Attachment 1.

ETHICAL REVIEW COMMITTEE, ICDDR,B.

RRC File copy

Date May 02, 1988
25/5/88

Principal Investigator Dr. Nancy Strockbine

Trainee Investigator (if any) _____

Application No. 88-016

Supporting Agency (if Non-ICDDR,B) _____

Title of Study DNA probe analysis of Selected Bangladeshi Pediatric Diarrheal Patients for Five Virulence-associated Characteristics of Escherichia coli.

Project status:
( ) New Study
( ) Continuation with change
( ) No change (do not fill out rest of form)

Circle the appropriate answer to each of the following (If Not Applicable write NA).

1. Source of Population:
   (a) Ill subjects — (Yes) No
   (b) Non-ill subjects — Yes No
   (c) Minors or persons under guardianship — Yes No
2. Does the study involve:
   (a) Physical risks to the subjects — Yes (No)
   (b) Social Risks — Yes (No)
   (c) Psychological risks to subjects — Yes (No)
   (d) Discomfort to subjects — Yes (No)
   (e) Invasion of privacy — Yes (No)
   (f) Disclosure of information damaging to subject or others — Yes (No)
3. Does the study involve:
   (a) Use of records, (hospital, medical, death, birth or other) — (Yes) No
   (b) Use of fetal tissue or abortus — Yes (No)
   (c) Use of organs or body fluids — Yes (No)
4. Are subjects clearly informed about:
   (a) Nature and purposes of study (Surveillance Hc) — (Yes) No
   (b) Procedures to be followed including alternatives used — (Yes) No
   (c) Physical risks — (Yes) No
   (d) Sensitive questions — (Yes) No
   (e) Benefits to be derived — (Yes) No
   (f) Right to refuse to participate or to withdraw from study — (Yes) No
   (g) Confidential handling of data — (Yes) No
   (h) Compensation &/or treatment where there are risks or privacy is involved in any particular procedure — (Yes) No
5. Will signed consent form be required:
   (a) From subjects — Yes (No)
   (b) From parent or guardian (if subjects are minors) — Yes (No)
6. Will precautions be taken to protect anonymity of subjects — (Yes) No
7. Check documents being submitted herewith to Committee:
   - N/A Umbrella proposal - Initially submit an overview (all other requirements will be submitted with individual studies).
   - ✓ Protocol (Required)
   - ✓ Abstract Summary (Required)
   - N/A Statement given or read to subjects on nature of study, risks, types of questions to be asked, and right to refuse to participate or withdraw (Required)
   - N/A Informed consent form for subjects
   - N/A Informed consent form for parent or guardian
   - N/A Procedure for maintaining confidentiality
   - N/A Questionnaire or interview schedule *

* If the final instrument is not completed prior to review, the following information should be included in the abstract summary:

1. A description of the areas to be covered in the questionnaire or interview which could be considered either sensitive or which would constitute an invasion of privacy.
2. Examples of the type of specific questions to be asked in the sensitive areas.
3. An indication as to when the questionnaire will be presented to the Cttee. for review.

(PTO)

agree to obtain approval of the Ethical Review Committee for any changes volving the rights and welfare of subjects before making such change.

Principal Investigator — Trainee

SECTION I - RESEARCH PROTOCOL

1. Title : DNA probe analysis of Selected Bangladeshi Pediatric Diarrheal Patients for Five Virulence-associated Characteristics of Escherichia coli

2. Principal Investigator : Dr. Nancy Strockbine, Scientist, CDC

   Co-investigators : Dr. Bradford A. Kay
   Dr. I. Kaye Wachsmuth
   Dr. Khaleda Haider
   Dr. Anwarul Huq
   Dr. Gary Hlady
   Dr. A.N. Alam

3. Starting Date : March 1, 1988

4. Completion Date : December 31, 1988

5. Total Direct Costs : US$ 5,330.00

6. Scientific Programme Head :

   This protocol has been approved by the Laboratory Sciences Div. Working Group.

   Signature of the Scientific Program Head

   Date Apr. 12, 1988

7. Abstract Summary

Diarrheal disease remains the single most serious threat to life of children under two in the developing world. Escherichia coli, a common intestinal bacterium, is known to play a major role in diarrheal disease. Most of the assays to identify diarrheogenic E. coli are time consuming, expensive and often require special expertise or resources.

DNA probes which can hybridize with the genes mediating pathogenicity in E. coli are now available and make it possible to easily screen large numbers of organisms in a large patient population. We propose to use the gene probes for enteropathogenic E. coli, enterohemorrhagic E. coli and enteroinvasive E. coli (all previously known to cause diarrheal diseases in children) to examine E. coli isolated from 400 children with diarrhea, under one year, coming to the ICDDR,B Treatment Centre in Dhaka. This protocol is designed to determine the potential significance of these diarrheogenic E. coli and to transfer the DNA probe technology to ICDDR,B where it should be useful in many diarrheal disease investigations.

8. Reviews :

(i) Ethical Review Committee : _____

(ii) Research Review Committee : _____

(iii)Director : _____

SECTION II - RESEARCH PLAN

A. INTRODUCTION

1. Objective : The objective of this protocol is to determine the frequency and hence epidemiologic significance of pathogenic Escherichia coli in 400 consecutive pediatric diarrheal patients one year of age and under seen at the ICDDR,B Treatment Centre in Dhaka. Assessment of the relative importance of E. coli-mediated diarrheal disease in this population will aid in a better understanding of the etiology and mechanisms of the pathogenesis of diarrheal disease. This will facilitate the identification of prevention and treatment modalities which may impact an morbidity and mortality in this and similar populations. It may also help to establish research priorities to be addressed in future ICDDR,B investigations.

2. Background : Escherichia coli may present one of the most challenging problems in bacterial pathogenesis. The organism is a common inhabitant of the human (and animal) intestine, and based on routine laboratory tests, the saprophytic strains are virtually indistinguishable from the inherently pathogenic strains (5). As pathogenicity mechanisms and markers have been identified, we are beginning to understand the complicated etiology of E. coli infections. There are apparently 4 distinct groups now recognized within this species: (1) Normal flora E.

coli which may cause opportunistic infections such as wound or lower urinary tract infections and various manifestations in the immunocompromised host. (2) E. coli causing septicemia or meningitis in neonates. (3) E. coli causing upper urinary tract infections or pylonephritis, and (4)E. coli causing diarrhea, dysentery, hemorrhagic colitis and hemolytic uremic syndrome.

