganisms, especially pathogens, in any environmental water sample, is the expected variability and low concentration of the target microorganism in environmental water. This can be addressed by taking larger volumes of water sample to give greater assurance that your water sample will contain the target microorganism. However, by sampling larger volumes of water, there is a need to reduce the volume of water while retaining the target microorganism so the sample can be analyzed by culture or by molecular methods. It should be noted here that samples to be cultured is generally limited to 1-10 ml although up to 100 ml samples can be assayed using some methods. For molecular methods, the

sample to be assayed is limited to very small volumes generally in 1-100 microliter volumes where 1 ul equals 0.001 ml. Thus, there is a need to use a method which can effectively reduce the volume of water in the sample and at the same time to efficiently retain (concentrate) all the target microorganism in a smaller volume of water, without damaging that target microorganism.

1. Method One: the Membrex Vortex Flow Filtration Concentrator.

A decision was made to use a new technology called Vortex Flow Filtration (VFF) to concentrate low concentrations of pathogens such as viruses in large volumes of water because the precision and efficiency of all other existing methods were unsatisfactory. As a result a Membrex VFF Concentrator was purchased since this concentrator had been reported in research journals to be effective in concentrating microorganisms in environmental samples. This technology has several advantages. First, it uses a re-usable membrane and continuous vortex flow which ensures maximum but gentle filtration of water through a small defined area of a specialized membrane resulting in a liquid retentate sample in which the physical shape and the biological activity (infectivity) of microorganisms are maintained. Second by selecting the membrane (100,000 molecular weight MW cut-off) it can retain and concentrate all microorganisms (bacteria, viruses, protozoa) to a volume of approximately 100 ml while allowing other smaller micromolecules (salts, proteins, heavy metals) which may be inhibitory to future steps in the process to pass through the membrane. Third, it can reduce large volumes (15-50 liters) of sewage or ocean water in a timely manner to a manageable 50-100 ml sample without the addition of any chemicals and without forcing the microorganisms to be adsorbed or compacted onto the membrane. Since the final concentrate is now reduced to 100 ml, this sample can be easily treated using many other techniques to further concentrate or purify the sample in preparation for analysis by either culturable or molecular methods. In this regard, the detailed procedure for the use of Membrex Vortex Flow Filtration Concentrator are included in Appendix C and D.

2. Method Two: Two Phase Method to Concentrate Viruses From Sewage.

Some samples such as sewage can be expected to contain relatively high concentrations of viruses and therefore large volumes of samples need not be processed. For these samples, simple methods which do not require special equipment can be used. One such method is the two-phase polymer separation method which can easily process up to one gallon samples with minimal time and effort by the laboratory analyst. In this method, polyethylene glycol (PEG) and sodium dextran sulfate are simply dissolved into one liter or one gallon of sewage and the entire content added to a separatory funnel. The funnel is kept in the cold room overnight to allow the separation of the top aqueous phase from the smaller bottom sodium dextran phase. During this phasing, viruses in the sample will migrate from the aqueous, top-phase to the bottom sodium dextran-phase, which can be easily collected by opening the stop cock located at the bottom of the funnel. Salts are then added to this sodium dextran sample which precipitates the sodium dextran and causes water and viruses to remain in the supernatant. The supernatant is usually dialyzed to remove excess salts and assayed for virus. The protocol describing the detailed steps of this method is outlined in Appendix E.

IV. STEP TWO: Extraction and Purification of Genetic Material from Sample.

Having concentrated the target microorganisms from a large volume of water to a smaller workable volume of sample, the next step is to extract the nucleic acid from the microorganisms and to purify this extracted genetic material so it can be analyzed by a molecular genetic method. This nucleic acid extraction method must be effective and gentle to recover enough nucleic acid in its native state. Moreover, this step can effectively remove many substances in the sample concentrate which can interfere with assays by culturable or molecular methods. Separate methods may be required to extract DNA and RNA. Protocols describing detailed steps to extract DNA and to extract RNA are outlined in Appendix F.

V. STEP THREE: Gene Probe Assay using the Polymerase Chain Reaction.

The polymerase chain reaction (PCR) method is the most feasible gene probe method to detect and identify the different microorganisms because it relies on finding a short but unique sequence of nucleotides and rapidly amplifying that unique sequence using a high temperature replicating enzyme (taq polymerase) and a thermal cycler equipment which can accurately raise and lower temperature. However, in order for target nucleotide sequences to be amplified by the polymerase chain reaction, a number of parameters must be optimized to generate PCR products which are specific for the target sequence. It should be noted that each PCR reaction must be optimized by varying the concentration of magnesium and PCR primers because these two parameters control the specificity and sensitivity of the primers to bind to the template DNA and to initiate the amplification of the target genes.

In all PCR reactions, positive and negative controls must be included with every set of samples. The positive control should be a pure culture of the target microorganisms. The negative control should include a negative reagent control which contain all the reagents used to resuspend the extracted DNA and a negative sample control in which a sterile water sample undergoes the same treatment as the test sample.

The key to PCR reaction is selection of the primers, which are the unique sequences of nucleotides specific to that microorganism. In most applied use of PCR, the primers for a given microorganism have already been identified and evaluated. However, one can find and select specific primers for most microorganism by consulting computer software programs which are designed to select the PCR primers for a specific microorganism. In this study, the computer software Oligo was used to search GeneBank for candidate sequences and subsequent primer selection. Once primer sequences were selected, these primers can be ordered and produced. The Molecular Biology Instrumentation and Training Facility at the University of Hawaii can produce the primers required.

Protocols which describe detailed steps for PCR methods to detect bacterial DNA genes and to detect viral RNA genes (enteric virus, hepatitis A virus) are outlined in Appendix C and G.

VI. STEP FOUR: Detection of Verification of PCR Products

After the PCR reaction, there is a need to determine whether the targeted nucleotide sequence was present in the sample and was amplified. Since the targeted gene has a known molecular weight, the easiest way to detect for this product is to add the PCR reaction sample to an agarose gel and to observe the migration of the products through the gel by electrophoresis. This method separates out DNA sequences based on their molecular weight. Visualization of PCR products on agarose gels is often aided by staining the DNA products on the gel with ethidium bromide. Under ideal conditions, all the products of the PCR reaction will have the same predicted molecular weight and will show up as a single band on the gel where that molecular weight DNA is expected. Use of known lengths of DNA called DNA ladders which band at proper places on the gel is used as comparative markers. Based on these results, it is safe to assume that the gene sequence targeted by the PCR reaction was present and was correctly amplified. Although this is strong presumptive evidence that the targeted genes has been synthesized, additional test such as Southern Hybridization should be conducted to verify that the product of the PCR actually contains the expected sequences of nucleotides.

Protocols which describe the detail steps in methods to detect PCR products are lined in Appendix C and H.

CHAPTER THREE: APPLICATION OF PCR TECHNOLOGY TO ADDRESS A WATER QUALITY PROBLEM IN HAWAII

Development of a PCR Detection System for the "*Bacteroides fragilis* Group"

I. Introduction to and Identification of a Water Quality Problem in Hawaii

Currently, recreational water quality standards are based on concentrations of fecal indicator bacteria (fecal coliform, *Escherichia coli*, enterococci). These bacteria are normally present in high concentrations of feces of human and other warm-blooded animals. Thus, their presence at concentrations exceeding recreational water quality standard is used to indicate that the water is contaminated with feces or sewage and that the risk for sewage-borne diseases for people who may be using that water for primary contact (swimming) is unacceptably high (Cabelli, 1983). In the application of recreational water quality standards, the basic assumption is made by USEPA and all health authorities that feces from human and animals are the only significant sources of these fecal indicator bacteria. However, it has been well established in Hawaii, Guam and Puerto Rico that these same fecal indicator bacteria are naturally present in the environment in the absence of fecal contamination (Carrillo et al.,1985; Lopez-Torres et al., 1987; Valdes-Collazo et al., 1987, Hardina and Fujioka (1991), Fujioka et al. (1988). In Hawaii and Guam, strong evidence have been obtained to show that these fecal indicator bacteria are multiplying in the soil environment and is the environmental source of these bacteria responsible for the persistently high concentrations of these fecal indicators in all of the streams, storm drains and other land-based run-off which enters coastal waters. Since, pathogens such as human enteric viruses and protozoans such as Giardia cannot multiply under environmental conditions, the increased concentrations of fecal indicator in environmental waters in tropical islands do not necessarily indicate that the water may be contaminated with sewage-borne pathogens.

In Hawaii, regulations require that environmental waters be monitored for fecal indicator bacteria. However, when fecal indicator bacteria are recovered from coastal waters one is not sure whether the source of these fecal bacteria is from the environment (soil) or from sewage. In ocean disposal of sewage, the fecal indicator bacteria die off so rapidly that these fecal indicator bacteria are not reliable markers of sewage. Thus, there is a need for another test which is reliable, fast and sensitive to determine whether environmental waters are or are not contaminated with sewage.

II. *Bacteroides*: A Group of Fecal Bacteria Suitable for PCR Technology

A genus of fecal bacteria which is present in higher concentrations in feces than fecal indicator bacteria is *Bacteroides*. Bacteria in this genus are nonsporulating, obligate anaerobic gram-negative bacteria which are present in the human intestinal tract at concentrations 100 to 1000 fold higher than *E. coli* (Oliveri, 1980). Four species of *Bacteroides* are dominant in the human intestinal tract, *B. vulgatus, B. thetaiotaomicro, B. fragilis*, and *B. distasonis*. Collectively these four species are referred to as the "*Bacteroides fragilis* group" (BFG) and are excellent

indicators of human fecal pollution since their habitat is restricted to the feces of humans and some warm-blooded animals (Allsop and Strikler, 1985). Moreover, *Bacteroides* spp. are found in higher numbers in human than a variety of warm blooded animals making this group more human specfic (Allsop and Strickler, 1985.). Although *Bacteroides* spp. have been cultured from some water samples (Allsop and Stickler 1984), this method is not reliable because BFG are strict anaerobes and dies when exposed to the aerobic conditions of environmental waters (Fikdal et al. 1985).

In summary, *Bacteroides* spp. represent a more specific marker of feces than any other fecal indicator. Thus, its presence in environmental water can be used as direct evidence that the water is contaminated with sewage. Although *Bacteroides* spp cannot be monitored for by culture methods, molecular genetic methods such as the Polymerase Chain Reaction (PCR) method can be used to detect for the presence of this group of bacteria in water samples.

III. Objective and Experimental Design of This Study

The goal of this study was to apply PCR technology to address a problem which cannot be solved using standard cultural method. The objective of this study was to develop a PCR test for "*Bacteroides fragilis* group" and to evaluate this test as a means to determine whether environmental water is contaminated with human feces or sewage.

The experimental design of this study was to measure sewage and sewage contaminated water for culturable concentrations of *E. coli* and to measure the same water samples for presence of *E. coli* and for *Bacteroides* spp. by PCR methods. Using this approach, the effectiveness of measuring environmental water samples for *Bacteroides* spp. can be evaluated.

IV. Material and Methods

A. Bacterial Strains and Culture Media. Bacterial isolates representing the "*Bacteroides fragilis* group" (*B. fragilis, B. vulgatus, B. thetaiotaomicron* and *B. distasonis*) and a variety of other Gram- negative and Gram-positive isolates were obtained from the American Type Culture Collection, Rockville, Md. *Bacteroides* spp. were grown in reinforced clostridial medium (Oxoid), under anaerobic conditions (90% N, 10% CO2) at 37°C. Non-Bacteroides species were grown in brain heart infusion broth (Difco Laboratories, Detroit, Michigan) at 37°C for 24 hrs. For survival experiments *B. thetaiotaomicron* was grown in reinforced clostridial medium to late log phase growth. Viable *B. thetaiotaomicron* was isolated using membrane filtration and WCGP agar as described by Allsop and Stickler (1984). *Escherichia coli* was isolated from human feces using mTEC agar and characteristic colonies were confirmed by API20 E strips. Human derived *E. coli* was grown in brain heart infusion broth until late log phase. For sewage and field samples, *E.coli* was detected by membrane filtration using mTEC agar in accordance with *Standard Methods* (1992).

B. Sample Locations and Preparation. A variety of environmental waters were sampled (both marine and fresh waters). Manoa and Makiki Streams, are located on the leeward side of the island of Oahu and are fed by rainwater. Samples were collected at locations above human contact (Manoa A and Makiki). In addition, one sample was collected from Manoa Stream at a site under the influence of urban storm water drainage (Manoa B). Manoa Stream discharges into Ala Wai Canal which serves as an urban drainage system for the Waikiki area of of Honolulu. In addition, samples were collected from Kaelepulu stream and Kawainui Canal located on the windward side of Oahu. These waterways receive stream run-off and also serve as urban drainage for the City of Kailua. Ala Moana beach is a popular bathing beach and is located in the City of Honolulu. Primary sewage samples were collected from the Sand Island treatment facility located on the island of Oahu. For sewage sensitivity testing, a series of ten-fold dilutions (10^{-2} to 10^{-7}) of primary treated sewage were made using phosphate buffered saline (pH 7.2) as the diluent. For survival experiments 5 ml of a late log culture of *B. thetaiotaomicron* were pelleted at 3,000 X *g* for 10 min. and washed twice with PBS and resuspended in PBS or ocean water at a final concentration of 10^9 cells per ml. This suspension was then added to 1000 ml ocean water or 1000 ml PBS to achieve a final concentration of approximately 10^6 cells/ ml. For PCR sensitivity experiments, a series of ten-fold dilutions (10^{-2} to 10^{-7}) of these seeded samples were made using phosphate buffered saline as the diluent.

C. DNA Extraction. For specificity testing, DNA was extracted from exponential phase cultures by lysis with 0.5 % sodium dodecyl sulfate treatment as described in *Short Protocols in Molecular Biology 2nd ed.* (Wiley and Sons, 1992). Following alkaline lysis, 0.007 M NaCl-1% hexadecyltrimethyl ammonium bromide was used to complex polysaccharides. Proteins and other impurities were removed by chloroform-isoamyl alcohol (24:1), and DNA was further purified by phenol-chloroform-isoamyl alcohol (24:24:2) extractions. DNA was then precipitated by 2.5 volumes of isopropyl alcohol and pelleted by centrifugation at 12,000 x *g* for 15 min. The DNA pellets were washed once with cold 70 % alcohol and dried under vacuum. (Difco Laboratories, Detroit, Michigan) for 24 hrs at 37°C. For field samples total DNA was extracted by filtering 50 ml of the respective water samples through a 0.45 µm pore-size HVLP filter (Millipore). The filter was than placed in 500 µl of sterile water containing 5% chelex (w/v), mixed thoroughly and placed in a boiling water bath for 15 min. Samples were allowed to cool to room temperature and then were concentrated using a centricon 100 (Amicon) concentrator to a final volume of 30 µl.

D. DNA Primers and PCR Amplification. Primer BF410 (5'-GTG AAG GAT GAA GGC TCT AT-3') and BF800(5'-CGT TTA CTG TGT GGA CTA CC-3') were chosen using Olgio 4.0 computer softeware (National Biosciences, Inc.) from a prevoiusly published *Bacteroides fragilis* 16s RNA sequence (Weiburg, et al., 1985). These primers generated a 410 bp PCR product. PCR detection of *E. coli* was performed using primers UAL-754 (5'-AAA CGG AAG AAA GCA G-3') and UAR-900 (5'- ACG CGT GGT TAC AGT CTT GCG-3'), which have previously been described by Bej. et al. (1991). They were used to amplify a 147-bp coding region of the *uid*A gene in *E. coli*. All primers were produced with an automatic DNA synthesizer (ABI 381A; Applied Biosytems International, Foster City, Calif.) PCR reaction mixture contained 1 x PCR buffer (10 mM Tris-HCL [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphate, 0.3 µM (each) primer, and 0.5 µl Taq DNA polymerase per 100 µl

(Perkin-Elmer Cetus, Norwalk, Conn.). The PCR temperature cycling profile for the detection of *Bacteroides fragilis* group (Primers BF410 & BF800) was initially denatured at 94°C for 5 min and then subjected to 30 cycles each consisting of 120 sec. at 94°C, 30s at 53 °C, and 60s at 72°C; then synthesis was completed at 72°C for 7 minutes. For the detection of *E. coli* a two step temperature profile, described by Bej.et al. (1991), was used and consisted of an initial denaturation at 94°C for 3 min followed by 25 cycles consisting of a 94°C denaturation step for 60 sec and primer annealing and extension at 50°C for 60 sec. PCR products were analyzed by gel electrophoresis on a 2 % SeaKem GTG agarose gel (FMC BioProducts, Rockland, Maine) stained with ethidium bromide (0.5 μg/ml) and were identified using a model 6-63 transilluminator (Ultra-Violet Products, San Gabriel, Calif.).

