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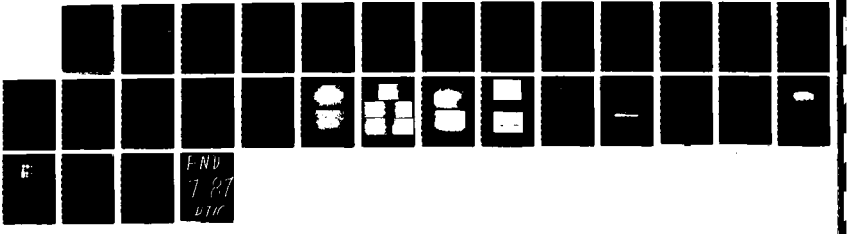

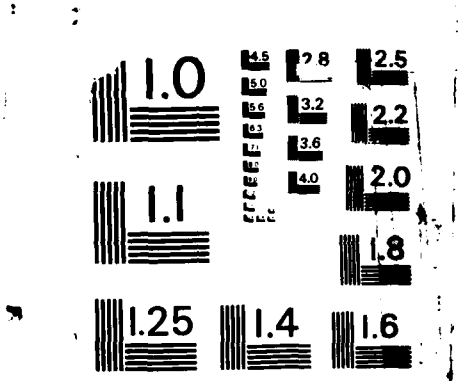

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DEVELOPMENT AND APPLICATION OF NUCLEIC ACID HYBRIDIZATION TECHNIQUES TO ARBOVIRUS SURVEILLANCE AND DIAGNOSIS

FEBRUARY 27, 1987

ANNUAL REPORT

MARCH 1, 1986 - FEBRUARY 28, 1987

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Sandwich hybridization techniques have been developed for detection of dengue virus RNA. Capture and detector probe constructs have been prepared in M13 and in vitro transcription plasmids, respectively. In preliminary trials to determine optimal concentrations of catcher and detector probe, nanogram levels of analyte RNA were readily detected.

Type and group specific hybridization probes for detection of California group virus RNA have been developed. The type specific probe hybridizes to snowshoe hare virus infected cells but not to La Crosse infected cell. The probe is a cDNA of a 2161 bp portion of the middle RNA segment. The group specific probe hybridizes to cells infected with any of the California group viruses. The probe is a cDNA of the small RNA segment of La Crosse virus.

RNA transcript probes have also been prepared to detect La Crosse virus RNA immobilized on nitrocellulose. The LAC cDNA of the small RNA segment was cloned into pSP65 and labeled with ^{32}P or ^{35}S. The probes were of equivalent senstivity, detecting nanogram quantities of analyte on nitrocellulose.

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Summary

In the LaCrosse virus hybridization system, specificity of the small RNA cDNA probe was determined by using ^{35}S labeled DNA in an in situ hybridization procedure. This probe was hybridized to cells infected with California group viruses. The small RNA cDNA probe hybridized to all of the viruses tested. This indicates it is not specific for LaCrosse virus. A cDNA of snowshoe hare (SSH) virus middle (M) RNA was obtained and used as a probe in an in situ hybridization procedure. This probe hybridizes to SSH infected cells, but does not hybridize to LAC infected cells. Thus, the M RNA cDNA is a type-specific probe. RNA transcript probes have been developed and used to detect LAC RNA extracted from infected cells. The sensitivity of the RNA probe has been determined.

Sandwich hybridization procedures were developed and tested for detection of dengue virus RNA in cell or virus extracts. The 3 clones of cDNA of the nonstructural region of dengue-2 virus, pVV-1, pVV-9, and pVV-17, were demonstrated to contain dengue-specific inserts. All or part of the pVV-17 1.95 kb cDNA was subcloned into in vitro transcription plasmids or single stranded (SS) DNA producing phage to construct 8 highly specific nucleic acid probes. Hybridization probes using cDNA from pVV-1 and pVV-9 are also being constructed. ^{32}P labeled probes have been used to lay the groundwork for hybridizations employing nonradioactive probes. The radiolabeled probes detect less than 100 pg of genomic dengue RNA or approximately 1×10^6 genome equivalents of dengue nucleic acid. To increase sensitivity, a novel sandwich hybridization technique has been developed to detect dengue RNA sequences in unprocessed clinical specimens. This hybridization technique utilizes SS RNA and SS DNA probes to function as detector and capture molecules, respectively, in the sandwich assay. The results of the first trials using sandwich hybridization are most encouraging; nanogram levels of viral RNA were readily detected. Studies are currently in progress to determine the sensitivity and specificity of the technique as well as its applicability to detection of viral nucleic acid in crude specimens.

Foreword

In conducting this research, the investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA molecules (May, 1986).

TABLE OF CONTENTS

I. Statement of the problem

Studies were proposed to develop, to evaluate, and to apply nucleic acid hybridization probe methodology to the surveillance and diagnosis of arboviruses in vectors or vector populations. Special attention was to be devoted to development of methods that can be applied in field circumstances. In particular, biotin-labeled hybridization probes (BLHPs) which can be detected by enzyme immunoassay techniques, were to be emphasized. Sensitivity and specificity of BLHP methods were to be compared and contrasted with conventional radioisotopic hybridization probe procedures as well as virus isolation and antigen detection techniques.