Because E. coli strains are common inhabitants of the human gastrointestinal tract, diarrheogenic strains were not recognized as intestinal pathogens until the 1940's (11). At that time, certain strains were identified as the cause of outbreaks of explosive, severe (often fatal) gastrointestinal illness in infants. Our knowledge about these pathogenic E. coli has expanded greatly since that time, especially in the past 10 years. There are now four recognized classes of E. coli that cause diarrhea : (a) enteropathogenic, EPEC; and (b) enterotoxigenic, ETEC; (c) enteroinvasive, EIEC; and (d) enterohemorrhagic, EHEC. Each class is composed of various, and often unique, O:H serotypes and possesses characteristic virulence properties. The serotypes which seem to be "inherent" pathogens are listed within this classification as Appendix I.

Evidence from the 1950's suggested that specific serotypes of E. coli were distributed worldwide and were important causes of diarrhea particularly in nursery outbreaks (9). The list of EPEC serotypes increased until specific mechanisms of pathogenicity were discovered for ETEC and EIEC strains. As certain "one-time EPEC" serotype strains were found to produce enterotoxin or invade the epithelium, these strains were dropped from the EPEC classification (11). At present, we can say that many EOEC strains seem to be enteroadherent as defined by adherence to HEp2 or HeLa cells in culture or by DNA probes which detect the presence of genes coding for HEp2 cell adherence (3,14). Other EPEC serotype strains produce a cytotoxic effect on Vero or HeLa tissue culture cells (13). The pathogenicity of many of these strains has been confirmed on several occasions in the ultimate infection model, the human volunteer (4).

The only commercially available EPEC diagnostic tools are antisera directed against the O group antigens. Data from several field studies indicate that less that 50% of the EPEC serogroup strains are actually EPEC serotypes. This means that presumptive EPEC strains, as determined by serogroup and clinical manifestations must be further characterized. One recent report describing the use of commercial diagnostic antisera, was rather discouraging. None of 118 E. coli that agglutinated in the commercial sera were identified as EPEC serotypes by the reference laboratory (6). Approximately 30% were confirmed as EPEC serogroups, but had a different H antigen (Echeverria-

personal communication). The investigators also tested 105 of these strains with the adhesiveness DNA probe and found that none hybridized. It is important therefore to use caution when interpreting serogroup results and to use these reagents only when there are well-documented and compelling clinical reasons (6,11).

Nearly all episodes of EPEC diarrhea occur in children under 2 years of age. Illness is uncommon in older children and adults even though they often carry the organism (4,11,23). Many investigations have suggested that the transition from breast-feeding to bottle-feeding or weaning foods in areas with high environmental contamination is a frequent antecedent to the development of EPEC diarrhea (26). It is likely that age-specific variations in peak attack rates for EPEC diarrhea in different areas are related partly to age at weaning. EPEC can cause large outbreaks of diarrhea in infant care institutions, and affected infants can represent a means of spread of this organism into the community (4). It is also noteworthy that past as well as present EPEC strains have been extremely resistanty to antimicrobial agents (7).

Volunteer studies have demonstrated that ingestion of 10^7 to 10^9 organisms cause disease in adults and infants (11). The early EPEC outbreaks in industrialized countries occurred in summer months, but by the 1960's, the seasonality appeared to have shifted to the cooler months. In developing countries today,

diarrhea due to EPEC is found most frequently in the warm season (11).

EPEC are an important cause of infant morbidity and mortality worldwide; upto 30% of acute sporadic infant diarrhea in Brazil and South Africa is attributed to these organisms. Even in the industralized countries, both outbreaks and sporadic cases continue to be reported (16).

ETEC

Appendix II describes some of the available toxin assays for LT and/or ST. In general, the biological assays measure intestinal fluid influx (rabbit loop, infant mouse, infant rabbit) or tissue culture response to adenyl cyclase activation. The application of these assays to diagnose ETEC infections is limited by the inherent problems and expense involved in acquiring and maintaining animal or tissue culture systems. The detailed methods, sensitivities and requirements for most of the assays listed are reviewed elsewhere (17,27). Agglutination assays for LT and several ELISA's for ST have been published more recently and offer procedures potentially more adaptable to the diagnostic microbiology laboratory (24). Many of these assays are currently available and routinely performed at ICDDR,B.

EIEC

There are certain strains of E. coli which cause an illness indistinguishable from shigellosis. These enteroinvasive strains

listed in Appendix I may be similar to (and occasionally indistinguishable from) shigellae. The first recognized outbreak of diarrhea due to EIEC occurred in Italy in 1944 and 1945. The causitive organism was initially reported as a paracolon bacillus. The organism was later identified as an E. coli 0124:H30. This same serogroup has been implicated in diarrheal disease outbreaks in the United States as late as 1981 (8). Most of the enteroinvasive strains do share O antigenic determinants with the shigellae and some appear to have identical O antigens (2).

This class of diarrheogenic E. coli may be the most difficult to diagnose. Like ETEC strains, they are identified by their pathogenic mechanism, which has been extremely difficult to demonstrate in vitro. The pathogenicity of EIEC is similar, or identical, to that of shigellae and is similarly mediated by a large-molecular-weight plasmid (10).

In general, EIEC has been identified by invasiveness of the guinea pig or rabbit conjunctiva (Sereny Test) (19). This means that 10 guinea pigs or rabbits would be required to test 10 E. coli colonies picked from a primary isolation plate of one patient's stool culture. With the advent and use of an ELISA (15a) and gene probes (1) for enteroinvasiveness, investigators learned that in acute illness there may be only 1 or 2 enteroinvasive colonies out of 20 colonies tested per patient (25). It is apparent that these newer and more sensitive assays could provide much-needed epidemiologic information of EIEC strains and infections.

For unknown reasons other than the potential limitations of laboratory identification, illness due to EIEC appears to be rare both in developed and developing areas, therefore little epidemiologic or public health information is available.

EHEC

Much like the history of EPEC, 0157:H7 strains were defined as pathogens on the basis of epidemiologic data (30). These strains possessed none of the traditional E. coli virulence properties, and initial attempts to infect a variety of laboratory animals were unsuccessful. In 1983, it was reported that 0157:H7 strains caused diarrhea in infant rabbits (6a). However, this is not an assay most laboratories would use, and the infection did not mimic the human illness. The most recently-described infection model is the gnotobiotic piglet (6b). The piglets develop some symptoms and pathology similar to those observed in humans, however they did not develop the grossly bloody diarrhea characteristic of human infections. This is also a model which most laboratories could not use due to expense and the necessity for specialized animal facilities.