E. DNA Hybridization. PCR products generated from *Bacteroides* spp. primers were confirmed using an internal probe, BFI 5'-GGA TTT ATT GGG TTT AAA GGG-3'. PCR products generated from *E. coli* -specific primers were confirmed with an internal probe UAP-1 (5'-TGC CGG GAT CCA TCG CAG CGT AAT G-3') as described by Tsai et al. (1993). The internal probes were 3' labeled with digoxigenin-11-ddUTP using a Genius 5 nonradioactive DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.). PCR products were transferred onto Hybond-N+ positively charged nylon membranes (Amersham Arlington, Heights, Ill.) using a PosiBlot pressure blotter (Stratagene, La Jolla, Calif.) for the Southern analysis. DNA was fixed to the nylon membranes by UV irradiation at 254 nm for 2 min using a CL-1000 ultraviolet crosslinker (UVP, San Gabriel, Calif.) Hybridization was perfromed at 55°C in presence of a labeled oligonucleotide internal probe (2 pmol/ml). The hybridized filters were washed twice with a high-salt solution (2 x SSC in 0.1% sodium dodecyl sulfate) at 50°C. A Genius 3 nucleic acid detection kit (Boehringer Mannheim) was used to prepare the hybridized filters for chemiluminescent detection. The hybridization signals were visualized on X-OMAT film (Eastman Kodak, Rochester, N. Y.) using autoradiography (Sambrook et al.).

IV. Results and Discussion

A. Specificfty of BFG Primers. In order for PCR to demonstrate a high degree of specificity, primers and target sequences must be conserved among BFG and not share similarities with other Gram-negative and Gram-positive bacteria. Primers BF410 and BF800 were initially tested to show they amplified the anticipated target sequence with all four members of the *Bacteroides fragilis* group (BFG) which includes *B. fragilis, B. vulgatus, B. thetaiotaomicron* and *B. distasonis* (Table 1). In order to determine the specificity of primers BF410 and BF800, 27 different enteric bacteria (e.g. *E. coli, Shigella* spp. and *Enterobacter* spp.) were utilized to test the specificity of these primers (Table 1). As an example, Figure 1 shows the PCR amplification results for the members of the *Bacteroides fragilis* group and other non-Bacteroides isolates. As seen in Table 1 and Figure 1 all four members of the *Bacteroides fragilis* group tested, generated a 410 bp PCR product while no products were detected for any of the non *Bacteroides* isolates. These results indicate that the primers BF410 and BF800 consistently generated a 410 bp product for all of the members of the *Bacteroides fragilis* group and the sequence targeted by these primers appears to be conserved for all of these members.

Table 1. Isolates Tested with Primer Set BF410 and BF800

Organism	Source	410 bp PCR Product
Bacteroides fragilis	ATCC 25285	+
Bacteroides vulgatus	ATCC 8482	+
Bacteroides thetaiotaomicron	ATCC 29741	+
Bacteroides distasonis	ATCC 8503	+
Salmonella typhimurium	ATCC 14028	-
Escherichia coli	ATCC 25922	-
Escherichia coli	ATCC 35401	-
Escherichia coli	ATCC 43886	-
Escherichia coli	ATCC 43889	-
Escherichia coli	ATCC 43890	-
Escherichia coli	ATCC 43894	-
Escherichia coli	ATCC 43895	-
Escherichia coli	ATCC 43896	-
Enterobacter aerogenes	ATCC 13048	-
Citrobacter freundii	ATCC 8090	-
Klebsiella pneumoniae	ATCC 13883	-
Shigella flexneri	ATCC 12022	-
Shigella sonnei	ATCC 25931	-
Bacillus cereus	ATCC 14579	-
Bacillus subtilis	ATCC 6051	-
Pseudomonas aeruginosa	ATCC 27853	-
Staphylococcus aureus	ATCC 25923	-
Streptococcus faecalis	ATCC 29212	-
Acinetobacter calcoaceticus	ATCC 19606	-
Serratia marcescens	ATCC 8100	-
Streptococcus pyogenes	ATCC 19615	-
Enterobacter cloacae	ATCC 23355	-
Proteus vulgaris	ATCC 13315	-
Staphylococcus epidermidis	ATCC 12228	-

B. Sensitivity of PCR Primers in Serially Diluted Sewage. Since the levels of BFG in the human intestinal tract are at least 100 to 1000 fold higher than the levels of *E. coli* they should be found at higher level in sewage than *E. coli* (Post et al. 1967). This would imply that BFG gene sequences should also be higher in concentration than *E. coli* gene sequences. In this experiment primary-treated sewage was serially diluted with PBS. These dilutions were submitted to PCR amplification using primers BF410 and BF800 targeting BFG. In addition a set of *E. coli* specific primers was utilized to detect *E. coli* gene sequences and viable *E. coli* was detected using mTEC agar. As seen in Figure 2, BFG PCR products could be detected by hybridization with an internal probe at a 10^{-6} dilution and *E. coli* could be detected at a 10^{-4} dilution. The concentration of *E. coli* in the sewage used for this experiment had a starting concentration 5.8×10^5 CFU/ml. Based

on these results PCR detection of *E. coli* had a sensitivity of 58 CFU/ ml. These results are similar to studies by Tsai et al. (1993) that showed a similar sensitivity using these same primers to detect *E. coli* in seeded sludge (80 CFU/g of sludge). These results indicate that BFG gene sequences appear to be in higher concentrations than *E. coli* sequences in sewage and that the PCR test for BFG is a more sensitive test for the presence of sewage in a sample.

These results are consistent with cultural data indicating that BFG levels are at least 100 to 1000 fold higher than *E. coli* in human feces (Allsop and Stickler, 1985). In summary, PCR detection of BFG appears to be a sensitive means of detecting low levels of sewage and is more sensitive than PCR detection of *E. coli*.

C. Survival of *Bacteroides thetaiotaomicron* in Ocean Water and PBS. As demonstrated by the serially diluted sewage experiment, PCR amplification using primers BF410 and BF800 is able to detect low levels of sewage in a sample. To be a useful marker of sewage, the survival and structural integrity of *Bacteroides* spp. in environmental waters should be known. To obtain this information an early stationary phase culture of *B. thetaiotaomicron* was seeded into ocean water and sterile phosphate buffer. Samples were collected at periodic intervals and submitted to cultural isolation. As seen in Table 2 there was a rapid decline in the number of cultural viable *B. thetaiotaomicron*. At time zero there was 1.4×10^6 CFU in ocean water and 2.6×10^6 CFU in sterile PBS. After four hour of exposure to these waters the number of culturally viable *B. thetaiotaomicron* in ocean water and PBS fell to 3.2×10^2 and 2.5×10^2, respectively. After 8 hour of exposure no *B. thetaiotaomicron* was detected by culture in ocean water or PBS. Thus, evidence was obtained that the anaerobic *B. thetaiotaomicron* can survive for 4 hrs but not 8 hrs in environmental waters.

In order to determine persistence of BFG gene sequences in ocean water, ocean water samples seeded with *B. fragilis* were collected at regular intervals throughout the survival study and submitted to PCR etection targeting the 16s RNA gene of *Bacteroides fragilis*. This was accomplished by filtering samples through a 0.45 μm pore-size filter. DNA was extracted from these filters and submitted to PCR. As seen in Table 2 and Figure 3, PCR products were detected for the entire eight day exposure period. This would indicate that although cultural viablity was lost after 4 hrs of exposure, PCR-targeted gene sequences persisted for at least 7 days.

These results are consistant with previous research by Fiksdal et al. (1985) who demonstrated that *B. fragilis* cells remained intact after eight days of exposure to environmental waters. These results indicate that although culture viability is lost after 4 hour of exposure to PBS and ocean water, gene sequences persist for at least 7 days. This would indicate that in ocean water PCR detection of BFG could serve as a marker for sewage for at least seven days. This is significant, since many pathogenic viruses and protozoans are capable of surviving for long periods of time in ocean water (Elliot and Colwell, 1985).

Table 2. Survival Study of *B. thetaiotaomicron*: Plate Counts and PCR Products

	Ocean Water		Sterile PBS[a]	
Time	CFU/ml	PCR Product	CFU/ ml	PCR Product
0	1.4×10^6	+	2.6×10^6	+
4 hrs	3.2×10^2	+	2.5×10^2	+
8 hrs	< 1	+	< 1	+
22 hrs	< 1	+	< 1	+
48 hrs	ND[b]	+	ND[b]	+
72 hrs	ND[b]	+	ND[b]	+
4 days	ND[b]	+	ND[b]	+
5 days	ND[b]	+	ND[b]	+
6 days	ND[b]	+	ND[b]	+
7 days	ND[b]	+	ND[b] human intestinal tract, *B. vulgatus*, *B. thetaiotaomicron*. *B. fragilis*, and *B. distasonis*. Collectively	+
8 days	ND[b]	+	ND[b]	+

[a]Phosphate buffered saline
[b]Not done

D. Detection of BFG and *E. coli* in Environmental Waters. In tropical climates, *E. coli* appears to be naturally occurring in tropical fresh waters and therefore may not be indicative of human wastes. Since the PCR method for BFG was shown to be a more sensitive test of sewage than *E. coli*, the presence or absence of BFG genes in environmental waters would indicate that these waters are or are not contaminated with sewage. To test this hypothesis, various environmental water samples were tested by PCR for *E. coli* and BFG gene sequences as well as for cultural concentration of *E. coli*. As seen in Table 3, *E. coli* was isolated from all 9 sources and ranged from 4.3×10^6 CFU/100ml in primary treated sewage to 12 CFU at Ala Moana beach Park. PCR-products for *uid* amplification were detected for 8 of the 9 samples (Figure 4). One sample, Ala Maona beach Park, did not show the presence of the *uid* gene sequence. This may be a reflection of the low numbers of *E. coli* (12 CFU/100ml) detected by culture in this sample. Since 50 ml of this sample was used for PCR there would be a theoretical 6 CFU which is close to the detection limit (1-2 cells of *E. coli*) described by Bej. et al. (1991) using these primers. As seen in Table 3 and Figure 4, BFG gene sequences were detected in one (primary sewage) of the 9 samples tested. In the remaining eight samples no BFG PCR products were detected. In summary these findings are consistent with previous studies by Hardina and Fujioka (1991) who

found high levels of *E. coli* in Manoa Stream in the absence of any known sewage contamination. These results indicate that PCR-based detection of BFG may be a more accurate means of detecting the presence or absence of fecal material in tropical waters.

Table 3. *E. coli* and BFG PCR Products Detected in Environmental Waters

Location	*E. coli* Culture CFU/100 ml	*uid* PCR Product	BFG PCR Product
1'Treated Sewage	4.3×10^6	+	+
Ala Wai Canal	2.1×10^3	+	-
Manoa Stream	2.8×10^3	+	-
Makiki Stream	8.2×10^2	+	-
Kaelepulu Stream	4.6×10^2	+	-
Kawainui Canal	6.8×10^2	+	-
Ala Moana Beach	12	-	-
Lower Manoa Stream	1.8×10^3	+	-

VI. Summary and Conclusions

Because of the ubiquitous nature of *E. coli* in tropical climates, alternative water quality indicators are needed. Previous research has proposed the use of the *Bacteroides fragilis* group as a water quality indicator. Unfortunately, this group does not survive for long periods of time in environmental waters and therefore cultural isolation is not an adequate means of detecting this group in environmental waters. In this study, a new PCR-based detection method was developed which decreases the time for detection and does not require cultural isolation. A set of PCR primers targeting the 16s RNA gene of *B. fragilis* was chosen. These primers generated the anticipated 410 bp PCR product for all 4 members of the *Bacteroides fragilis* group and did not cross react with any of the 27 *non-Bacteroides* isolates. In order to determine the sensitivity of these primers, primary treated sewage was diluted and submitted to PCR amplification using primers BF410 and BF800 and a set of primers targeting the *uid* gene of *E. coli*. BFG PCR products could be detected at a 10^{-6} dilution as compared to 10^{-4} dilution for *E. coli*. These results indicate that primers BF410 and BF800 are able to detect the presence of sewage at levels at least 100 fold lower than the *uid* primers. Samples collected from a variety of environments with no known sewage impact were positive for the presence of *E. coli* while negative for BFG sequences. This would indicate that PCR detection of BFG is a more accurate means of detecting the presence or absence of sewage in tropical waters.

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CHAPTER FOUR: PROJECT ASSESSMENT AND RECOMMENDATIONS

I. Benefit and Usefulness of the CCH Matching Study

The obvious benefit for the CCH matching study was that NWRI paid for most of the direct cost for funding the study. Thus, much of the scientific information obtained from this study was funded by and evaluated by NWRI. The usefulness of this CCH matching study are as follows: First, molecular methods were developed and used to address environmental water quality problems in Hawaii. Second, laboratory personnel from the CCH laboratory were trained in the use of molecular methods and the CCH laboratory furnished with the necessary equipment to perform molecular genetic experiments. Third, the capability of the CCH laboratory to independently perform molecular genetic methods at the CCH Sand Island Laboratory was established.

With this new capability, the CCH is in a position to gain additional benefits. First, since the laboratory staff members are trained and experienced in performing molecular genetic methods, they can act as staff resources to critically assess research results based on the use of molecular genetic methods. Second, the CCH laboratory can now design experiments using molecular genetic methods to obtain data which traditional monitoring methods have not been able to achieve. Finally, with this new capability, the CCH can function as an agency which is pro-active by using state of the art methods as compared to many monitoring agencies which limit their activity to only USEPA approved methods and as a result appear ignorant and reactionary when asked to respond to research results based on molecular genetic methods.

II. Assessment of PCR Technology

Application of molecular genetic methods such as PCR is clearly the state of the art technology which has been responsible for the development of new hypothesis in all disciplines of science. Since the use of molecular genetic methods is still in its developmental stage, the potential productivity of this technology is still incalculable. There is a clear trend that these methods will continue to be applied to address environmental water problems. Significantly, commercial equipment and pharmaceutical companies have recognized the application of these molecular genetic methods and are manufacturing equipment and preparing reagents to simplify this technology and expand its use to monitoring, non-research laboratories. Although this activity is currently being focused on clinical laboratories it will soon be applied to water monitoring laboratories. It should be noted that when these new developments are made, only agencies such as the CCH which understand the application of molecular genetic methods and which have the capability to perform these methods will be in a position to take advantage of these new developments. Finally, by adopting the strategy to use of PCR technology, specific answers to address water problems can be obtained. In comparison, most of the traditional microbiological methods currently approved to monitor water samples provide only indirect data and therefore only indirect answers.

Although PCR technology is at the cutting edge of science it should be recognized that there are problems related to appropriate situations where PCR should be applied and situations where other tests would be more appropriate. In the application of molecular methods, the most controversial issue is proper interpretation of the data. Too often, the potential benefits of the method is emphasized to the point that over interpretation of the data naturally follows. The issues that must be resolved before PCR can make the transition from a research tool to a water quality monitoring tool are as follows: a) consistent and efficient recovery of PCR-targeted gene sequences from different environmental water samples with the complete removal of inhibitory compounds, b) simpler and more rapid methods of detecting and confirming PCR products, c) development of methods to quantitate the initial amount of the target gene, d) development of methods to differentiate and enumerate between viable and non-viable microorganisms as well as the special group of microorganisms categorized as viable but non culturable, and e) scientifically based guidelines on interpreting the public health significance of detecting PCR-targeted gene sequences in various environmental samples.

III. Recommendations

Despite the promise and potential in the use of molecular methods, agencies must be very careful in the application of molecular methods and in the interpretation of the data obtained. As stated earlier, careful controls must be performed to ensure that the methods are done correctly. Moreover, personnel must keep up with the literature and understand the reactions well. It is well known that laboratory analysts need time and opportunity to perform several variations of molecular genetic methods to become proficient in the use of this technology and more importantly to understand the limitations and potential usefulness of these new methods.