All wild type 0157:H7 strains and a limited number of other E. coli serotypes appear to produce large quantities of a Shiga-like toxin which is cytotoxic for HeLa or Vero cells (13). The toxin is also referred to as Vero toxin. Although we do not know the exact role of toxin in the pathogenesis, it does seem to play some role in hemorrhagic colitis and hemolytic uremic syndrome.

We know now that there are at least two immunologically distinct Shiga-like toxins; they appear to be mediated by phage DNA and have the same biological activities as those of Shiga toxin from S. dysenteriae type 1 (48,21). DNA probes have now been developed for both toxins (15).

The six reported outbreaks of hemorrhagic colitis due to E. coli 0157:H7 in which detailed information is available have together involved almost all age groups. Laboratory studies have indicated that E. coli 0157:H7 is an important pathogen. It was isolated from the stools of 15% of patients with sporadic bloody diarrhea in two reports. In another study of 1,425 stool specimens, E. coli 0157:H7 was isolated more frequently than Salmonella.

PROBES

The fidelity of complementary base pairing in nucleic acid hybridization reactions is the basis for exquisitely specific DNA probe assays (31). DNA probes for enteric bacterial pathogens can identify a genus, species, bacteriophage, or virulence trait and can vary considerably in size and origin (28,29). Synthetic oligonucleotides offer several advantages over the native restriction fragment probes.

Generally, DNA probes for toxins, enteroadherence, and enteroinvasiveness have been comparable or superior to bioassays or immunoassays. For example: (1) DNA probes for Shiga-like/Vero toxin genes (EHEC) were found to be as specific and sensitive as assays to detect toxin in stool filtrates, colony sweeps and

individual colony lysates. (2) In two separate outbreaks of nosocomial neonatal diarrhea in Ohio and in Burma, probes were used successfully to identify EPEC serotypes 0114:NM and 0114:H2. (3) In a community study of bloody diarrhea, there was excellent correlation between culture results and hybridization analysis using the ipaC probe for enteroinvasiveness (22). In summary, a growing list of new DNA probes, the routine processing of large numbers of isolates, and continuing technological advances in all aspects of probing should make hybridization a practical approach to the diagnosis of many enteric diseases. A list of some of the available enteric probes is presented in Appendix III (29).

3. Rationale : DNA probe technology has rapidly advanced and has allowed the development of useful investigative tools with broad applicability in the study of diarrheal diseases. The exquisite specificity of binding of complementry DNA base pairs under stringent conditions is the foundational principle of this methodology. A variety of DNA probes now exist which test for the specific gene sequences responsible for the expression of a diversely of virulence-related characteristics of E. coli. The elucidation of these characteristics by DNA probes eliminates the need for using the expensive, time consuming and technically difficult in-vivo and in-vitro techniques formerly required. This in turn allows a much greater number of organisms to be tested with a greater number of patients, yielding results which give a

more accurate estimate of the true prevalence, and significance of these pathogenic mechanisms in the patient population studied.

Escherichia coli can be isolated from more than 90% of the ICDDR,B diarrheal patients. At present we are able to test only a small number of these E. coli isolates, and then only for a maximum of one or two characteristics; most commonly for ST and LT production. Several additional virulence-associated characteristics of E. coli have been recognized which can now be easily identified by DNA probes. Using these probes, it is possible for us to screen a large number of E. coli isolates for a wide range of virulence characteristics; an undertaking that was previously not possible for economic and logistical reasons.

B. SPECIFIC AIMS

We propose to examine five E. coli isolates from all surveillance patients one year of age and under seen at the Dhaka Treatment Centre between March 1, 1988 and August 31, 1988. Each isolate will be tested by DNA probes for the following characteristics :

1. Localized adherence (an EPEC characteristic)
2. Diffuse adherence (an EPEC characteristic)
3. Enteroinvasiveness
4. Shiga-like toxin 1
5. Shiga-like toxin 2

C. METHODS OF PROCEDURE

The DNA probes for localized and diffuse adhesiveness, Shiga-like toxins I and II, and enteroinvasiveness will be prepared from the following recombinant vectors : pJMR100, pSLM852, pJN37-19, pNN111-19, and lambda gt11-W22 respectively.

Plasmid DNA will be prepared using a commercially available spin column according to the procedure outlined in Appendix IV. Phage DNA will be prepared as described by Silhavey et al. (20). After digest of the plasmid or phage DNA with the appropriate restriction endonucleases, the probe DNA fragments will be purified by agarose gel electrophoresis and labelled by random priming with alpha^{-32}P dCTP and a commercially available kit (see Appendixes V and VI, respectively, for DNA fragment purification and labelling procedures).

Five identical filters containing five presumptive E. coli isolates from each child will be prepared as described in Appendix VII (12). Control strains consisting of known positive and negative strains for each virulence trait will be included on every filter. Each of the filters will be probed with one of the probes as described in Appendix VIII.

D. SIGNIFICANCE

DNA probe results will be analized with information routinely available on surveillance patients which includes

Age,
Sex,
Clinical presentation (fever, vomiting, dehydration status)

Type of diarrhea (watery, non-watery, bloody)
Duration of diarrhea
Pathogens isolated (bacterial, parasitic, rotavirus)
Nutritional status
Treatment history
Clinical outcome

This information will be used to assess the following

1. The frequency of each of the probed characteristics in this patient population.
2. The epidemiologic and clinical coorelations with each of the probed characteristics with this population.
3. The overall significance of E. coli expressing these characteristics in this patient population.

E. FACILITIES REQUIRED

All work will be done in the existing laboratories of the ICDDR,B in Dhaka.

F. COLLABORATIVE ARRANGEMENTS

DNA probes will be prepared at the CDC by Drs. Strockbine and Wachsmuth and will be brought to Dhaka for use by Dr. Strockbine. Dr. Strockbine will be the team leader for the Probe experiments in Dhaka. Drs. Kay, Alam, and Hlady will be responsible for acquisition of patient samples, patient history information, and the prepartion of filters to be probed. Drs. Huq and Haider will be responsible for the processing of the E. coli filters in the Dhaka laboratories and will directly supervise the personnel involved with all phases of bacterial isolation, storage and filter preparation.

REFERENCES

1. Buysse JM, Stover CK, Oaks EV, Venkatesan M, Kopecko D: Molecular cloning of invasion plasmid antigen (ipa) genes from Shigella flexneri: analysis of (ipa) gene products and genetic mapping. J Bacteriol 169:2561-2569, 1987.