Due to the above precautionary statements, the recommendation is made that the CCH continue to seek the advice of the WRRC and to work with the WRRC for the next several years in the application of PCR technology to monitor environmental waters. It is always prudent to analyze the same water sample assayed by PCR methodology with approved monitoring methods which measures the viable concentrations of relevant microorganisms such as fecal bacterial indicators. Some recommended and future uses of PCR technology are listed below:

A. Use PCR as a Sensitive Test for the Presence or Absence of Sewage in Environmental Water Samples.

Chapter 3 of this final report describes the development and application of the PCR test for bacteroides fragilis group of bacteria (BFG). Of the many PCR assays, this assay is relatively simple and sensitive. Moreover, personnel from the CCH have been trained in the use of this method.

1. *Analysis of surface waters and soil leachates*. There are many instances when sewage is suspected as the source or to have contaminated surface waters (streams, storm drains). Alternatively the source of water flowing directly from a soil site (soil leachate) is believed to be

sewage. Under these conditions, culturable assays for fecal indicator bacteria are not useful since WRRC has shown that these same fecal indicator bacteria are naturally present in the soil environment of Hawaii. The presence or absence of BFG in these samples may provide the information to determine whether the source of this water is or is not sewage.

2. *Analysis of ocean water samples.* The movement of sewage from ocean outfall is still controversial and culturable tests for fecal bacterial indicators are too insensitive. Application of PCR methodology to the samples near ocean outfall which are already being measured for fecal bacteria indicator shows great potential. In this regard, WRRC has already proposed that such a study should be initiated. WRRC has received a mini-grant from Sea Grant Program to determine if this PCR technology can be useful in determining whether coastal waters are polluted. The CCH laboratory is in a position to initiate this study.

B. Use of PCR to Detect for Pathogens and Other Microorganisms That Cannot be Feasibly Measured by Culturable Tests.

1. *Assay for human enteric viruses.* Methods to assay for viable concentrations of human enteric viruses in sewage and environmental waters are available but these tests are too insensitive, too complicated and too costly. However, there is great value in testing waters for the presence of human enteric viruses because the source of human enteric viruses is human and not from animals. Thus, detection of human enteric viruses in environmental samples is direct evidence that the water is contaminated with human feces. If these viruses are viable, the data clearly indicate a public health risk. Although PCR technology will measure both dead and live human enteric viruses, the ability of PCR to detect for human enteric viruses in any environmental water sample will provide information that is not generally possible by other methods. Although the CCH laboratory personnel has been trained in the RT-PCR test for human enteric viruses, this PCR test must be modified to initially convert the RNA of human enteric viruses to DNA. Thus, this PCR test is more expensive, has an additional step and is more prone for mistakes. To address this concern, WRRC is determining whether tests for human adenovirus in sewage by PCR will be an effective alternative test. The major advantage for selecting adenovirus is because it is a DNA virus and therefore the efficient direct PCR method can be used to detect this human virus in sewage.

2. *Assay for other pathogens in sewage.* WRRC is planning to use PCR to assay for other pathogens which may be present in sewage and are difficult to detect by culture method. This includes protozoans such as Giardia and Cryptosporidium and some selective bacteria such as *Vibrio cholerae* and pathogenic *E. coli* including 0157:H7. CCH should continue to consult with WRRC on measurement of specific pathogens in sewage or sewage contaminated water.

C. PCR assay to Detect Viable Concentrations of Human Enteric Viruses. The CCH Sand Island Laboratory will soon be analyzing environmental waters for human enteric viruses using the standard culturable method. Culturable assays for human enteric viruses requires the establishment and use of cell culture and monitoring these inoculated cell cultures for weeks to observe for the growth of human enteric viruses. One inherent problem with the use of cell culture is the death or

contamination of cell culture before the observation of growth of human enteric viruses. To address this limitation, PCR test for human enteric viruses can be applied to cell cultures two days after inoculation and before cell death and cell contamination can ruin the experiment. The PCR test can be used to determine for the presence of small numbers of human enteric viruses in cell culture. Thus, this application of the PCR test can be integrated with the tedious cell culture method to provide information on viable levels of human enteric viruses in a very short time. Since WRRC is already using this test, the CCH should consult with WRRC on applying this test.

D. Fingerprinting of Major Types of Effluents for Pollution Analyses of Environmental Waters.

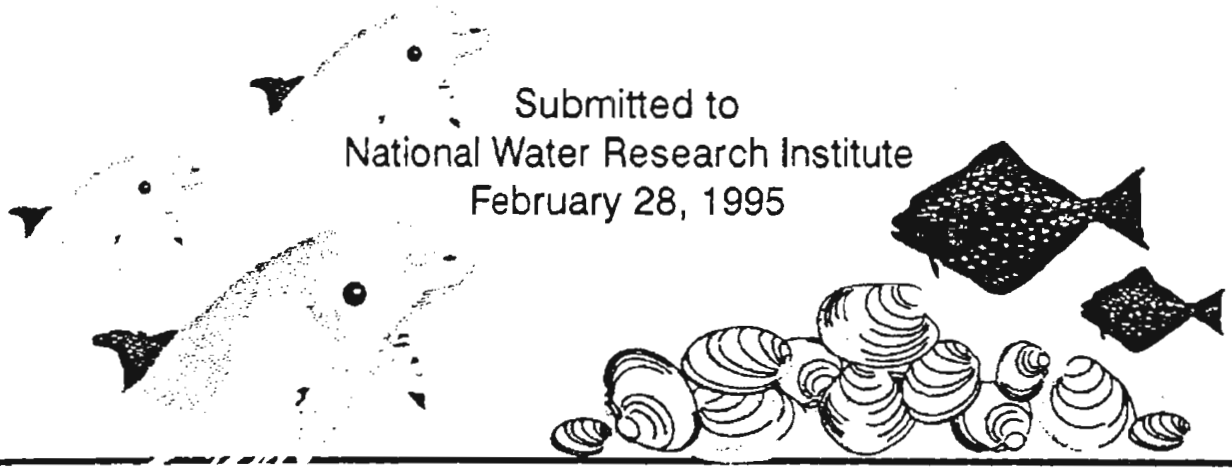
In Chapter 3, we developed a test for a specific group of bacteria characteristically found in human feces. When environmental waters become contaminated, the traditional test using fecal indicator bacteria is not very useful because these fecal bacteria are found in most other major known effluents such as feces or effluents from chicken, horses, cattle and pig farms as well as from the soil of Hawaii. However, each of these sources can be expected to have some group of bacterial species which are found in high concentrations in one effluent but not in other effluent. For example, cattle effluent should contain high concentrations of bacteria which digest cellulose and these bacteria are not expected to be found in humans or chicken. By developing a PCR assay specific for all of these effluents, one can fingerprint these effluents based on concentrations of one or more different types of unique bacteria. By applying this data base and this PCR method, the source of the pollution which is impacting environmental waters can be determined. The CCH should continue to consult with WRRC in the development of these tests.

FINAL REPORT
NWRI GRANT HR-92-06

Collaborative National Study Using Molecular Techniques to Detect Hepatitis A Virus and Virulence Factor Genes in *E. coli*

Principal Investigators

Dr. Carol J. Palmer[1]
Dr. Yu-Li Tsai[1]
Dr. Louis Sangermano[1]
Dr. Roger S. Fujioka[2]
Dr. Mark D. Sobsey[3]



Submitted to
National Water Research Institute
February 28, 1995

[1]Environmental Sciences Laboratory - County Sanitation Districts of Orange County, California
[2]Water Resources Research Center and Dept. of Microbiology - University of Hawaii at Manoa
[3]Dept. of Environmental Sciences and Engineering - University of North Carolina Chapel Hill

waters receiving treated sewage for recreational pursuits. An exciting offshoot of this study was the fact that we discovered that polio infects PBMC cells. This could explain how polio enters the central nervous system and thus could shed more light on the method of polio pathogenesis.

6. According to seeding studies, HAV survives much longer than polio in ocean water and was the predominant virus detected in the California field study. Hepatitis A was not detected in Hawaii water in any of the water types sampled. Enteroviruses were not found in Hawaii ocean water but were recovered from streams entering coastal waters. Of interest in North Carolina was the finding that three different estuarine areas, one with human waste, one with cattle waste and one with no waste, all contained enteroviruses.

7. There were no differences between ocean water from the three study sites in virus seeding experiments. Although we suspected that the different physical components (salinity, pH, etc) would affect virus survival, it did not. Sunlight, however, was shown to impact the ability to culture viruses, since after two 6 hour periods of sunlight exposure, viruses could only be detected by PCR and they no longer infected cells lines. We also showed that naked viral RNA does not survive well in ocean water without its protective outer protein coat. This information combined with the knowledge that our concentration methods eliminate cellular debris from burst organisms gives us confidence that PCR is detecting intact organisms and not artifacts or pieces of nucleic acid from dead or lysed organisms.

In conclusions, we obtained all major goals set forth in our grant proposal plus completed six additional goals. Our findings represent and portray some of the pioneering efforts in developing and applying molecular technology to the field of water quality. Because PCR is not currently able to differentiate live from dead organisms or viral infectivity, our findings can not be used as the basis for public health decisions. However, that fact that these organisms were detected indicates that additional research should be completed to better define the public health implications and to assist in further understanding the complexity of microbial systems.

Genetic Analysis
DNA Methods Workshop

Dr. Carol J. Palmer, Senior Scientist
Dr. Yu-Li Tsai, Scientist
Dr. Louis R. Sangermano, Laboratory Manager

August 31 - September 15, 1992
University of Hawaii, Snyder Hall 303

ENVIRONMENTAL SCIENCES LABORATORY
COUNTY SANITATION DISTRICTS OF ORANGE COUNTY, CALIFORNIA

SCHEDULE

Week 1 - Use of gene probes to detect toxigenic *E. coli*

Date	Time	Session
Monday, August 31	10:00 A.M.	Theory & Methodologies Prepare reagents, label probes
Tuesday, September 1	9:00 A.M.	DNA extractions
Wednesday, September 2	9:00 A.M.	DNA hybridization
Thursday, September 3	9:00 A.M.	Detection of tox genes-*E.coli*
Friday, Sept. 4	9:00 A.M.	Stripping and Reprobing blots

Week 2 - PCR to detect *Legionella pneumophila*

Monday, September 7	9:00 A.M.	HOLIDAY
Tuesday, September 8	9:00 A.M.	PCR - to amplify *Legionella* DNA
Wednesday, September 9	9:00 A.M.	Hybridization and detection of *Legionella*
Thursday, September 10	9:00 A.M.	Ken Tenno's group in environmental meetings
Friday, September 11	9:00 A.M.	Thurs/Fri Our group will set up control sites and collect water from control sites on these two days

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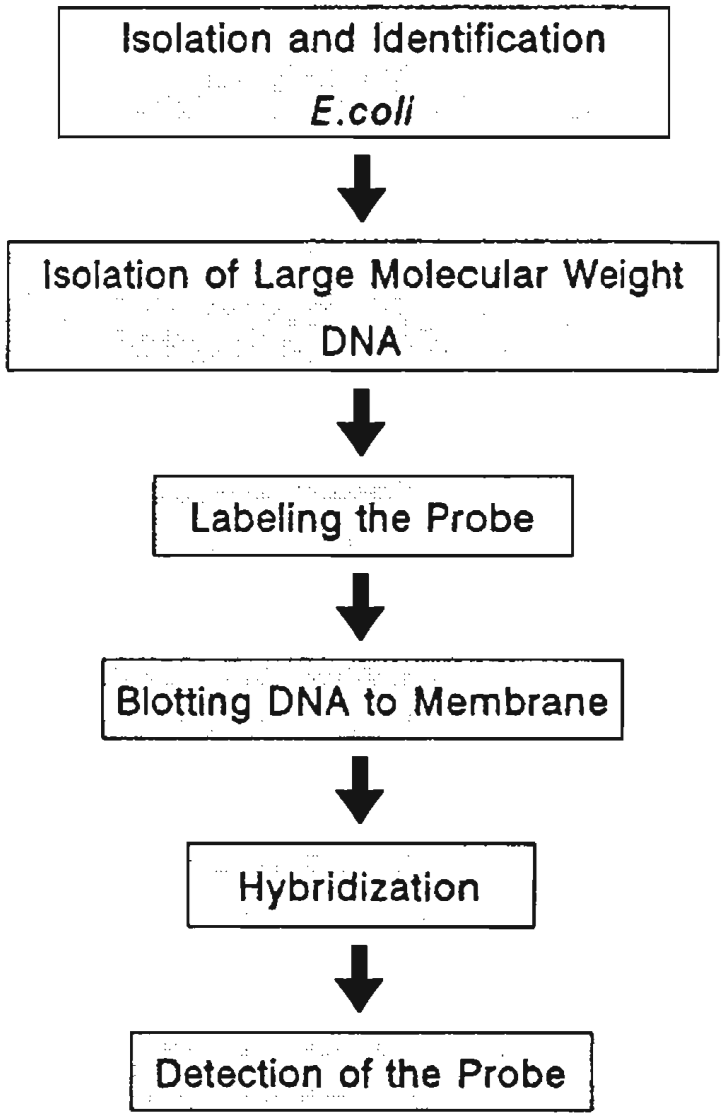
Week 3 - Theory on Enterovirus Recovery, Trouble shooting, Wrap-up

Monday, September 14	9:00 A.M.	Run new samples for *Legionella*
Tuesday, September 15	9:00 A.M.	Preview virus recovery, Wrap-up