2. Cheasty T and Rowe B: Antigenic relationships between the enteroinvasive Escherichia coli O antigens 028ac, 0112ac, 0124, 0136, 0143, 0144, 0152, and 0164 and Shigella O antigens. J Clin Microbiol. 17:681-684, 1983.

3. Cravioto A, Gross RJ, Scotland SM, Rowe B: An adhesive factor found in strains of Escherichia coli belonging to the traditional infantile enteropathogenic serotypes. Current Microbiol 3:95-99, 1979.

4. DuPont HL: Escherichia coli diarrhea. In Bacterial Infections of Humans. Evans AS and Feldman HA, (eds.). Plenum Medical Book Company, New York, pp 219-234, 1982.

5. DuPont HL, Formal SB, Hornick RB, Snyder MJ, Libonati JP, Sheahan DG, LaBrec EH, and Kalas JP: Pathogenesis of Escherichia coli diarrhea. New England J of Med 285:1-9, 1971.

6. Echeverria P, Taylor DN, Leksomboon U, Blacklow NR, Pinnoi S, Nataro JP, Kaper J, and Rowe B: Identification of enteric pathogens in the small and large intestine of children with diarrhea. Diagn Microbiol Infect Dis. 4:277-284, 1986.

6a. Farmer JJ. Potter ME, Riley LW, Barrett TJ et al. Letter, Lancet i:702-703, 1983.

6b. Francis DH, Collins JE, and Duimstra JR: Infection of gnotobiotic pigs with an Escherichia coli 0157:H7 strain associated with an outbreak of hemorrhagic colitis. Infect Immun 51:953-956, 1986.

7. Gross RJ, Ward LR, Threlfall EJ, King H and Rowe B: Drug resistance among infantile enteropathogenic Escherichia coli strains isolated in the United Kingdom. Br Med J 2985:472-473, 1982.

8. Harris Jr, Mariano J, Wells JG, Payne BJ, Donnell HD, and Cohen ML: Person-to-person transmission in an outbreak of enteroinvasive Escherichia coli. Amer J Epi 122:245, 1981.

9. Kauffmann F, and DuPont A: Escherichia strains from infantile gastro-enteritis. Acta Pathol Microbiol Scand 27:552-564, 1950.

10. Kopecko DJ, Baron LS, and Buysse J: Genetic determinants of virulence in Shigella and dysenteric strains of Escherichia coli: Their involvement in the pathogenesis of dysentery. In: Current Topics in Microbiology and Immunology 118:71-95. Springer-Verlag. Berlin. Heidelberg, 1985.

11. Levine MM and Edelman R: Enteropathogenic Escherichia coli of classic serotypes associated with infant diarrhea: Epidemiology and pathogenesis. Epidemiologic Reviews. 6:31-51, 1984.

12. Maas R: An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid 10:296-298, 1983.

13. Marques LR, Moore MA, Wells JG, Wachsmuth IK, and O'Brien AD: Production of Shiga-like toxin by Escherichia coli. J Infect Dis 154:338-341, 1986.

14. Nataro JP, Baldini MM, Kaper JB, Black RE, Bravo N, and Levine MM: Detection of an adherence factor of enteropathogenic Escherichia coli with a DNA probe. J Infect Dis 152:560-565, 1985.

15. Newland JW, Strockbine NA, Miller SF, O'Brien AD, and Holmes RK: Cloning of Shiga-like toxin structural genes from a toxin-converting phage of Escherichia coli. Proceedings from the 21st Conference on Cholera. October 21-23, 1985.

16. Paulozzi LJ, Johnson KE, Kamahele LM, Clausen CR, Riley LE, and Helgerson SD: Diarrhea associated with adherent entropathogenic Escherichia coli in an infant and toddler center, Seattle, Washington. Ped 77:296-300, 1986.

17. Sack RB: Human diarrheal disease caused by enterotoxigenic Escherichia coli. Ann Rev Microbiol 29:333-353, 1975.

18. Scotland SM, Smith HR, Willshaw GA, and Rowe B: Vero cytotoxin production in strains of Escherichia coli is determined by genes carried on bacteriophage. Lancet ii:216, 1983.

19. Sereny B: Experimental keratoconjunctivitis Shigellosa. Acta Microbiol Hung 4:367-376, 1957.

20. Silhavey TJ, Berman ML and Endquist LW: Large scale isolation of DNA, In Shilhavey TJ, Berman ML and Endquist LW (ed.). Experimens with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 1984.

21. Strockbine NA, Marques LRM, Newland JW, Smith HW, Holmes RK and O'Brien AD: Two toxin-converting phages from Escherichia coli 0157:H7 strain 933 encode antigenically distinct toxins with similar biological activities. Infect Immun 53:135-140, 1986.

22. Strockbine NA, Puhr N, Venkatesan M, Shaffer N, Tauxe R, Wells J, and Wachsmuth IK: Survey of patients with bloody diarrhea on the Navajo Indian Reservation using culture techniques and DNA probes for detecting bacterial pathogens. Ann Meet Am Soc Microbiol, Miami Beach, FL, 1988.

23. Summary of a workshop on Enteropathogenic Escherichia coli. J Infect Dis 147:1108-1118, 1983.

24. Svennerholm AM and Lindblad M: GM 1 ELISA method for demonstration of Escherichia coli heat-stable enterotoxin. FEMS Microbiol Ltrs 30:1-6, 1985.

25. Taylor DN, Echeverria P, Pal T, Sethabutr O, Saiborisuth S, Sricharmorn S, Rowe B, and Cross J: The role of Shigella spp., enteroinvasive Escherichia coli, and other enteropathogens as causes of childhood dysentery in Thailand. J Infect Dis 153:1132-1138, 1986.

26. Thoren A: The role of enteropathic E. coli in infantile diarrhea. Scand J Infect Dis (Suppl) 37:1-51, 1983.

27. Wachsmuth K: Laboratory detection enterotoxins. In: Infections Diarrheal Diseases : Current Concepts and Laboratory Procedures. Ch 6:93-115. Marcel Dekker, Inc, New York and Basel, 1984.

28. Wachsmuth K: Molecular epidemiology of bacterial infections: Examples of methodology and of investigations of outbreaks. Rev Infect Dis 8:682-692, 1986.

29. Wachsmuth K, Strockbine NA, Aye T, Cook WL, Barrett TJ, Sack DA and Wells JG: DNA probes for enteric bacterial pathogens. Proceedings from the Fifth International Symposium on Rapid Methods and Automation in Microbiology and Immunology. Florence, Italy, 4-6 November 1987 (in press).

30. Wells JG, Davis BR, Wachsmuth IK, Riley LW, Remis RS, Sokolow R, and Morris GK: Laboratory investigation of hemorrhagic colitis outbreaks associated with a rare Escherichia coli serotype. J Clin Microbiol 18:512-520, 1983.

31. Wetmur JG, Davidson W: Kinetics of renaturation of DNA. J Molec Biol 31:349-370, 1968.

ICDDR,B
1988 BUDGET PROPOSAL (IN US $)

DIVISION: LSD
PROTOCOL/BRANCH NAME:
NAME OF P. I./BRANCH HEAD/DIVISION HEAD: Dr. Nancy Strockbine
BUDGET CODE: STARTING DATE: March 1, 1988
PROTOCOL NO: COMPLETION DATE:August 31, 1988
DONOR NAME: GRANT AMOUNT:

EXPENSE CATEGORY			Column A	Column B	Column C
A/C Code	Description	Refer to Page No.	Actual Jan.- June '87	Estim. Whole Yr 1987	Proposed 1988
3100	Local Salaries	02			352
3200	Intl. Salaries	08			0
3300	Consultants	14			0
3500	Travel Local	15			0
3600	Travel Intl.	16			2500
3700	Supplies & Mat.	18			390
4000	Other Costs	19			688
4800	Inter Deptl. Ser.	21			1400
	Total Direct Operating Cost		0	0	5330
0300	Capital Expenditure (P.22)				0
	TOTAL DIRECT COST		0	0	5330

Description	No. of Positions	No. of Man Months	$ Amount
A. Direct Project/Protocol/ Branch Staff at 01.01.1988 (Source: Page 3)	0	0	0
Add:			
B. New Recruitments (Source: Page 4)	0	0	0
C. Staff allocated from other area (Source: Page 5)	1	1	352
(i) Sub Total	1	1	352
Less:			
D. Separations (Source: Page 6)	0	0	0
E. Staff allocated to other area (Source: Page 7)	0	0	0
(ii) Sub Total	0	0	0
(i) - (ii) TOTAL	1	1	352

	A	B	C	D	E	F=(D x E)
Job Title	Level	Budget Code Of Other Area	No.of Positn	No.of Man Months	Rate Per Month	$ Amount
1. Research Officer	GS-5	210210	1	1	352	352
2.						0
3.						0
4.						0
5.						0
6.						0
7.						0
8.						0
9.						0
10.						0
11.						0
12.						0
13.						0
14.						0
15.						0
16.						0
17.						0
18.						0
19.						0
20.						0
21.						0
22.						0
23.						0
24.						0
25.						0
26.						0
27.						0
28.						0
29.						0
TOTAL			1	1		352

Name & Job Title	Travel From-To-from	Estim. Travel Days (A)	Per Diem Rate (B)	Per Diem Amount (C = AXB)	Transportation Air (D)	Transportation Ground (E)	Other Cost (F)	Total $ Amount G=(C+D+E+F)
Dr. N.Strockbine	Atlanta-Dhaka-Atlanta	0	0	0	2500			2500
2.				0				0
3.				0				0
4.				0				0
5.				0				0
6.				0				0
7.				0				0
8.				0				0
9.				0				0
10.				0				0
11.				0				0
12.				0				0
13.				0				0
14.				0				0
15.				0				0
16.				0				0
17.				0				0
18.				0				0
19.				0				0
20.				0				0
21.				0				0
21				0				0
22.				0				0
23.				0				0
24.				0				0
25.				0				0
26.				0				0
27.				0				0
28.				0				0
29.				0				0
TOTAL				0				2500

A/C Code	Item Description	$ Amount
3701	Drugs (used for medicationin the hospitals and field stations)	
3702	Glassware (bottle, beaker, cylinder, petridish, aluminium seal, slides stopper, tube etc.)	
3703	Hospital Supplies (bandage, gauge blade, bowl, catheter, cotton, needle syringe, solution, leukoplast, towel etc.)	
3704	Stationery and Office Supplies (Battery, book register, binders, files, pencil, fastener, paper, ribbon, stapler etc.)	
3705	Chemicals and Media (Acid, reagent dextrose, sodium, bactoagar etc.)	
3706	Materials for Uniform (Cloth, button etc required for making uniforms)	
3707	Fuel, Oil and Lubricants (Diesel, mobil, petrol, kerosene etc.)	
3708	Laboratory Supplies (Aluminium foil, bag blade, brush, cap, container, X-ray etc.)	200
3709	Housekeeping SUpplies (Aerosol, battery, wiping cloth, duster, lock and key etc.)	
3710	Janitorial Supplies (Bleaching powder, brush, detol, detergent, insecticide, soap etc.)	
3811	Tools and Spares (Automobile spares, tyres, tubes, battery, stores required for maintenance services etc.)	
3712	Non-stock Supplies (Materials not normally kept in stock and purchased only against specific requisitions)	100
	Sub Total	300
3713	Freight and other charges (Add 30% to above sub total)	90
	TOTAL	390

A/C Code	Item Description	$ Amount
3800	Repairs and Maintenance (Maintenance and repairs of vehicles, equipments, furniture and building)	100
3900	Rent, communication and utilities (Postage, telephone, telegram, electricity etc.)	
4100	Bank charges	
4200	Legal and Professional Expenses (Professional membership fee, legal fee, audit fee etc.)	
4300	Printing and Publication (Printing of forms, books, journals, reprints etc.)	
4400	Hospitality and Doantion (Guest house accommodation, donations, hospital food, lunch, refreshment etc.)	588
4500	Service Charges (porter, labour, washing, lauindry and other misc. expenditure)	
4600	Staff Development and Training (Training course fee, training materials, stipend, scholarship, subsistance paid to the staff)	
	TOTAL	688

A/C Code	Service Area	$ Amount
4801	Computer	
4802	Transport Dhaka	
4803	Transport Matlab	
4804	Water Transport Matlab	
4805	Transport Teknaf	
4806	Xerox and Mimeograph	
4807	Pathology	
4808	Microbiology Tests	1200
4809	Biochemistry	
4810	X-Ray	
4811	I.V. Fluid	
4812	Media	100
4813	Patient Hospitalization study	
4814	Animal Research	100
4815	Medical Illustration	
4817	Telex	
4818	Out Patient Care	
4819	Maintenance Charges	
4820	Vehicle Maintenance Charges	
4821	Library Service Charges	
4822	Staff Clinic Charges - Dhaka	
4823	Staff Clinic Charges - Matlab	
4824	Bacteriology Test	
4830	Transport Subsidy	
	TOTAL	1400