MAJOR STEPS IN E.COLI TOX GENE PROJECT



ISOLATION OF DNA

Prepare Cells for Extraction

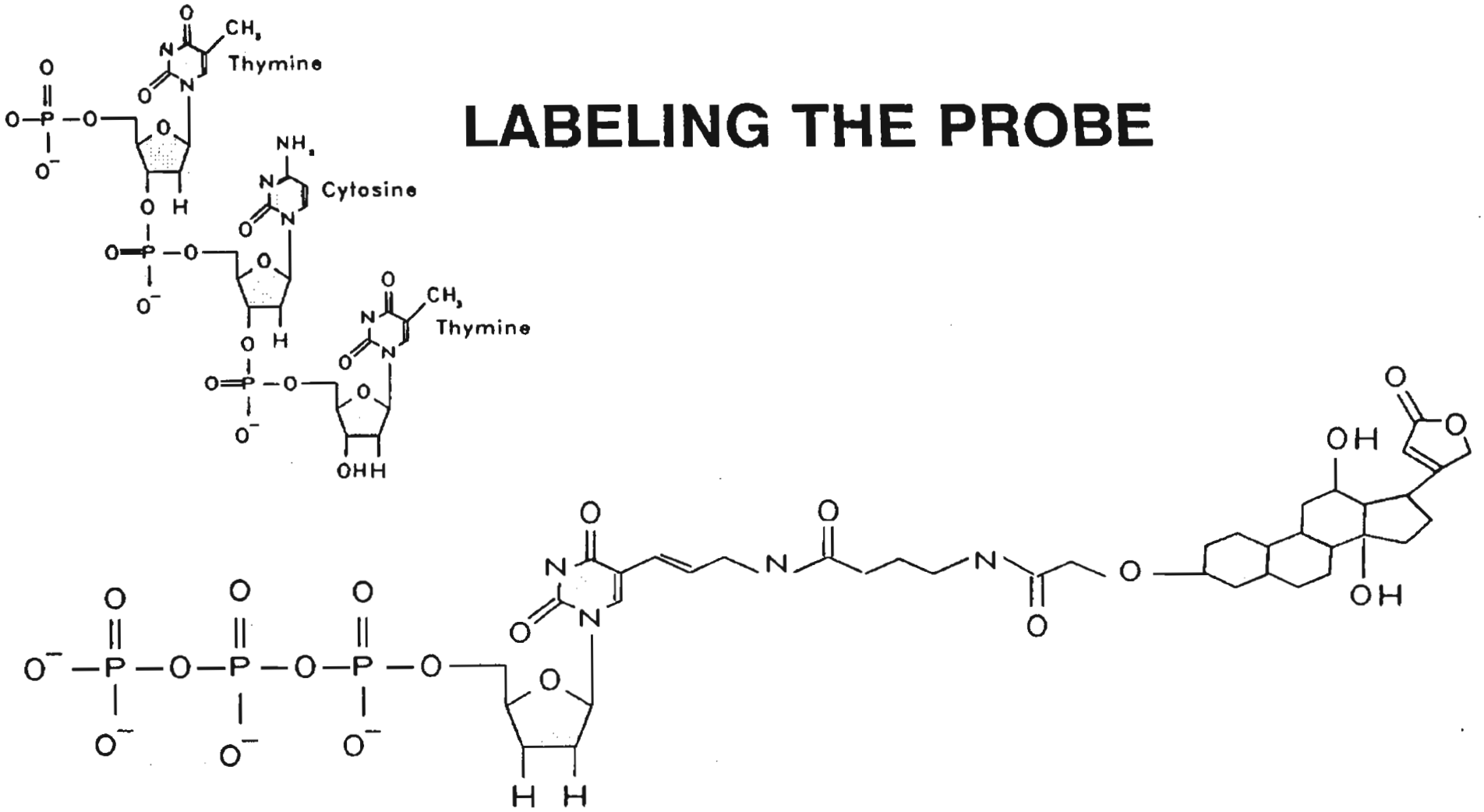
Lyse Cells and Digest RNA and Proteins

Remove Cellular Debris

Precipitate DNA

Remove DNA

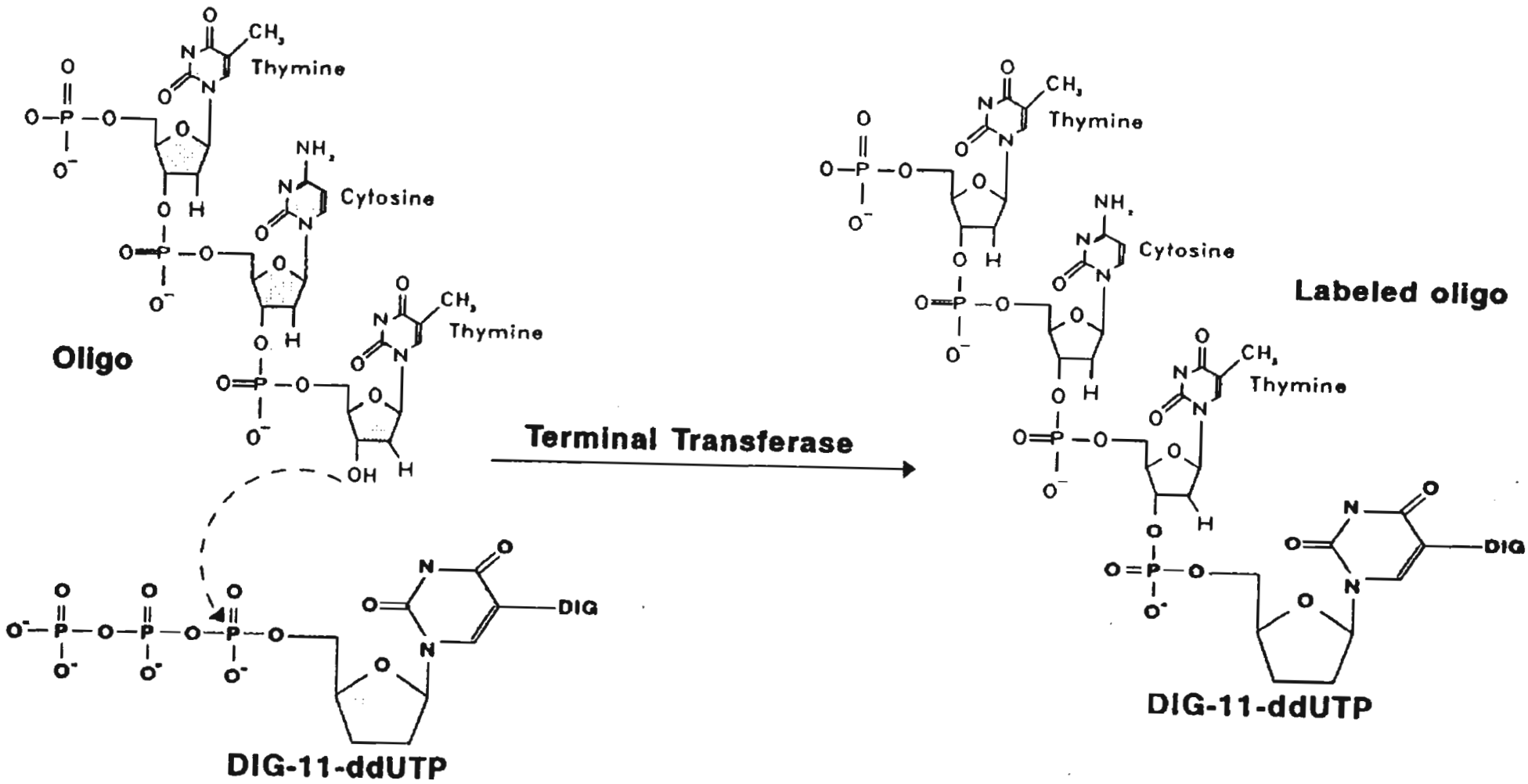
LABELING THE PROBE



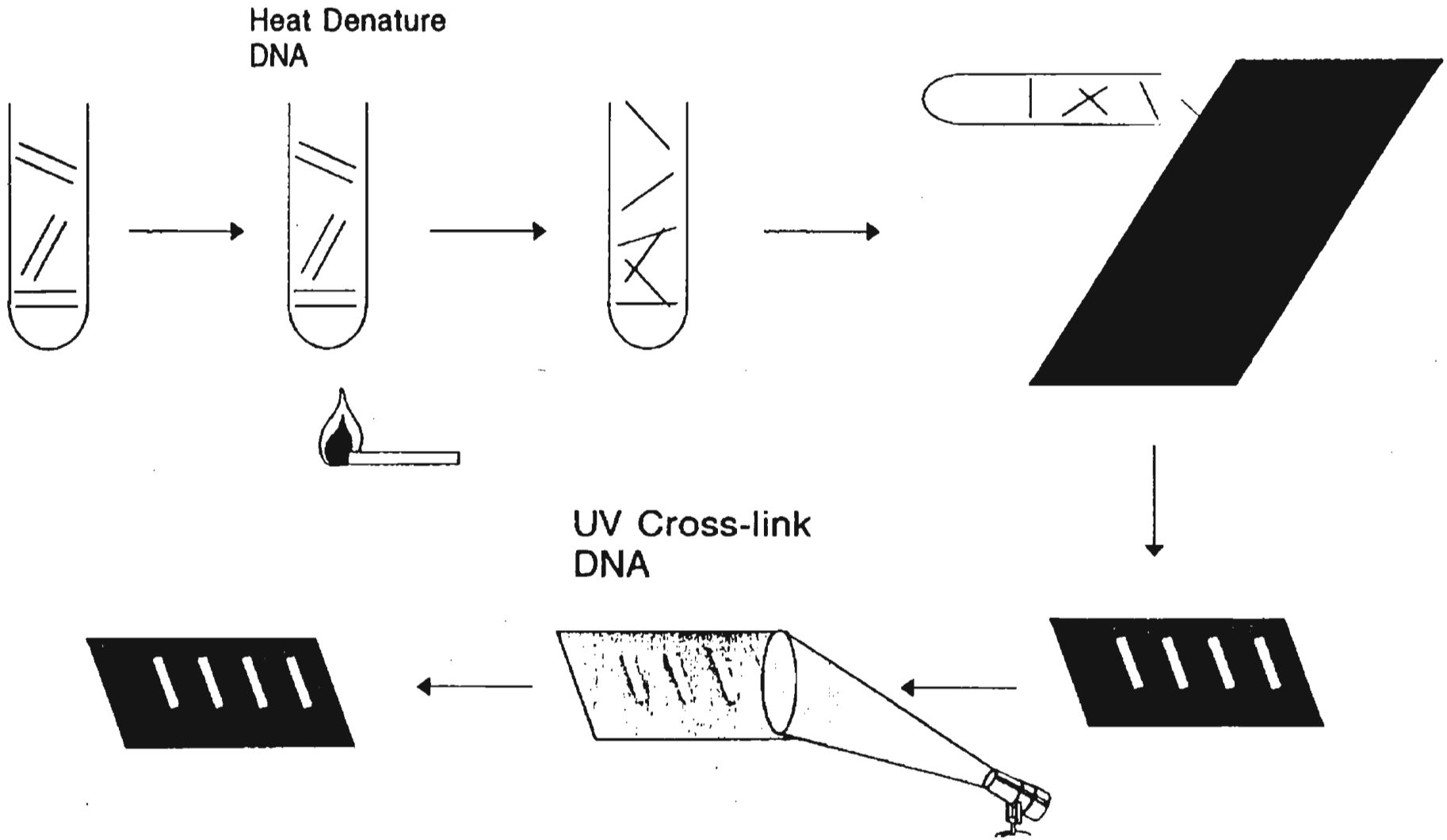
Digoxigenin-11-dideoxyuracil-tripho sphate

DIG-11-ddUTP

THE LABELING REACTION



BLOTTING DNA TO MEMBRANE



Optimization of PCRs

3. Oligonucleotide primers

0.1 - 0.5 μM

Too high:

- mispriming - nonspecific products
- primer-dimer
- low yield of desired product

Too low:

- insufficient product

18 to 28 nucleotides

50 to 60 %G+C

Optimization of PCRs

4. Magnesium concentrations

   0.5 - 2.5 mM
   Primer annealing
   Strand dissociation temperature
   Product specificity
   Formation of primer-dimer
   Enzyme activity and fidelity

Optimization of PCRs

5. Thermal profiles

Three-temperature
- denaturation
- annealing
- extension

Two-temperature
- denaturation
- annealing and extension

Optimization of PCRs

6. Denaturation temperature and time

95°C, 30 sec.
97°C, 15 sec.
Half-life of *Taq* polymerase:

2 h	92.5°C
40 min	95°C
5 min	97.5°C

Too high and/or too long:
loss of enzyme activity

Optimization of PCRs

7. Primer annealing

Annealing temperature:
- 5°C below T_m

55 - 72°C yield best results
- increase specificity
- reduce misextension

Using two-temperature scheme

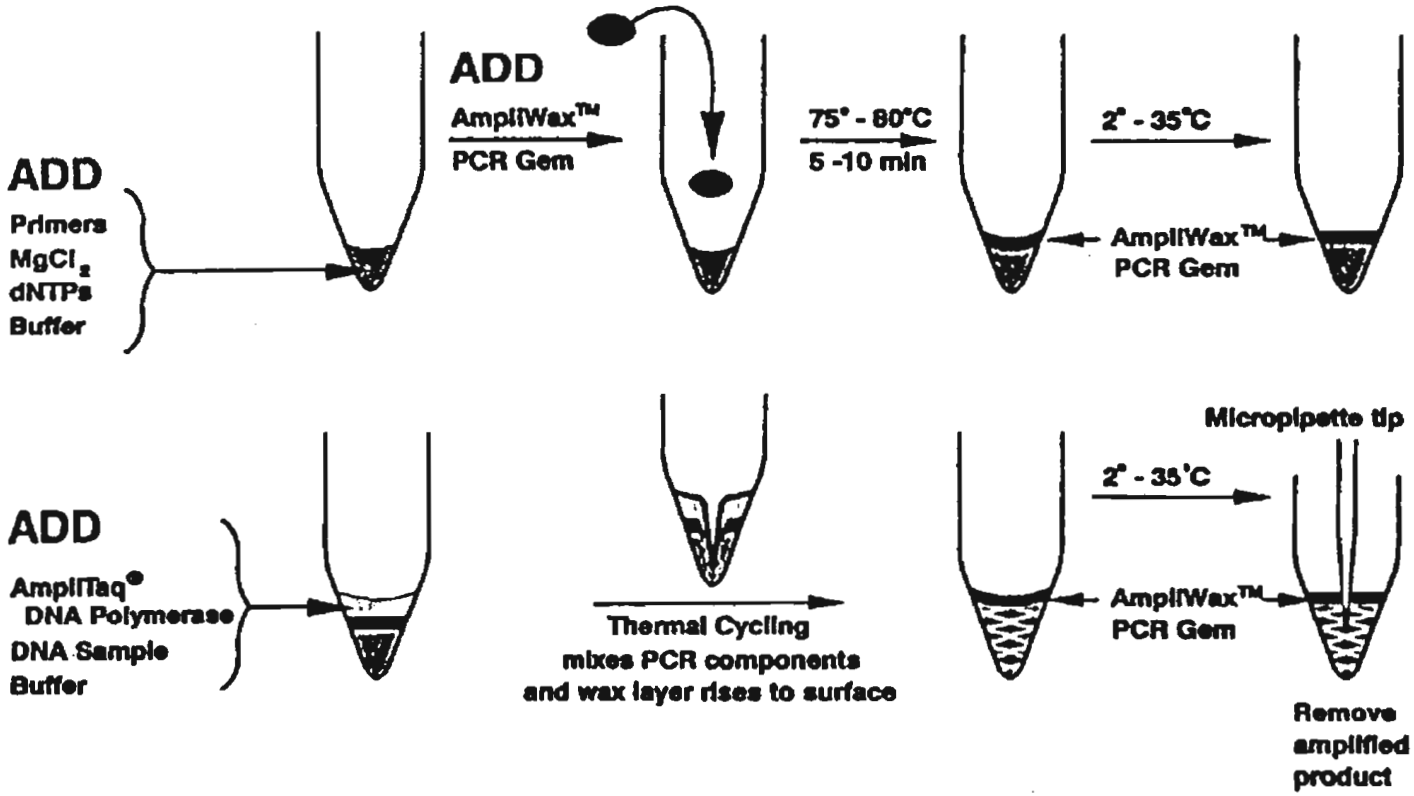
Optimization of PCRs

9. Cycle number

No. of target molecules	No. of cycles
3×10^5	25 to 30
1.5×10^4	30 to 35
1×10^3	35 to 40
50	40 to 45

Too many cycles:
- increase nonspecific background products

Hot Start Technique with AmpliWax™ PCR Gems



RNA Amplification

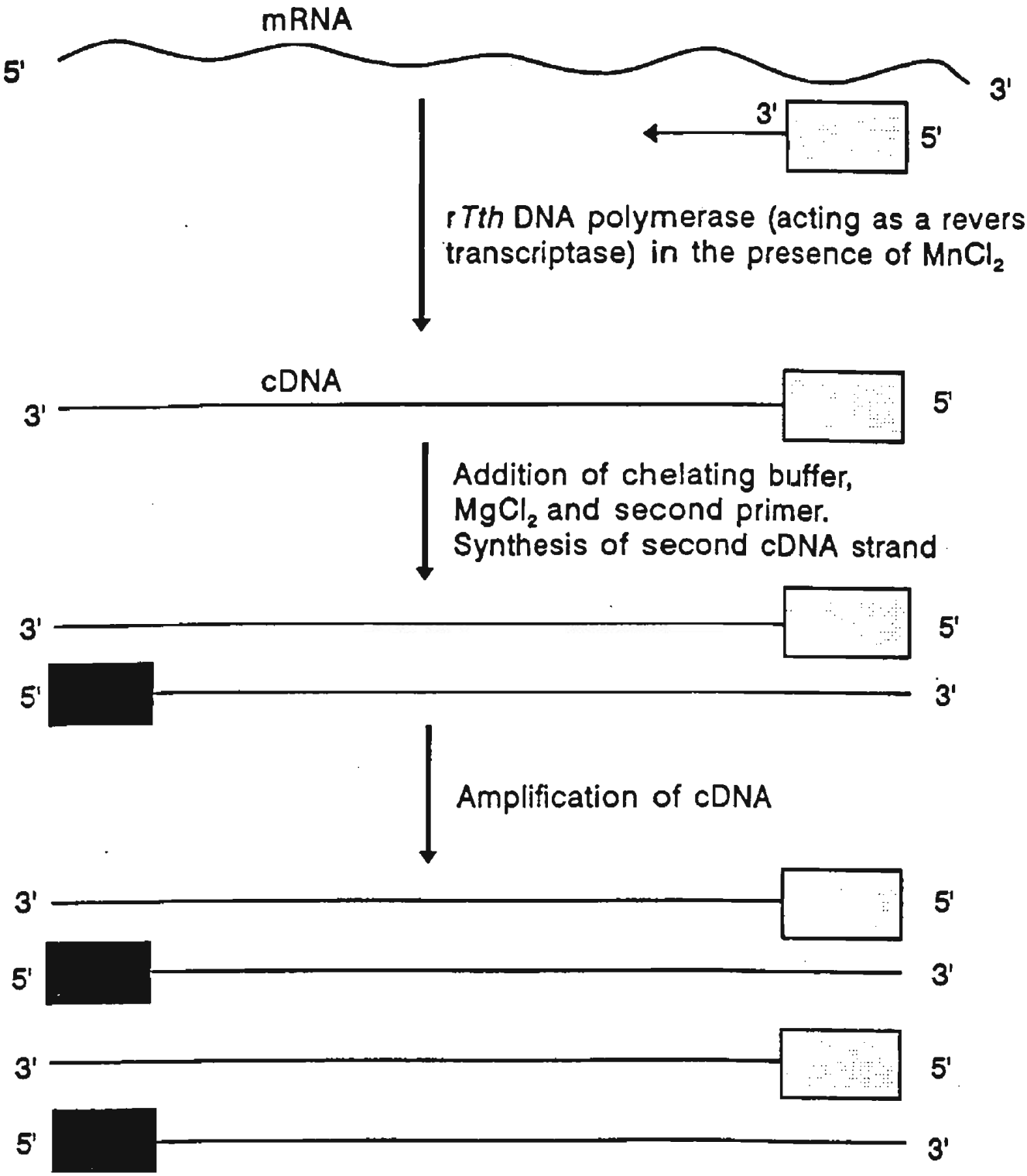
- Reverse transcription
  - Reverse transcriptase
    (e.g., moloney murine Leukemia virus)
  - RNase inhibitor (e.g., RNasin)
  - Random hexamer
  - dNTPs