APPENDIX I

SEROTYPES OF *ESCHERICHIA COLI* ASSOCIATED WITH HUMAN DIARRHEAL DISEASES

Enteropathogenic (EPEC)		Enteroinvasive (EIEC)	Enterohemorrhagic (EHEC)
026:NM	0114:H2	028ac:NM	0157:H7
026:H11	0119:H6	029:NM	
055:NM	0125ac:H21	0112ac:NM	
055:H6	0127:NM	0115:NM	
055:H7	0127:H6	0124:NM	
086:NM	0127:H9	0124:H7	
086:H2	0127:H21	0124:H30	
086:H34	0128ab:H2	0135:NM	
0111ab:NM	0142:H6	0136:NM	
0111ab:H2	0158:H23	0143:NM	
0111ab:H12		0144:NM	
0111ab:H21		0152:NM	
		0164:NM	
		0167:NM	

APPENDIX II

ASSAYS FOR ENTEROTOXINS OF *ESCHERICHIA COLI*

Assay	Heat labile enterotoxin	Heat stable enterotoxin
Rabbit ileal loop		
6-7 h	+[a]	+
18 h	+	+ or -
Infant mouse (4 h)	-	+
Infant rabbit	+	+
Rabbit skin	+	-
Y1 mouse adrenal cells	+	-
Chinese hamster ovary cells	+	-
Enzyme-linked immunoassay	+	+
Radioimmune assay	+	+
Agglutination	+	-
Staphyloccocal coagglutination		
Latex particle agglutination		
Reverse passive latex agglutination		
Genetic probe	+	+

[a] + = positive result in assay, - = negative result,
+ or - = variable result.

APPENDIX III

BIOASSAYS AND DNA PROBES FOR SEVERAL ENTERIC BACTERIAL PATHOGENS

Pathogen and Assay	Probes
Vibrio cholerae and Enterotoxigenic *E. coli*	
Rabbit ileal loop (CT, LT I, ST Ia and Ib)	CT*, HLY
Pig ileal loop (ST II)	LT I*
Infant mouse, intragastric (ST Ia and Ib)	LT IIa and IIb
Rabbit skin permeability test (CT, LT I)	ST II*
Y-1 adrenal and CHO cells (CT, LT I, LT IIa and IIb)	ST Ia* and Ib*
	CFA I, CFA II
Enteropathogenic *E. coli*	
Gnotobiotic piglet model	EAF
HeLa and HEp-2 cell adherence	
Enteroinvasive *E. coli*	
Sereny, conjunctivitis	Inv
HeLa cell invasion	*ipa* A,B,C,H
Enterohemorrhagic *E. coli*	
Gnotobiotic piglet	EHEC
HeLa and Vero cytotoxin	SLT I
Henle 407 adherence	SLT II
Yersinia enterocolitica	
Mouse lethality	Ca^{++} dep.
Sereny	
Vibrio parahemolyticus	
Kanagawa hemolysin	Kp*

*Natural and synthetic oligonucleotide probes have been used

APPENDIX IV

5 Prime → 3 Prime, Inc.
19 East Central Avenue
Paoli, Pennsylvania 19301
(215) 644-4710

pZ523 PROTOCOL

Catalog Number: 5305-523325 3 Columns
5305-523523 10 Columns

FOR RESEARCH USE ONLY

STORAGE: Store columns refrigerated between 2°-8°C. **Do Not Freeze.**

GENERAL: Please read instructions completely before proceeding:

1. Each pZ523 column comes complete with one processing tube and one sample collection tube.

2. The column resin has been equilibrated in TE, pH 8.0 (10mM Tris, 1mM EDTA, pH 8.0).

INTENDED USE: pZ523 columns are intended for the purification and recovery of plasmid DNA away from bacterial chromosomal DNA. This spun column procedure offers an attractive alternative to the use of Etbr-CsCl density gradient purification procedures.

PERFORMANCE: Plasmid DNAs prepared by pZ523 spun column chromatography have been extensively tested and shown to be of purity suitable for restriction analysis, ligation, transfection of mammalian cells and transformation of bacteria.

NOTE: The pZ523 column has been designed for use with low speed table top centrifuges with swinging buckets. It will give optimum performance in this type of centrifuge when centrifuged at 1100 xg as described in the protocol below. Although the use of high speed centrifuges is not recommended, the pZ523 column will still function adequately when used in swinging bucket rotors at 1100 xg in such centrifuges. If a high speed centrifuge is used, one must take into account the time required for the rotor to reach speed and the time required for the rotor to decelerate to a stop.

QUALITY ASSURANCE:

Each lot of pZ523 columns is performance tested using E. coli HB101 transformed with pBR322. The resulting plasmid DNA is evaluated for purity by agarose gel electrophoresis to insure the absence of contaminating chromosomal DNA or cellular materials.

Each lot of pZ523 columns is tested to insure the lack of detectable DNases in the following assay:

Hind III digested lambda DNA is incubated with column eluate for 14-16 hours at 37°C. The entire reaction is resolved by agarose gel electrophoresis and stained with ethidium bromide. Any loss or tailing of the DNA bands is indicative of DNase activity.

MATERIALS REQUIRED:

1. Solution A: 50mM Glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA
2. Solution B: 0.2N NaOH, 1% SDS
3. Solution C: To 60 ml of 5M potassium acetate add 11.5 ml glacial acetic acid and 28.5 ml H_2O.
4. Solution D: 10mM Tris-HCl, 1mM EDTA, 1M NaCl, pH 8.0
5. Lysozyme: Highest purity available.

PREPARATION OF SAMPLE[1]:

Please note: The following suggested protocol is designed for an amplified or non-amplified 1 liter culture. Reagent volumes should be scaled down proportionately for smaller culture volumes. Incubation times remain the same.

1. Pellet bacteria by centrifugation at 8000 xg for 20 minutes at 4°C.
2. Resuspend bacterial pellet from a 1 liter culture in 10 ml of Solution A containing 5mg/ml lysozyme. Lysozyme should be added as a solid to Solution A just prior to use. Incubate for 5 minutes at room temperature.
3. Add 20 ml of Solution B. Mix by inversion (do not vortex). Incubate on ice for 10 minutes.
4. Add 15 ml of ice-cold Solution C. Mix by inversion (do not vortex). Incubate on ice for 10 minutes.
5. Centrifuge at 47,000 xg for 20 minutes at 4°C. Note: Lower speeds may be used with proportionately longer centrifugation times.

6. Centrifuge the column/collection tube assembly at 1100 xg for 24 minutes.

7. Recover the column effluent containing your purified plasmid DNA.

8. Add 0.6 volumes of 100% isopropanol, mix and allow to stand at room temperature for 20 minutes.

9. Centrifuge at 12,000 xg for 30 minutes at room temperature. Discard the isopropanol supernatant.

10. Wash the DNA pellet in cold 70% ethanol once at room temperature. Discard as much of the ethanol as possible, then dry the pellet.

11. Plasmid DNA pellet may then be resuspended in a suitable buffer, "Ready for Use".

REMOVAL OF RNA FROM PREPARATIONS OF PLASMID DNA

For some applications, it is desirable or necessary to obtain plasmid DNA which is free of all contaminating RNA. RNA may be removed by either of the following methods:

I. RNase Digestion

1. Resuspend the plasmid DNA pellet from "Preparation of Sample" step 9 above in 2 ml of TE, pH 8.0.

2. Add DNase-free RNase[2] to a final concentration of 50-100µg/ml. Incubate at room temperature for 1/2-1 hour.

3. Extract once with an equal volume of phenol/chloroform/isoamyl alcohol [redistilled, buffer equilibrated phenol containing 0.2% 8-hydroxyquinoline/ chloroform/isoamyl alcohol (25:24:1)].

4. Extract the upper aqueous phase with an equal volume of chloroform/isoamyl alcohol (24:1). After each extraction transfer the upper aqueous phase to a clean tube.

5. Add 10M ammonium acetate to the recovered aqueous phase to a final concentration of 2M and add 2 volumes of 100% ethanol. Incubate at -20°C for at least 2 hours.

6. Recover plasmid DNA by centrifugation at 12,000 xg for 30 minutes.

7. Wash the pellet once with cold 70% ethanol, dry the pellet

and resuspend in 1.8 ml of Solution D.

8. Proceed to step 1 of the "Column Procedure" section.

II. Chromatography through Bio-Gel™ A-150m[1]

1. Treat the plasmid DNA preparation with DNase-free RNase as described in steps 1 through 3 in the above procedure.

2. Extract the solution once with an equal volume of phenol equilibrated in TE, pH 8.0.

3. Layer up to 1 ml of the aqueous (upper) phase on a column of Bio-Gel A-150m (1cm x 10cm) equilibrated in TE, pH 8.0 and 0.1% SDS.

4. Wash the DNA into the column, apply a reservoir of TE with 0.1% SDS, and immediately begin to collect 0.5 ml fractions.

5. When 15 fractions have been collected, clamp off the bottom of the column. Analyze 10µl of each fraction by electrophoresis through 0.7% agarose gel or by ethidium bromide fluorescence in order to locate the plasmid DNA.

6. Pool the fractions that contain plasmid DNA. Recover the DNA by precipitation with ethanol as described in steps 4-6 in the Method I procedure above.

7. Resuspend the plasmid DNA in a suitable buffer.

Note: It is very difficult to remove all traces of plasmid DNA from Bio-Gel A-150m columns. To eliminate the possibility of cross contamination, it is suggested that use each column be used only once.

[1] Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. Molecular Cloning, Cold Spring Harbor Laboratory.

[2] **DNase-free RNase:**

1. Dissolve pancreatic RNase A at 10mg/ml in 50 mM Citrate, pH 5.0-5.5.
2. Vortex to dissolve.
3. Heat for 1 hour at 90°C in 1 ml aliquots.
4. Store at -20°C.
5. Test for DNase activity by incubating lambda DNA with an aliquot of RNase for 12-14 hours at 37°C. A loss of lambda DNA as determined by agarose gel electrophoresis would be indicative of DNase activity.

APPENDIX V

PURIFICATION OF DNA FRAGMENTS

Preparation of paper:

1. Soak Whatman DE81 DEAE - cellulose paper strips in 2.5 m NaCl several hours then wash several times with dH_2O.
2. Store in 1 mm EDTA pH8.0 at 4°C.
3. Soak in gel buffer before use.

Procedure:

1. Run gel - remove edge and stain to locate bands.
2. Cut gel and insert DEAE paper.
3. Electrophoresis until bands enter paper.
4. Remove paper - rinse several times in dH_2O (optional, briefly in low salt 0.3 M NaCl).
5. Blot with filter paper.
6. Place in siliconize glass tube or microfuge tube
   300-700 ul/50 mm^2 paper (20 mm Tris-HCl, pH7.5
   1 MM EDTA
   1.5 M NaCl)
7. Shread by vortexing and incubate 2 hours at 37°C with occassional agitation.
8. Spin down DEAE paper - if required filter supernatant through 0.5 - 1.0 cm Bio Rad P-60 in pasteur pipette.
9. ETOH precipitate DNA from supernatant.

International Biotechnologies, Inc.
275 Winchester Avenue
P.O. Box 9558
New Haven, CT 06535
Telephone: (203) 562-3878
Toll Free: (800) 243-2555
Telex: 643993

April 24, 1987

APPENDIX VI

PRIME TIME "C"

IBI
Certificate of Analysis
Product Systems for Molecular Biology

Product: PRIME TIME, an oligo-labelling biosystem for high specific activity DNA probes utilizing radioactive dCTP

Catalog #: 77800

Lot #: E715-02

Contents:

1.	5X PT-C Reaction Buffer	Lot # H611-01	200 ul
2.	BSA Mix	Lot # D601-02	20 ul
3.	Klenow Fragment	Lot # I504-A3	100 units
4.	STOP Solution	Lot # C712-01	4 ml

DNA probes (1-5 x 10^9 dpm/ug). These probes are suitable for a wide range of techniques including those which require a high signal to noise ratio (e.g., the detection of single copy genes in eukaryotic DNA by Southern analysis or filter hybridization). IBI's Klenow fragment (a cloned source) enhances both the overall efficiency of incorporation as well as the stability of the labelled probe.

April 24, 1987

PRIME TIME "C" BIOSYSTEM

INTRODUCTION

A method for the *in vitro* incorporation of radioactive label into specific DNA sequences has been developed by Feinberg and Vogelstein (*Anal. Biochem.* (1983). 132,6). This procedure is based on the priming of second strand DNA synthesis using random primers in the presence of Klenow, dNTPs, and ^{32}P-dCTP.

The original protocol utilized oligonucleotide primers generated by pancreatic deoxyribonuclease digestion of calf thymus DNA, the rationale being that this mixture should contain a large array of oligonucleotides (hexamers) with random sequences complemetary to the templates being primed.