- PCR amplification
  - uses the same buffer as RT

Thermostable r*Tth* RT-PCR

- *Thermus thermophilus* (r*Tth*) DNA polymerase
- Optimum at 70 -72°C
- Requires $MnCl_2$ for RT
- Chelates Mn^{2+} prior to PCR
- Requires $MgCl_2$ for PCR

RNA PCR with r*Tth* DNA Polymerase



Quantitative PCR

- Low-copy-number mRNA transcripts
- Internal standard
- Exponential co-amplification of internal standard and target RNA
- Gene expression determination

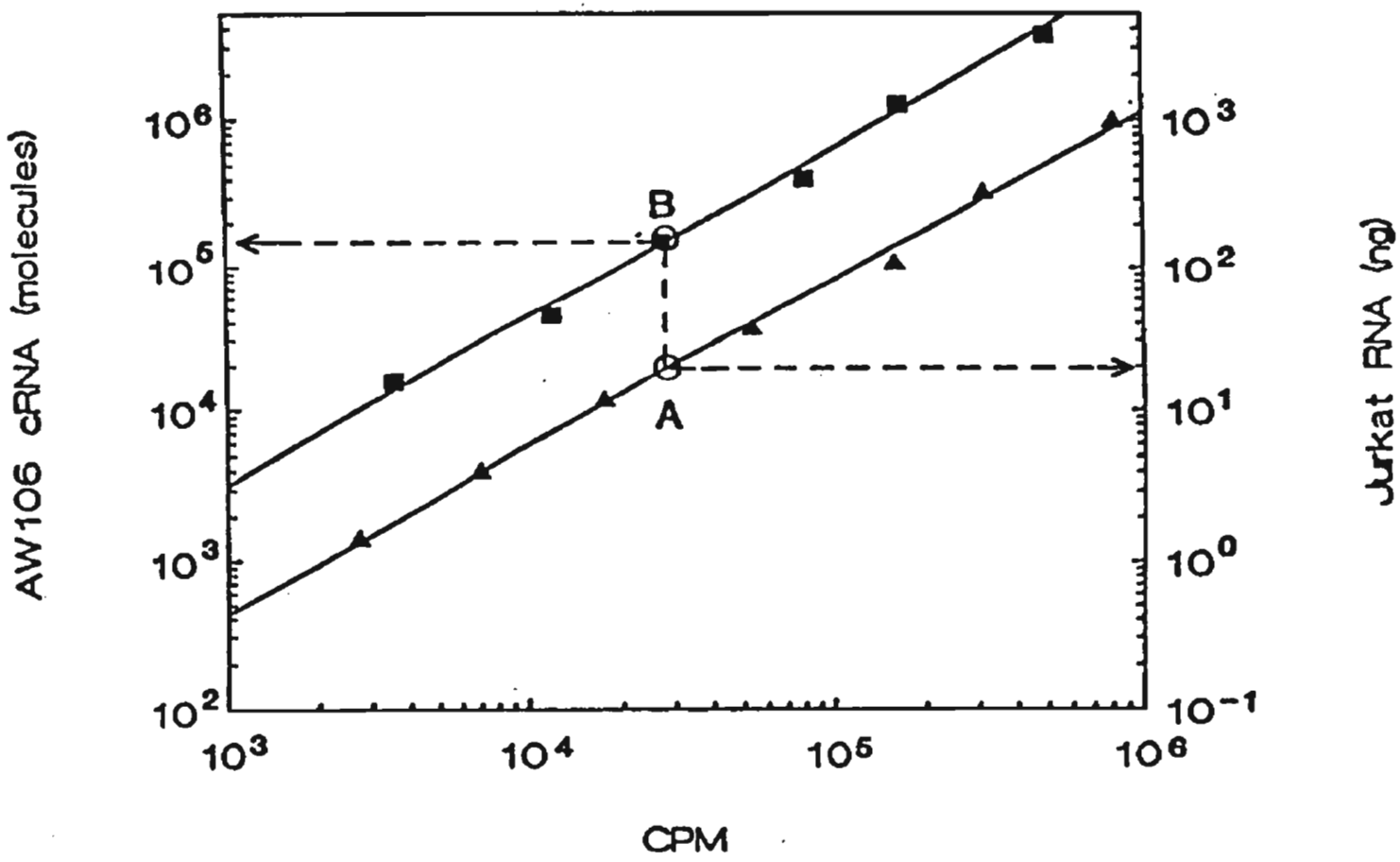
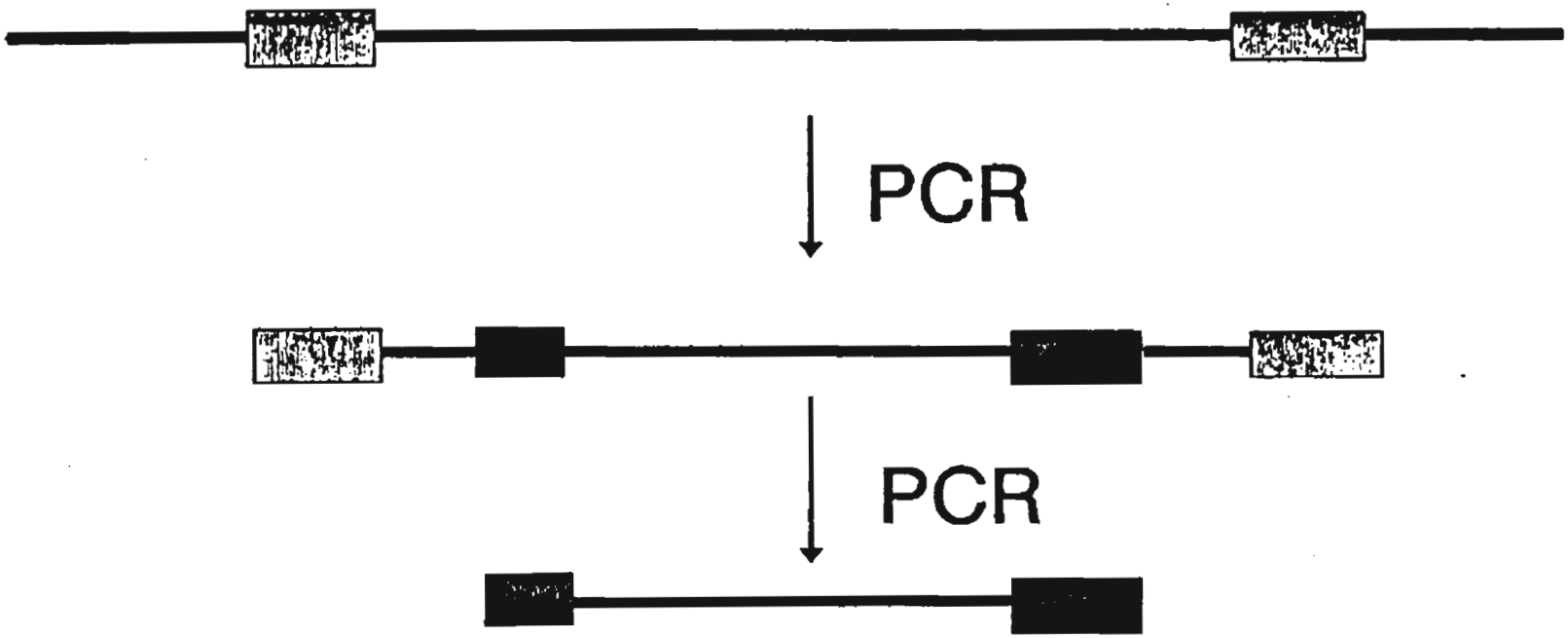


Figure 2 Quantitation of IL-2 mRNA in Jurkat cells by co-amplification with internal standard AW106 cRNA. The variable template concentrations of the internal standard AW106 cRNA and Jurkat RNA were plotted against the radioactivity of their PCR product. The PCR analysis was performed with IL-2 primers. (■) PCR product of AW106 cRNA. (▲) PCR product of Jurkat RNA.

Nested PCR

increases the sensitivity and specificity of both DNA and RNA amplification



Asymmetric PCR

- Unbalanced priming
- Synthesis of a single strand DNA
- Generating template DNA for sequencing

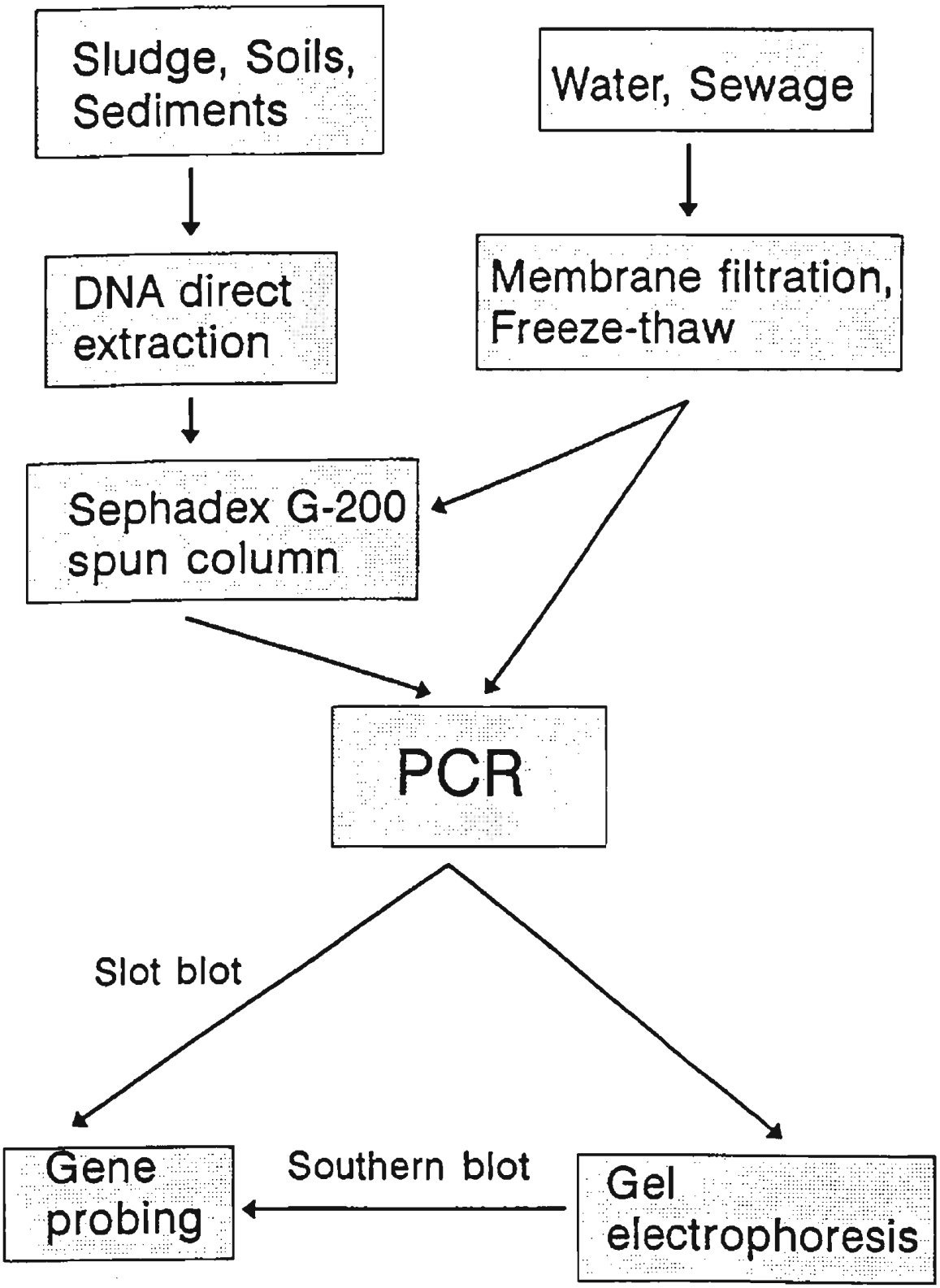
Applications of PCR

* Genetic disease
* Gene cloning
* Forensic analysis
* Archival materials
* Environmental microbiology

Environmental Applications of the PCR

- GEMs in soils
- Indicator bacteria in water
- Viruses in water
- Pathogenic protozoa in water
- *Legionella* in water
- Activities of microorganisms

PCR on Environmental Samples



PROTOCOLS FOR VIRUS CONCENTRATION AND DETECTION BY RT-PCR

Environmental Virus Detection Workshop

Dr. Carol J. Palmer, Senior Scientist
Dr. Yu-Li Tsai, Scientist
G. Fred Bonilla, Research Technician

August 1 - 7, 1993
University of Hawaii
Honolulu, HI

ENVIRONMENTAL SCIENCES LABORATORY
COUNTY SANITATION DISTRICTS OF ORANGE COUNTY, CALIFORNIA

MEMBREX METHOD FOR CONCENTRATION OF SEWAGE AND OCEAN WATER (Ref. 1)

1. Place the feed tubing and the retentate tubing into the container holding the sample you want to concentrate.

2. Turn on the pump and the Membrex and adjust the flow rate to approximately 120 ml/min (about #5 on the Manostat peristaltic pump). We use a 100 KD pore size filter for virus concentration.

3. The LED will begin to scroll the options:
   1=Set RPM/RUN
   2=Set Max. PSI
   3=Set Run Time
   4=Recall Setup
   5=Save Setup

   Press 1 to enter the RPM/RUN mode.

4. After you press the "1" key you are presented with your first choice: Are you running the 200 cm^2 or the 400 cm^2 cartridge. If the incorrect cartridge size apears press "Change" then "Enter". If the correct size appears simply press "Enter".

5. After you select the cartridge size you are asked to select the run time "Routine = 0 min". Press "Enter" to allow the filtration to occur without an interruption or press "Change" and use the key pad to select a desired time then press "Enter".

6. Next you are asked to select the rotation rate "Speed = ?". If the desire speed appears (the Membrex recalls the last value used) press "Enter". If you wish to change the speed press "Change" then select the desired speed and press "Enter". The average speed used for ocean water is 1000 to 1250 RPM. The average speed used for sewage is 1500 to 1800 RPM.

7. The cartridge will start to rotate at 200 RPM and the screen will prompt you to "Adjust pressure then press enter" and will show you a pressure readout. **Make sure the Rotary Separation Unit is filled with liquid before the cartridge starts spinning.** Adjust the pressure with the nut which clamps the retentate line. When you have established the pressure you want to run press "Enter". The average pressure to begin a run is 5 to 6 psi. The pressure may increase as the run proceeds. Make sure it does not exceed 16 psi.

8. The cartridge will begin to spin at the speed you selected and the screen will display the system pressure ("P:"), the torque on the motor ("T:"), and the speed ("R:").

Clean Up and Storage

9. Upon completion of the run, collect the concentrated sample from the container, Separation Chamber, and tubing. Clean the system by running approximately 4 liters of 0.25% Sodium Hypochlorite followed by 4 liters of DIH_2O.

10. Store the membrane in a 0.1% Sodium Azide solution at 4°C. The membrane can be reused several times depending on the nature of the samples. To test the filter run DIH_2O through the membrex with the pressure valve completely open. A new filter will read about 2 psi. at a flow rate of 120 ml/min. If a reused filter reads over 10 psi. we recommend you change to a new filter.

DETECTION OF ENTEROVIRUSES AND HEPATITIS A VIRUS IN SEWAGE AND OCEAN WATER BY REVERSE TRANSCRIPTASE - POLYMERASE CHAIN REACTION

1. Concentration of Sewage for RT-PCR (Ref. 1)

1.1 Concentrate 100 ml of sewage to 4 ml using Centriprep-100 (Amicon, Inc., Beverly, Mass.) at 2,200 rpm (1,000 x g) at 4°C. Transfer the concentrate into 15-ml Falcon tubes.

1.2 Add an equal volume (4 ml) of chloroform-isoamyl alcohol (24:1) [Amresco] to each of the concentrated sewage samples. Vortex briefly. Centrifuge at 2,000 rpm (800 x g) at 4°C for 10 min and carefully collect the supernatant (upper aqueous phase). **Note:** Do not disturb the interphase and chloroform layer (lower organic phase).

1.3 Concentrate the 2 ml of supernatant to 100 μl (retentate) using a Centricon-100 (Amicon) at 2,200 rpm at 4°C.

1.4 Use 2 μl of retentate as template in RT-PCR. Store the retentates at -20°C.

2. Concentration of Ocean Water for RT-PCR (Ref. 1)

2.1 Concentrate 15 l of ocean water sample to 100 ml by vortex flow filtration (Membrex, Inc., Garfield, N.J.) as described on page 1.

2.2 Further concentrate the condensed (100 ml) samples to 100 μl using the above sewage protocols (steps 1.1-1.4).

3. Reverse Transcription of Viral RNA of Enteroviruses and Hepatitis A Virus

3.1 Prepare a cocktail mix by adding the reagents (RNA PCR Kit, Perkin-Elmer, Norwalk, Conn.) in the order and proportion shown. N = the total number of reactions performed.

Component	Volume	Final Concentration
$MgCl_2$ solution (25 mM)	4 μl x N	5 mM
10X PCR Buffer II (pH 8.3)	2 μl x N	1X
500 mM KCl		50 mM
100 mM Tris-HCl		10 mM
Sterile distilled water*	1 μl x N	--
dGTP (10 mM)	2 μl x N	1 mM
dATP (10 mM)	2 μl x N	1 mM
dTTP (10 mM)	2 μl x N	1 mM
dCTP (10 mM)	2 μl x N	1 mM
Random hexamers (50 μM)	1 μl x N	2.5 μM

3.2 Vortex the cocktail briefly and dispense 16 μl into each reaction tube.

3.3 Dispense 1 μl x N of reverse transcriptase (50 U/μl) and 1 μl x N of RNase inhibitor (20 U/μl) into a separate tube and store on ice.

3.4 Add 2 μl ($\leq$ 1 μg) of experimental samples (sewage or ocean water retentate). Heat at 99°C for 5 min. (Program #___)

3.5 Chill tubes immediately on ice for 2 minutes and add 2 μl of reverse transcriptase and RNase inhibitor mixture to each tube. Vortex the tubes and centrifuge them briefly.

3.6 Heat at 25°C for 10 min and then incubate at 42°C for 30 min. Heat to 99°C for 5 min then chill on ice. (Program #___)

4. Polymerase Chain Reaction on cDNA of Enteroviruses and Hepatitis A Virus

4.1 Prepare a cocktail mix (RNA PCR Kit, Perkin-Elmer) from the following:

Component	Volume	Final Concentration
$MgCl_2$ solution	4 µl x N	2 mM
10X PCR Buffer II (pH 8.3)	8 µl x N	1X
Sterile distilled water	66.5 µl x N	--
AmpliTaq DNA Polymerase	0.5 µl x N	2.5 U/100 µl
5' primer (EV-L; HAVC-L)	0.5 µl x N	0.3 µM
3' primer (EV-R; HAVC-R)	0.5 µl x N	0.3 µM
Total volume per sample	80 µl x N	

4.2 Dispense 80 µl of the PCR cocktail mix into each reverse transcription reaction tube. Change tips between additions to avoid sample carryover.

4.3 Vortex and centrifuge the tubes briefly and then add tubes to the thermal cycler.

4.4 Temperature cycling for sample amplification:

Program # _____

HOLD:	2 min at 95°C
CYCLE:	1 min at 95°C 1 min at 55°C 1 min at 72°C 40 cycles
HOLD:	7 min at 72°C
HOLD:	4°C forever

5. Agarose Gel Electrophoresis

Run the RT-PCR products (20-30 μl each) on a 2% SeaKem GTG agarose (FMC BioProducts, Rockland, Maine) gel in 1X TBE (50 mM Tris base, 50 mM Boric acid, 1 mM EDTA) buffer. Set constant voltage at 90-110 v and run for 1-2 h. (Time will vary based on the size of the gel.)

6. Southern Transfer

6.1 Nick amplified DNA in the ethidium bromide stained agarose gel by UV irradiation for 5 min.

6.2 Denature the gel with 300 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for two 25-min with shaking on a rotary shaker at 120 rpm.

6.3 Rinse the gel 3-4 times with distilled water.

6.4 Wash the gel for two 25-min with shaking using 300 ml of neutralizing solution (1.5 M NaCl, 0.5 M Tris, pH 8.0).

6.5 Soak the gel in 10X SSC (1X SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) for 25 min.

6.6 Soak Hybond-N+ positively charged nylon membrane (Amersham, Arlington Heights, Ill.) cut to gel size in 10X SSC for 10 min before blotting.

6.7 Use either a VacuumBlot (Millipore, Bedford, Mass.) or a PosiBlot (Stratagene, La Jolla, Calif.) blotting apparatus to transfer DNA onto the membrane as described by the manufacturer.

6.8 UV cross-link the DNA on the membrane for 2 min.

7. Slot Blotting

7.1 Denature 10 μl of the amplified DNA by heating at 99°C for 5 min and cooling at 4°C for 5 min via a programmed PCR thermocycler.

7.2 Add 100 μl of 20X SSC to the DNA samples and mix.

7.3 Add 100 μl of 20X SSC to the slots to wash the membrane.

7.4 Place the full amount (110 µl) of each DNA sample into a slot blot well, and pull the sample through the membrane using a vacuum.

7.5 Wash each well with 100 µl of 20X SSC.

7.6 Remove the membrane from the slot blotter and UV cross-link the DNA on the membrane for 2 min.

8. DIG Oligonucleotide 3'-end Labeling: Genius 5 Kit

8.1 Mix: 4 µl tailing buffer (Vial 1)
4 µl $CoCl_2$- solution (Vial 2)
5 µl oligonucleotide (100 pmol oligonucleotide added)
1 µl DIG-ddUTP solution (Vial 3)
1 µl terminal transferase [50 units] (Vial 4)
Make up to final volume of 20 µl with distilled water.

8.2 Incubate at 37°C for 15 min.

8.3 Mix 1 µl of glycogen solution (Vial 8) with 200 µl of 0.2 M EDTA, pH 8.0. Add 2 µl of the dilution to the reaction mixture.

8.4 Add 2.5 µl of 4 M LiCl and mix.

8.5 Add 75 µl EtOH (100 %).

8.6 Store at -20°C for 2 h or -70°C for 30 min.

9. Hybridization Protocol for EV-IN and HAVC-IN Probes (Ref. 1)

9.1 **Prewash**: Seal the membrane with 20 ml (10 ml/100 cm^2) of 0.1X SSC/0.5% SDS and incubate at 65°C with shaking for 1 h (for Southern blotted membrane only).

9.2 **Prehybridization**: Prehybridize with 30 ml of hybridization solution (5X SSC, 0.1% Sarkosyl, 0.02% SDS, 2% blocking reagent) at 60°C for 2 h.

9.3 **Hybridization**: Hybridize the membranes overnight with 8 ml of hybridization plus 100 µl of EV-IN or HAVC-IN probe (100 pmol) at 60°C.

9.4 **Wash**: Wash with 50 ml of 2X SSC/0.1% SDS for 5 min twice at room temperature, followed by two 15-min washes of 50 ml 0.1X SSC/0.1% SDS at 50°C for EV-IN or at 47°C for HAVC-IN.

9.5 Air dry the membranes for detection.

10. Detection of EV-IN and HAVC-IN Probes: Genius 3 Kit

Note: All the following steps are carried out in a hybridization oven at room temperature

Solutions:

Buffer 1: 100 mM Tris-HCl; 150 mM NaCl; pH 7.5 (20°C).

Buffer 2: Blocking reagent, 3% (w/v), (vial 11) in Buffer 1 (The blocking reagent does not dissolve very rapidly, so prepare the solution 1 h in advance and dissolve at 50-70°C. **Note:** The solution will remain turbid).

Buffer 3: 100 mM Tris-HCl; 100 mM NaCl; 50 mM $MgCl_2$; pH 9.5 (20°C).

10.1 Wash the membranes with 60 ml of Buffer 1 for 2 min.

10.2 Block the membranes with 60 ml of Buffer 2 [1% blocking reagent (vial 11) in Buffer 1] for 30-60 min.

10.3 Pour out Buffer 2 and replace with 12 ml of dilute antibody conjugate [12 ml Buffer 2 + 2.4 μl conjugate (Vial 8)] for 30 min.

10.4 Wash twice with 50 ml Buffer 1 for 15 min.

10.5 Rinse with 50 ml Buffer 3 for 2 min.

10.6 Carefully cover the membranes with plastic wrap, minimizing the handling of the membrane as much as possible. **Wear powder free gloves.** Use a sterile transfer pipet to place several drops of Lumi-Phos 530 on the membrane (0.5 ml/100 cm^2). Rub the covered membranes with Kimwipes to remove any air bubbles from the membranes.

10.7 Expose to X-ray film (Kodak XAR) for 10-30 min.

10.8 Develop the X-ray film.

11. PCR Primers and An Internal Probe for Enteroviruses (Ref. 2)

EVR	5'-ACCGGATGGCCAATCCAA-3'
EVL	5'-CCTCCGGCCCCTGAATG-3'
EV-IN	5'-ACTACTTTGGGTGTCCGTGTTTC-3'

12. PCR Primers and An Internal Probe for Hepatitis A Virus (Ref. 3)

HAVCR	5'-CTCCAGAATCATCTCCAAC-3'
HAVCL	5'-CAGCACATCAGAAAGGTGAG-3'
HAVC-IN	5'-TTGCTCCTCTTTATCATGCTAT-3'

REFERENCES

1. **Tsai, Y.-L., M.D. Sobsey, L.R. Sangermano, and C.J. Palmer.** 1993. Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection using reverse transcriptase - polymerase chain reaction. Appl. Environ. Microbiol. 59: (in press).

2. **De Leon, R., C. Shieh, R.S. Baric, and M.D. Sobsey.** 1990. Detection of enteroviruses and hepatitis A virus in environmental samples by gene probes and polymerase chain reaction, p.833-853. In Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, San Diego, Calif. American Water Works Association, Denver.

3. **Schwab, K.J., R. De Leon, R.S. Baric, and M.D. Sobsey.** 1991. Detection of rotaviruses, enteroviruses and hepatitis A virus by reverse transcriptase-polymerase chain reaction, p.475-491. In Advances in water analysis and treatment. Proceedings of the Water Quality Technology Conference, Orlando, Florida. American Water Works Association, Denver.

Concentration of Viruses and Bacteria in Environmental Water Samples Using the Membrex Vortex Flow Filtration System

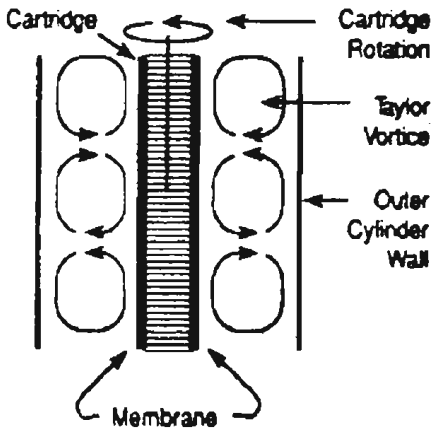
Purpose:

The following is a condensed version of the Membrex operators manual. This protocol describes a streamlined method for concentrating viruses, bacteria, genetic material and particulates with a molecular weight greater than 100, 000 kD from large volumes (1 to 40 liters) of water. This concentration method utilizes vortex flow filtration (VFF) which is useful for the both PCR and cultural analysis.

Introduction:

The Membrex (Benchmark Gx) vortex flow filtration system is a new type of filtering system that prevents filter membranes from becoming clogged. Because of the rotary motion of a special filter membrane, particulates do not come in contact with membrane surfaces. This is accomplished by Taylor vortices which are created by the rotational movement of the filter membranes which prevents particulates from contacting membrane surfaces (Figure 1). The combination of a special filter membrane (Membrex Ultra Filic) and a rotary mechanism, allows the Membrex system to separate agents having a molecular weight greater than 100, 000 kD from various types of environmental waters.

Figure. 1



The Membrex system is also semi-automated and is controlled by a microprocessor (Guardian), which constantly monitors system pressure. If the system pressures exceed or fall below certain user-selected pressure limits then the Membrex system will shut off. The microprocessor also monitors the

system torque and rotation rates which permits the Membrex system to respond to increasing feed viscosity.

System Description:

The Membrex system has two basic components, an electronic control unit (ECU) and a rotary separation unit (RSU)(Figure 2 & 3).

A. Electronic Control Unit:

The ECU contains all of the system electronics and software, the magnetic motor and its associated controls (Figure 1). The primary function of the ECU is to control the rotation speed of the membrane cartridge within the rotary separation unit and to monitor the pressure being applied the RSU.

Figure.2

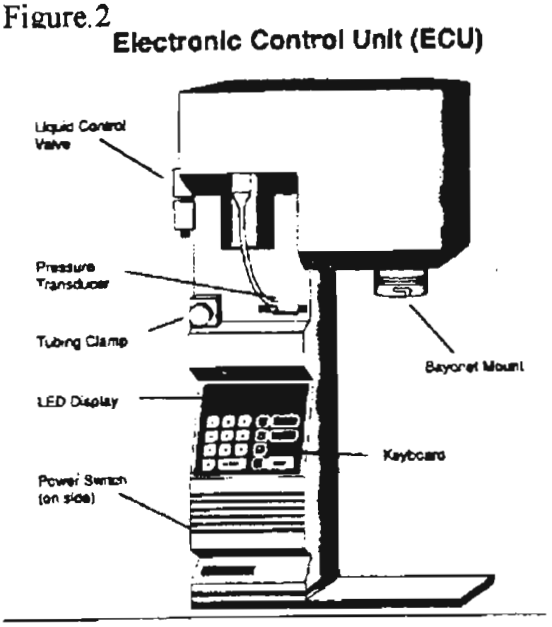
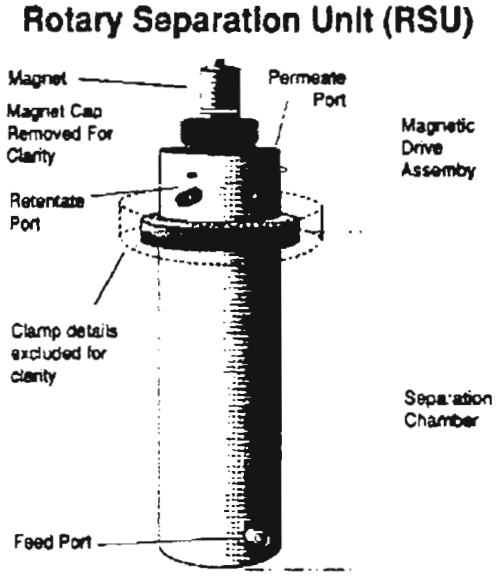


Figure. 3



B. Rotary Separation Unit (RCU) :

Separation takes place within the RSU. The RSU is comprised of four separate components: a magnetic drive assembly, a membrane cartridge, a separation chamber, and a sanitary fitting clamp (Figure 3). In addition the magnetic drive assembly housing contains the permeate and retentate ports and the separation chamber contains the feed port.