IBI has improved upon this strategy by utilizing synthetic hexameric primers of every possible sequence combination. This ensures the efficient and reproducible priming/labelling of any given DNA sequence in an easily standardized biosystem. Consequently, the specific activity achievable greatly exceeds that of either nick translation or end-labelling. **PRIME TIME C** offers the advantages of requiring fewer steps and less manipulation than other commonly used methods for radiolabelling DNA, limiting the user's exposure to radioactivity.

Both double- or single-stranded DNA can be used as the template. Cold dCTP may also be substituted on an equimolar basis when using single-stranded M13 DNA to screen for small inserts, an adaptation to a protocol by D. Young and A. Gottlieb (*Gene Anal. Techn.* (1984). 1,104-108).

April 24, 1987

PRIME TIME "C" BIOSYSTEM

METHODS

1. Mix double- or single-stranded template DNA with water such that 33 ul contains approximately 20 - 100 ng of DNA (0.6 - 3.0 ng/ul final concentration). If single-stranded DNA is being used as the template, steps (3) and (4) may be omitted. Most problems associated with secondary structure can be circumvented however, by including these steps when using single-stranded DNA in the reaction.

2. Add 10 ul of the 5X PT-C Reaction Buffer and vortex briefly.

3. Place the tightly capped microfuge tube containing the buffered DNA solution in a boiling waterbath and incubate for three (3) minutes.

4. Remove the microfuge tube from the waterbath and immediately place on ice (quick cool).

5. Microfuge the tube briefly (10 seconds) and return to ice.

6. Add the following components to the cooled solution:
   1 ul BSA Mix
   1 ul Klenow fragment (5 units)
   5 ul ^{32}P-dCTP (3000 Ci/mmole; 10 mCi/ml)

   50 ul total volume

7. Incubate at room temperature for 2 - 14 hours. Due to potential for DNA degradation caused by high incorporation of label, it is suggested that incubation times be kept under four hours. Greater than 80% of the radioactive label is incorporated into full length molecules after only 3 hours.

8. Add 200 ul of STOP Solution and vortex briefly.

9. Use the labelled DNA directly or remove unincorporated nucleotides by ethanol precipitation, Sephadex chromatography or spin dialysis.

We would like to thank Dr. C. Roberts and Dr. D. Graham, both of NIADDK, for their valuable input and advice in optimizing this biosystem.

APPENDIX VII

PREPARATION OF COLONIES FOR HYBRIDIZATION

1. Inoculate colonies in a grid pattern (30-50 colonies per plate on MacConkey agar using a wooden applicator stick on an inoculating needle. Inoculate the same colony in the same position on 5 plates. Incubate 16-18hr. at 35°C.

2. Mark a disc of Whatman #541 paper with an arrow to orient the position of the colonies. Place this disc over the inoculated MacConkey plate with the arrow between colonies 1 and 2. Remove any trapped air bubbles with a glass spreader.

3. Incubate for 1-2 hrs at room temperature.

4. Saturate a sheet of Schleichet & Schull Absorbent Paper #470 with Lysing solution in a pyrex petri dish (10ml/ each 10 x 10 cm sheet.)

5. Remove the Whatman paper from the MacConkey disc and place it on the saturated paper, the side without bacterial growth against the surface of the saturated paper. Remove any trapped air bubbles.

6. Place the papers in an open dish and steam (in the Arnold) for 3 min.

7. After removing the papers from the steamer, immerse the Whatman paper in fresh lysing solution for 1 min. (50ml lysing solution/ disc.)

8. Transfer the disc from the lysing solution to neutralizing solution (50ml/ disc) and incubate for 4 min.

9. Blot the disc on filter paper and air dry at 37°C. The disc is now ready for hybridization and can be stored wrapped in foil or in a petri dish until needed.

Lysing Solution (make fresh daily)

0.5 M NaOH (20g/1)
1.5 M NaCl (87.66g/1)

Neutralizing Solution

1 M Tris, pH 7.0 (121.1g/1, adjust the pH with concentrated HCl)
2 M NaCl (116.8g/1)

APPENDIX VIII

COLONY BLOT HYBRIDIZATION

1. Put filters in plastic bag (4 of the 8 cm diameter 541 filters per 8"x12" bag). Arrange filters so that filters are back to back and the DNA is on the outside of the filter set.

2. Add approximately 15 ml of prehybridization solution per bag and seal the bag with the Seal'n meal. Try not to trap bubbles in the bag. Seal the bag near the top so that there is room to cut open the bag, exhange the hybridization solution, and reseal the bag.

3. Incubate the filters at 68-70 °C for at least 1 hour. (Overnight will not hurt if this is convenient.) The purpose of this step is to block non-specific DNA-binding sites on the filter with the salmon sperm DNA which is in the prehybridization solution.

4. After blocking, cut open each bag and discard the prehybridization solution down the sink. Next add approximately 15 ml of hybridization solution which contains the denatured probe. The hybridization solution is merely the prehybridization solution plus the denatured probe DNA or RNA (approximately 10^6 cpm/bag). To denature the probe, boil the probe for 10 minutes and then rapidly cool by putting on ice. Briefly spin the tube (15 seconds) to get the label off the sides of the tube.

Add the denatured probe to the prehybridization mix and dispense into the appropriate bags and seal. Be careful not to trap bubbles in the bag with the filters. The bubbles can be manipulated with a pipette used as a rolling pin. I recommend that a primary seal be made near the top of the bag and that a second seal be made near the filters to keep the hybridization solution localized with the filters. Bubbles which remain after the first seal can be manuvered so that they are away from the filters when the second seal is made.

5. Incubate the filters submerged at 68-70 °C overnight.

6. The next day, cut open the bags and discard the hybridization solution (radioactive) carefully down the sink. Run water down the sink to help decontaminate the sink.

7. Put radioactive filters in plastic trays (filters hybridized with the same probe may be put in the same tray) with approximately 500 ml of the first wash solution (2X SSC, 0.1% SDS) for 10 minutes at room temperature. (First wash: 100 ml of 20X SSC and 10 ml of 10% SDS, qs to 1 liter with deionized water.)

8. Discard first wash and replace with approximately 500 ml of the second wash solution (0.1 X SSC, 0.1% SDS) and wash filters at 68 °C for 1 hour with gentle agitation. (Second wash: 5 ml of 20X SSC and 10 ml of 10% SDS, qs to 1 Liter with deionized water.) Leave lid off water bath and change settings a little higher since the cover if off.

9. Repeat step 8.