C. Accessories:

The primary accessories needed are a variable speed peristaltic pump which is used to recirculate feed material and generate system pressure, and an external gas pressure source (nitrogen) which is used to provide pressure during the small batch mode.

System Set-up:

A. Filter Storage:

The filter should be stored in 0.1% (w/v) sodium azide solution at 4°C. If the filter will be used within 1-2 days it can be stored in deionized water (Note: The filter should never be allowed to dry out).

B. Installation of Filter Cartridge:

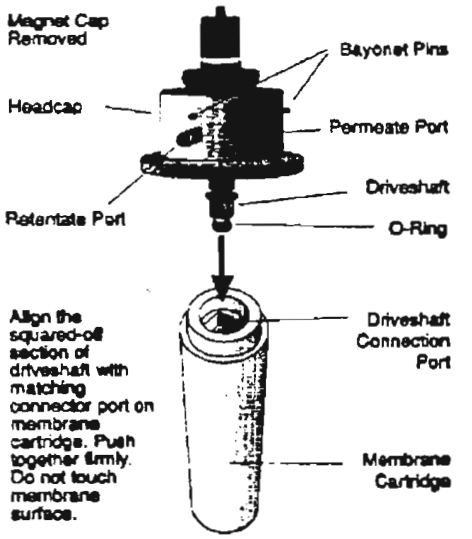
1. Wear gloves when handling the filter.

2. Open and remove filter from storage container. Avoid direct contact with the membrane surface by handling the driveshaft connection port on the top of the filter.

3. Stand the filter on a flat dry surface with the driveshaft connection port of the membrane cartridge facing up.

Figure. 4

Attaching Membrane Cartridge to Drive Assemby



4. Attach the drive assembly to the membrane cartridge by aligning the square end of the driveshaft with the square connection port on the filter and carefully insert the driveshaft into the port. Push down on the drive assembly to create a snug seal between the shaft and cartridge.

5. Position the gasket into the groove on the top edge of the separation chamber. Gently insert the filter into the separation chamber. Align the gasket with respective grooves on the bottom of the headcap. Align retentate port on the headcap with the feed port on the separation chamber.

6. Fasten the headcap to the separation chamber with the tri-cloved clamp. Position the clamp so that the handle does not block either the permeate port or the retentate port. Hand tighten the clamp until it is snug. Do not over tighten the clamp.

Attachment of the RSU to ECU:

1. The rotary separation unit attaches to the bayonet mount on the ECU. Orient the separation chamber so that the retentate port is facing forward.

2. Align the bayonet pins with the slots in the mount. Insert the drive assembly up to the left in order to lock the pins. A slight resistance should be felt when the headcap is lock. The pins should be all the way to the left in the slots.

System Operation:

The Membrex system can be configured to do four types of filtration. However, only two of these types of filtration are used for concentrating large volumes environmental samples for PCR and cultural analysis. The two types of filtration used are; 1) recirculation and 2) small batch, for samples volumes less than 70ml.

The recirculation mode is used for concentration when the feed solution is to be returned to the feed container during the operation. A pump is used to deliver the solution to the RSU. The small batch mode is used when the solution to be concentrated is less than 70ml. When using the small batch mode the solution is loaded into the RSU by a syringe. An external gas source (e.g. nitrogen) is required in order to provide the pressure for the concentration. The Table 1 below shows ranges of feed sample volumes achievable with each type of filtration.

Table 1. Feed Sample Volumes

Type of Filtration	Initial Feed Volume	Final Retentate Volume
Recirculation	100ml-25L	200ml
Small Batch	200ml	45-60ml

Environmental samples for PCR and cultural analysis are prepared by concentrating 5 to 40 liters volumes to approximately 50 ml. This is accomplished by using the recirculation mode followed by the small batch mode. The following section gives a detailed description of the recirculation and small batch modes as individual procedures.

Keyboard Operation:

The keyboard is divided into two parts: the light emitting diode (LED) and touch keypad. The LED displays the operating parameters and error messages, if any occur. The keypad is located below the LED and is used for setting the operating parameters and controlling the filtering process. The operating parameters of 1500rpm and 7-9 psi should already be saved in the memory of the Membrex and do not need to be set at each usage. If for some reason the operating parameters are not set, review the Membrex operators manual to set the proper parameters. Once the parameters are saved to the memory, only two keys, the "1" and "ENTER" are used during the filtration process.

System Start-Up:

1. Check that the power switch located on the left side of the Membrex is turned off. Connect the power cable to the male socket on the lower back panel of the system.

2. Connect the short 1ft. pump connector cable to the female socket on the lower back panel of the Membrex. The pump will receive its power from the ECU. In the event that there is a system shutdown due to low or high pressure, the pump will also shut off.

3. Turn the power switch on. The LED will display the system name and begin to scroll the operations menu.

RECIRCULATION

Tubing Attachment

Referring to the diagram (Figure 5), attach the permeate tubing , feed tubing, retentate tubing, pressure transducer tubing and valve connector tubing to the system in the following manor.

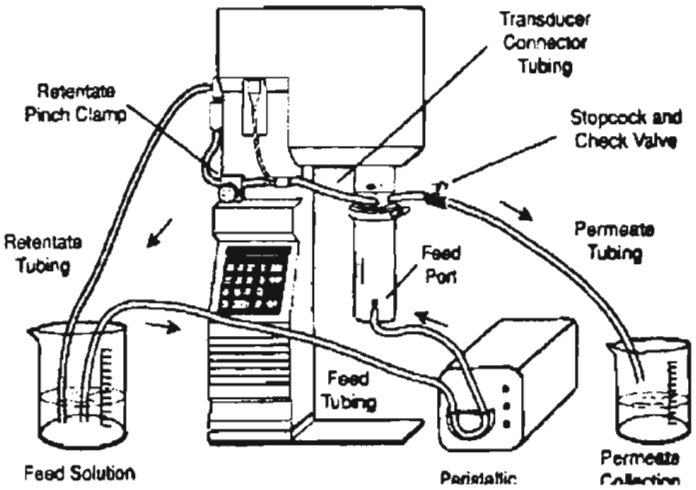
1. Locate the transducer connector tubing. It is a 5 1/2 inches long piece of Tygon tubing with one metal connector and one plastic connector. Attach the metal luer lock to the retentate port. Attach the plastic luer lock to the right port of the pressure transducer.

2. Locate the retentate tubing. It is about 48 inches long and has no connectors on either end. Push one end into the quick connector on the liquid valve. Put the other end into the feed vessel.

3. Locate the permeate tubing. It is about 48 inches long and has one metal luer connector and one end with no connector. Attach the metal luer lock on the permeate tubing to the permeate port on the headcap.

4. Locate the liquid valve connector tubing. It is about 5 1/2 inches long and has a female lure on one end and a male luer on the other end. Connect the female luer to the left side of the pressure transducer and the male luer to the bottom of the liquid pressure valve. Note: The direction of flow through the liquid pressure valve is in through the bottom and out through the front.

Figure 5 **Recirculation Configuration**



5. Locate the pump tubing. It is about 48 inches long and is made of Norprene. Mount this tubing into the pump head following the instructions provided by the pump manufacturer. Attach the plastic luer lock to the feed port. Adjust pump tubing around the pump head to avoid excessive wear on one area of tubing.

Operation

1. Check that all luer lock fittings are properly connected and connectors are tight.

2. Unscrew the top nut on the liquid pressure valve. There should be no pressure on the membrane when the system starts.

3. Fill the separation chamber with feed sample by turning the pump on. Make sure your pump is filling the cylinder from the bottom.

4. Set the pump to give a flow rate of 200ml/ min. This can be changed depending on the experiment.

5. Recirculate feed through retentate line for 1-2 min. Do not apply any pressure at this time.

6. Close the stopcock on the permeate tubing.

7. Start the system by pressing key "1" to enter the run mode. And watch the pressure display.

8. Slowly increase the pressure using the liquid pressure valve, until the display prompts a reading of 5-7 psi.

9. Press the "enter" key (the Membrex will ramp up to the selected sped of 1500 rpm) and open the stopcock in the permeate line. Start collecting permeate in a separate container. (If the "enter" key is not pressed within five minutes, the Membrex will shut off and display the message " Operator Timeout". To start up again press clear to shut off the alarm and the "1" key to enter the run mode again.)

IMPORTANT: Always keep a minimum of 5 psi in the system while the cartridge is rotating above 1000 rpm.

10. Increase the pressure to an appropriate level (between 7-9 psi). (If the system pressure exceeds 12 psi or falls below 5 psi, then the system will shut down.. To start up again press clear to shut off the alarm and the "1" key to enter the run mode again.)

Shutdown

1. Turn the power switch off to stop filtration.

2. Decrease the pressure by opening the liquid pressure valve.

3. Remove the retentate tubing from the sample container.

4*. Drain all retentate fluid from the rotary separation chamber by reversing the pump direction.

5*. Unlock the permeate tubing from the headcap and drain it.

6*. If a small amount of sample remains in the chamber, disconnect the pump tubing from the feed port. Connect a disposable syringe to the feed port and remove the small volume with the syringe.

7*. The membrane cartridge can now be cleaned (See filter cleaning section).

*(Note: When the recirculation mode is followed by the small batch (e.g. PCR concentrations) omit steps 4,5,6 and 7 in the recirculation mode shutdown.)

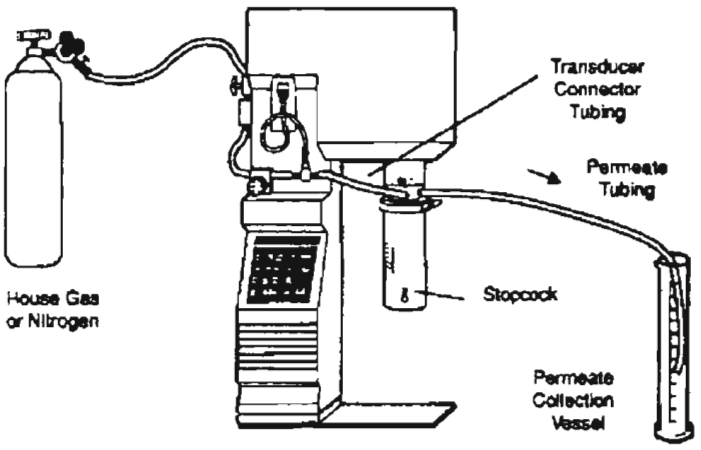
SMALL BATCH: Filtration for Samples Volumes Less that 70 ml

Tubing Attachment

Refer to the system diagram (Figure 6) and attach the permeate tubing, gas-inlet connection and transducer connection tubing to the system.

1. Locate the transducer connector tubing. It is about 5 1/2 inches long with one metal luer connector and one plastic luer connector. Attach metal luer to the retentate port. Attach plastic luer lock to the right port of the pressure transducer.

Figure 6 **Small Batch — Less than 70 ml**



2. Locate gas inlet connection tubing. It is about 5 1/2 inches long and has two plastic connectors on it. Attach plastic luer lock on gas inlet tubing to the left port of the pressure transducer. Run the tubing through the pinch clamp and attach the other plastic luer lock to base port on gas control valve on the left side of the system. Make sure the pinch clamp is open.

3. Locate the permeate tubing. It is about 48 inches long and has one metal connector on one end and no connector on the other end. Attach metal luer lock on permeate tubing to the permeate.

4. Attach house gas line to top of gas control valve.

5. Attach stopcock to feed port.

Operation

1. Check that all luer lock syringe.

5. Reattach gas inlet tubing to base of gas control valve.

6. Start the system by pressing key "1" to enter the run mode. Respond to the prompts in the run.

7. Slowly turn house gas on. Pressure should be 5-9 psi. Open gas control valve.

IMPORTANT: Always check fittings for proper connections. Disconnect gas inlet tubing connection at base of gas control valve.

2. Attach syringe to stopcock valve on the feed port.

3. Slowly inject feed material into RSU. Maximum feed sample capacity in the chamber and tubing is 80ml.

4. Close stopcock valve and remove keep a minimum of 5 psi in the system while the cartridge is rotating above 1000 rpm.

8. Press enter to get the Membrex to ramp up to the running speed. If the enter key is not pressed within five minutes, the Membrex will shut off and display " Operator Timeout". To start up again, press the clear key to shut off the alarm and the "1" key to enter the run mode again.

9. Concentrate to desired volume. A final volume of 50ml is approximately 1/3 of the separation chamber. (If the system pressure exceeds 12 psi or falls below 5 psi, then the system will shut down. ". To start up again press clear to shut off the alarm and the "1" key to enter the run mode again.)

Shutdown

1. Turn the power switch off to stop filtration.

2. Close the gas control valve.

3. Shut off the gas source.

4. Disconnect gas inlet tubing attached to base of gas control valve.

5. Unlock the permeate tubing and drain it.

6. Attach a syringe to the stopcock and drain all retentate fluid in separation cylinder by aspirating with the syringe.

7. The membrane cartridge can now be cleaned (See filter cleaning section).

CONCENTRATION OF ENVIRONMENTAL SAMPLES FOR PCR ANALYSIS

Preparing Filter for Use:

Set the system up in the recirculation configuration. After the filter cartridge is in place, clean with the system with 2 liters of deionized water. The first liter is not recirculated and is run through with conditions of no pressure or rpm. The second liter is recirculated through the system for 30 min. The parameters are 1500rpm and 7-9 psi, with a pump sitting of 200ml per min. The small batch mode is used to flush all but about 40mls out of the membrane housing. A syringe can be used to remove the remaining water from the membrane housing.

Filtration of Sample

This protocol is designed to concentrate 5 to 40 liter samples down to approximately 50ml. Follow the protocol for recirculation mode. Remember not to run the system over 1000rpm unless there is at least 5psi backpressure.

Start out with both the permeate tubing and the retentate tubing going back into the feed solution vessel. Pressure is adjusted with the retentate pinch clamp. When pressure has been adjusted correctly and the system is running smoothly, move the permeate tubing to the permeate collection vessel. This will eventually be discarded. Under this conditions the system will filter about 8 liters per hour. If the pressure goes above 12 psi it will automatically shut down and will have to be restarted.

Continue filtering until all of the sample is in the pump tubing, but turn the system off before air gets pumped into the filter housing. Turn off the Membrex unit first, then immediately followed by the peristaltic pump. Close the stopcock at the feed port and disconnect the feed tubing. Turn on the pump

and collect any sample remaining in the tubing. Collect this volume and the remaining in the feed vessel, in a 60ml syringe and attach to the feed port.

Set the unit according to small batch mode. Apply a small amount of back pressure to the system using the nitrogen gas, start the membrane on initial spin speed, open the stop cock and inject the sample from the syringe into the filter housing. Close the stopcock and run the Membrex at 1500rpm and 7-9psi using the nitrogen gas. Run until there is approximately 50ml remaining in the filter housing. This volume is about 1/3 of the filter housing. Turn off the Membrex and nitrogen tank and relieve the nitrogen pressure. Harvest the concentrated sample from the filter housing using a syringe.

Cleaning the System after a Sample

Cleaning with Bleach

Use 2 liters of laundry bleach solution (16ml bleach per liter deionized water) to sanitize the system when using the recirculation mode. The first liter is not recirculated and the first 500ml is run through with zero pressure and rpm. The remaining bleach is recirculated through the system at 1500rpm and 7-9psi. Remove all but 40ml using the small batch mode and a syringe.

Neutralizing with Sodium Thiosulfite:

Stock solution is 10g per liter deionized water.

Working solution is 10 mls of stock per liter of deionized water.

Rinse 1 liter though the system. The first 500ml with zero pressure and rpm and the remainder at 1500rpm and 7-9psi. Remove all but 40ml using the small batch mode and the remainder with a syringe.

Rinse with Distilled Water:

Rinse with 2 liters of distilled water. The first 500ml with zero pressure and rpm and the remainder at 1500rpm and 7-9 psi. Remove all but 40ml using the small batch mode and the remainder with a syringe.

Storage:

Using gloves, the filter should be removed from the headcap and stored in a 0.1% (w/v) sodium azide solution at 4°C. All tubing and the separation chamber should be drained and laid out at room temperature to dry until the next usage.

Appendix E

Two-Phase Separation Method for Concentrating Virus

Materials:
- 4 liters (1 gal.) sample
- Separatory funnel
- Conical centrifuge tubes
- Polyethylene Glycol - 6000 (PEG-6000)
- Sodium Dextran Sulfate (SDS)
- Sodium Chloride (NaCl)
- Potassium Chloride (Kcl)

Preparation: Weigh out appropriate amounts of PEG, SDS, and NaCl[1] as listed in procedures below.

Procedures:

A. Sewage Effluent

1. Clarify sample through AP25 filter.
2. Check pH. If it is not between 7.0 and 7.3, adjust using 0.6N HCl or 0.5N NaOH.
3. To 4 liter (1 gal) sample add:

258g PEG	(64.5g/L)
8g SDS	(2.0g/L)
70g NaCl	(17.0g/L)

4. Mix thoroughly until well dissolved.
5. Pour sample into separatory funnel. Let separate overnight at 4°C.
6. After separation, collect the lower phase (DS) and interphase (Int) in a conical tube.
7. Centrifuge at 2000 rpm for 10 min.
8. Remove and discard any PEG floating on top.
9. Further concentrate DS and Int phases by one of the following methods:

a. Sodium Chloride (separation)
   1) Add 50 mg NaCl to each 1 ml of DS + Int.
   2) Vortex to mix until NaCl is well dissolved.
   3) Refrigerate overnight.
   4) Centrifuge at 2000 rpm for 10 min. for clearer separation.
   5) Collect upper (NaCl) phase with Pasteur pipette.
   6) Dilute a portion 1:7 (.5 in 3ml) with sterile distilled water.
   7) Dialyze the remainder vs. PBS overnight, or quick dialyze (1 hr. dist. H_2O, 1 hr. PBS).

b. Potassium Chloride (precipitation)
   1) Add 50 mg KCl to each 1 ml of DS + Int.
   2) Vortex to mix until KCl is well dissolved.
   3) Refrigerate 2 hours.
   4) Centrifuge at 2000 rpm for 10 min.

[1] For seawater sample eluates or glycine eluates, do not weigh out NaCl until the conductivity of the sample has been measured.

5) Collect upper aqueous phase.
6) Dilute a portion 1:7 (.5 in 3ml) with sterile distilled water.
7) Dialyze remainder. (Sample may be concentrated further prior to this step by PEG dialysis).

10. HA filter and infect samples.

B. Seawater Eluate (Aquella-glycine)

1) Adjust pH as in A-2. (No clarification is necessary)
2) Measure conductivity of sample. (Cs)
3) Add x gm. NaCl according to the following equation:

$$x = \frac{130{,}000 - Cs}{130{,}000 - Cs} \times (v) \times (17.5)$$

where v is vol. of sample in liters.

4) Measure conductivity of the sample, if not in the vicinity of 130,000, repeat step 3.
5) Add PEG and SDS as in A-3. Follow procedures for A (4 - 10).

Appendix F

Protocol to Extract Bacterial DNA and Virus RNA

1. Cetyltrimethylammonium Bromide-Sodium Chloride (CTAB/NaCl) Method of Extracting Bacterial DNA

1.1 Add 300 μl of TE buffer to 260 μl of sample concentrate and shake.[1]

1.2 Add 36 μl of 10% SDS and 4 ul of 20 mg/ml proteinase K, mix, and incubate 1 hr at 37°C.

1.3 Add 100 μl of 5 M NaCl and mix thoroughly. Add 80 μl CTAB/NaCl solution, mix, and incubate 10 min at 65°C.

1.4 Add 800 μl phenol/chloroform/isoamyl alcohol[2] (25:24:1) , mix, and microcentrifuge 5 min at 12,000 x g.

1.5 Transfer supernatant (making sure none of the white interface is aspirated) to a fresh microcentrifuge tube containing 700 μl chloroform/isoamyl alcohol (24:1). Mix and centrifuge for 5 min at 12,000 x g. Transfer supernatant to a fresh tube.

1.6 Add equal volume 100% isopropanol, mix and incubate 30 min at -70°C or 1hr at -20'C. Centrifuge at 12,000 x g for 15 min at 4°C. The DNA pellet should be slightly visible.

1.7 Discard supernatant carefully, add 500 μl of 70% ethanol and centrifuge at 12,000 x g for 15 min at 4°C. Discard supernatant and resuspend in 100 μl TE buffer.

Ref. Ausubel, Fredrick M. *et. al.* 1989.
Short Protocols in Molecular Biology

[1]Adjust volume accordingly to obtain a final volume of 560µl but use a minimum 250µl TE.
[2]Phenol is very toxic and must be added in a fume hood. Avoid inhalation and contact with skin.

2. Total RNA Extraction from RNA Viruses

2.1 Add 3 ml of denaturing solution to virus concentrate (0.5 ml) and vortex vigorously.

2.2 Add 0.24 ml of 2 M sodium acetate (pH 4.0) and mix thoroughly by inversion.

2.3 Add 2.4 ml phenol:chloroform:isoamyl alcohol mixture, mix by inversion 2-3 times and shake vigorously for 10 sec. Aliquot the homogenate into 6 microfuge tubes and chill on ice for 15 min.

2.4 Microcentrifuge at 14,000 rpm for 10 min (or at 12,000 rpm for 20 min) at 4°C.

2.5 Transfer aqueous phase to a fresh tube. Mix with 1.0 ml isopropanol. Place at -20°C for at least 1 hr to precipitate RNA.

2.6 Spin at 14,000 rpm for 15 min at 4°C. Pool the RNA pellet into 2 microfuge tubes.

2.7 Resuspend the RNA pellet in 0.5 ml denaturing solution and vortex until the RNA is dissolved. In some instances such as seeded sewage, heating to 65°C may be required to resuspend the pellets. Heating should be done for as short of time as possible.

2.8 Precipitate with 0.5 ml cold isopropanol at -20°C for at least 30 min.

2.9 Centrifuge at 14,000 rpm for 10 min at 4°C.

2.10 Wash the pellet with 0.5 ml 75% ethanol, centrifuge at 14,000 rpm for 10 min.

2.11 Resuspend RNA in 100-200μl of DEPC-treated dH_2O.

Guanidine denaturing solution:
4 M Guanidium Thiocyanate
25 mM Sodium Citrate, pH 7.0
0.5% Sarcosyl
0.1 M 2-mercaptoethanol

Ref. Chomczynski, P and N. Sacchi. 1987. Anal. Bioch. **162,** 156-159

Appendix G

Protocol For DNA-PCR and RT-PCR

1. DNA-PCR: Bacteria

Lower Mix		Volume (ul/100uL) Total Reaction	Volume (uL) for ( ) Reactions		Final Concentration
10X AmpliTaq Buff.		5			1X
MgCl (25 mM)		10			2.5 mM
dATP (10 mM)		2			200 uM
dCTP "		2			"
dGTP "		2			"
dTTP "		2			"
Water		23			
Primer 1 (30 uM)		2			0.6 uM
Primer 2 (30 uM)		2			0.6 uM
Total Volume		50			
Upper Mix					
Water		34.5			
10 X AmpliTaq Buffer		5			1X
AmpliTaq DNA Polymerase		0.5			2.5 U/100ul
Total Volume		40			

1.1 Use the template above and make a lower master mix for the appropriate number of reactions (n).

1.2 Aliquot 50μl into each PCR thin-walled reaction tubes.

1.3 Add 10 μl of experimental sample and place in the thermocycler at 95°C for 5 min.

1.4 While the sample is the heat block, prepare the upper mix and keep on ice.

1.5 Remove the samples from the heat block and add 40 μl of the upper mix as quickly as possible.

1.6 Place tray back in the thermocycler (still at 95°C) and select a run program.

Bacteroides (Program # ___)

95°C 5 min.

94 °C 1 min
53 °C 30 sec
72 °C 1 min
35 cycles
72°C 7 min
4°C forever

E. coli (Program # ____)

94°C 3 min.

94 °C 1 min
50 °C 30 sec
35 cycles
4°C forever

2. Reverse Transcription PCR: RNA Viruses

2.1 Prepare a cocktail mix by adding the reagents (RNA PCR Kit, Perkin-Elmer, Norwalk, Conn.) in the order and proportion shown. N = the total number of reactions performed.

FOR 30 ul REACTIONS

Component	Volume	Final Concentration
$MgCl_2$ solution (25 mM)	6 μl x N	5 mM
10X PCR Buffer II (pH 8.3)	3 μl x N	1X
500 mM KCl		50 mM
100 mM Tris-HCl		10 mM
dGTP (10 mM)	2 μl x N	1 mM
dATP (10 mM)	2 μl x N	1 mM
dTTP (10 mM)	2 μl x N	1 mM
dCTP (10 mM)	2 μl x N	1 mM
Downstream primer (25μM) {or Random hexamers}[3]	2 μl x N	0.5 μM

2.2 Vortex the cocktail briefly and dispense 19 μl into each reaction tube.

2.3 Dispense 1.5 μl x N of reverse transcriptase (50 U/μl) and 1.5 μl x N of RNase inhibitor (20 U/μl) into a separate tube and store on ice.

2.4 Add 8 μl ($\leq$ 1 μg) of experimental sample to each reaction tube and heat at 99°C for 5 min. (Program #___)

2.5 Chill tubes immediately on ice for 2 minutes and add 3 μl of reverse transcriptase/RNase inhibitor mixture to each tube. Vortex the tubes and centrifuge them briefly.

2.6 Heat at 25°C for 10 min and then incubate at 42°C for 30 min. Heat to 99°C for 5 min then chill on ice. (Program #___)

---

[3]If Random hexamers are used be sure to add both primers in step 4.7.

2.7 Prepare a cocktail mix (RNA PCR Kit, Perkin-Elmer) from the following:

Component	Volume	Final Concentration
$MgCl_2$ solution	4 μl x N	2 mM
10X PCR Buffer II (pH 8.3)	8 μl x N	1X
Sterile distilled water	55.5 μl x N	--
AmpliTaq DNA Polymerase	0.5 μl x N	2.5 U/100 μl
5' primer (25 μM)	2 μl x N	0.5 μM
Total volume per sample	70 μl x N	

2.8 Dispense 70 μl of the PCR cocktail mix into each reverse transcription reaction tube. Change tips between additions to avoid sample carryover.

2.9 Vortex and centrifuge the tubes briefly and then add tubes to the thermal cycler.

2.10 Temperature cycling for sample amplification:

Program # _____

HOLD: 3 min at 95°C

CYCLE: 1 min at 95°C
45 sec at 55°C *(For Enteroviruses)*
45 sec at 72°C
35 cycles

HOLD: 7 min at 72°C
HOLD: 4°C forever

Appendix H

Protocol to Detect PCR Products

1. Agarose Gel Electrophoresis

1.1 Prepare a 2% SeaKem GTC agarose gel in 1X TBE (50 mM Tris base, 50 mM Boric acid, 1 mM EDTA) buffer. Microwave the agarose in the buffer for 2 ½ minutes or boil over a flame with constant stirring until agarose dissolves.

1.2 Add 0.5 µg/ml of ethidium bromide[4] and stir by swirling beaker lightly. Place in 60-65 °C water bath for 10 min.

1.3 Load the PCR or RT-PCR products (20-30 μl each) with 10% loading buffer into each well very carefully. Set constant voltage at 90-110 v and electrophorese for 1-2h. (Time will vary based on the concentration of the gel and the size of the fragments.)

1.4 View gel under UV irradiation[5] and potograph as necessary. If the amplified bands have not run sufficiently, do *not* re-expose gel to UV. Place back into electrophoresis chamber and continue the run quickly.

---

[4] Ethidium Bromide is a suspected mutagen. Handle with gloves and dispose of the gel and the buffer into a sealed container for proper decontamination. Also, keep EtBr in a dark container at 4°C.

[5] Be careful not to expose eyes nor skin to UV irradiation.

2. Southern Transfer

2.1 Nick the amplified DNA in the ethidium bromide stained agarose gel by exposing it to UV irradiation for 5 min.

2.2 Denature the gel with 300 ml of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 25 min. while shaking on a rotary shaker at 120 rpm. Discard solution.

2.3 Repeat step **6.2.**

2.4 Rinse the gel 3-4 times with distilled water.

2.5 Wash the gel with 300 ml of neutralizing solution (1.5 M NaCl, 0.5 M Tris, pH 8.0) for 25 min. while shaking on a rotary shaker at 120 rpm. Discard solution.

2.6 Repeat step **6.5.**

2.7 Soak the gel in 10X SSC (1X SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) for 25 min.

2.8 Soak the Hybond-N+ positively charged nylon membrane and two pieces of filter paper (cut slightly larger than the gel size) in 10X SSC for 10 min. before blotting.

2.9 Use either a VacuumBlot (Millipore, Bedford, Mass.) or a PosiBlot (Stratagene, La Jolla, Calif.) blotting apparatus to transfer DNA onto the membrane as described by the manufacturer.

2.10 UV cross-link the DNA onto the membrane for 2 min.

3. Slot Blotting

3.1 Denature 10 - 20 μl of the amplified DNA by heating at 99°C for 5 min and cooling at 4°C for 5 min with a programmed PCR thermocycler. (A boiling water bath may be substituted if necessary).

3.2 Add 100 μl of 20X SSC to the DNA samples and mix by vortexing.

3.3 Wash the membrane by adding 100 μl of 20X SSC to each slot and passing it through under vacuum.

3.4 Place the full amount (110-120 μl) of each DNA sample into a slot blot well, and pull the sample through the membrane using a vacuum.

3.5 Wash each well with 100 μl of 20X SSC.

3.6 Remove the membrane from the slot blotter and UV cross-link the DNA on the membrane for 2 min.

4. DIG Oligonucleotide 3'-end Labeling: Genius 5 Kit

4.1 Mix: 4 μl tailing buffer (Vial 1)
4 μl $CoCl_2$- solution (Vial 2)
5 μl oligonucleotide (100 pmol oligonucleotide added)
1 μl DIG-ddUTP solution (Vial 3)
1 μl terminal transferase [50 units] (Vial 4)
Make up to final volume of 20 μl with distilled water.

4.2 Incubate at 37°C for 15 min.

4.3 Mix 1 μl of glycogen solution (Vial 8) with 200 μl of 0.2 M EDTA, pH 8.0. Add 2 μl of the dilution to the reaction mixture.

4.4 Add 2.5 μl of 4 M LiCl and mix.

4.5 Add 75 μl EtOH (100 %).

4.6 Store at -20°C for 2 h or -70°C for 30 min.

4.7 Centrifuge at 12,000 × g for 15 min at 4°C. Discard supernatant and resuspend in 100 μl TE buffer.

5. Hybridization Protocol for Digoxigenin-labeled Probes

5.1 **Prewash**: Seal the membrane with 20 ml (10 ml/100 cm^2) of 0.1X SSC/0.5% SDS and incubate at 65°C with shaking for 1 hr. (This is for Southern blotted membranes only. For slot-blotted membranes begin with prehybridization step).

5.2 **Prehybridization**: Prehybridize with 30 ml of hybridization solution (5X SSC, 0.1% Sarkosyl, 0.02% SDS, 2% blocking reagent) at 60°C for at least one hour.

5.3 **Hybridization**: Hybridize the membranes overnight with 10 ml of hybridization plus 100 μl of EV-IN or HAVC-IN probe (100 pmol) at 60°C[6].

5.4 **Wash 1**: Wash twice with 50 ml of 2X SSC/0.1% SDS for 5 min at room temperature.

5.5 **Wash 2**: Wash twice with 50 ml 0.1X SSC/0.1% SDS for 15 min at 50°C for EV-IN or at 47°C for HAVC-IN.

5.6 Air dry the membranes for detection.

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[6] Check the optimal temperature for each probe other than EV-IN or HACV-IN.