II. Background

Surveillance programs permit identification of geographical areas of high risk for arbovirus infections. Surveillance programs for arboviruses usually entail monitoring of either infections in humans and/or other vertebrates and monitoring of vector populations and infection rates (1). Estimates of vector population densities and infection rates are particularly useful for predicting the risk of human and animal disease. Since such information can be gathered before the incidence of human disease, health practitioners can successfully intervene to prevent infections.

The conventional method for arbovirus surveillance involves isolation of virus from mosquitoes or mosquito pools by amplification in a bioassay such as suckling mice, cell cultures, or embryonated eggs (1). Virus isolates must then be serologically identified. These conventional techniques are labor intensive, slow, and may require elaborate laboratory tissue culture and containment facilities. Frequently samples must be collected and sent to distant central laboratories for processing. Results typically are not available in time to permit field practitioners to institute effective control or therapeutic measures. Further, samples must be protected from environmental conditions which could destroy virus infectivity. Thus elaborate cold-chains or other protective measures are necessary to ensure virus viability for amplification.

Second generation diagnostic techniques have circumvented several of these problems. In these tests, diagnosis is effected by detection and simultaneous identification of virus specific antigens. Enzyme immunoassays (EIAs) are examples of second generation diagnostic procedures which preclude many difficulties associated with conventional virus isolation and subsequent identification techniques (1, 2). However, certain technical and methodological difficulties are inherent in EIA techniques. Many of these can be precluded by the use of a third generation diagnostic test, nucleic acid (NA) hybridization. Since direct detection of NA is the method of diagnosis, concerns with antigen-antibody equivalence, antigen clearance or degradation, latent infections or other infections without substantial antigen production, and specificity of immunoglobulins are all eliminated. Nonetheless, the sensitivity and specificity of EIA may be retained. A drawback to the widespread use of hybridization in diagnosis has been the

need for radioisotopic labeling of probes. However, development of BLHPs by Ward and co-workers (3, 4) provides a technique that retains the exquisite sensitivity and specificity of nucleic acid hybridization without drawbacks associated with safety, shelf-life, and disposal of radioisotopes. After hybridization, the biotin reporter molecule incorporated into a probe can be detected immunologically by immunofluorescence (IF) or enzyme immunoassay (EIA). Biotinylated NA hybridization techniques have gained wide acceptance for detection of both integrated and extrachromosomal virus specified NA sequences (3, 5-7). However these techniques had not been applied to the detection of arboviruses in vectors.

The rationale for this proposal was that NA hybridization techniques should be applicable to detection of arbovirus genomes or specified NA sequences in vectors. Further, NA hybridization should provide an extremely sensitive and specific mechanism for detection of infected arthropods. With the substitution of biotin-labeled NA probes for radioisotopic probes, the technique should be adaptable to field situations and should permit rapid diagnostic capability. Further, immobilization of virus NA on nitrocellulose should permit storage and transport of diagnostic samples, thereby precluding problems of preservation of virus antigens or infectious virus.

Significant progress was made in the first year of this granting period in the areas of development and characterization of constructs and protocol development. Techniques for blotting and detecting LAC and dengue RNA species on nitrocellulose using both isotopic and biotinylated probes have been developed as well as techniques to detect LAC RNA in situ. Results thus far are most encouraging. However, two major problems or potential problems have been encountered which impede the use of hybridization in virus diagnosis and surveillance, especially in clinical or field situations. These are problems associated with sensitivity for detection of nucleic acid blotted onto nitrocellulose and the need for laborious, time consuming extraction procedures necessary to purify the RNA for processing in the current protocols. Because of these difficulties or potential problems, we have embarked upon the development of sandwich hybridization techniques to detect arbovirus NA in mosquito suspensions.

III. Methods and Results

A. LaCrosse (LAC) virus: Development of techniques for detection of virus nucleic acid (NA) species:

In the LAC virus system, progress has been made in development of type and group specific hybridization systems. In addition, studies have begun to develop SS RNA probes (SP6 transcripts) for detection of LAC nucleic acids (9).

In the previous report, biotin labeled hybridization probes were developed. However, we have found it necessary to return to radioactive probes temporarily for two reasons. First, these probes allow us to more accurately determine hybridization parameters in order to develop an extremely reliable test. Secondly, use of radioactivity will allow us to quantitate levels of RNA detected in cells by the in situ hybridization method. We plan to return to the use of biotin labeled probes once quantitation studies have been completed.

1. Hybridization - specificity of ^{35}S labeled S RNA cDNA probe.

Studies were conducted to determine the specificity of the LAC S RNA probe. The probe is a cDNA of the S RNA cloned into the PvuII site of the plasmid pBR322 (8). The construct was labeled with ^{35}S by nick translation. Cells were infected with the following California group viruses: LAC, SSH, California encephalitis virus (CEV), tahyna (TAH), Jamestown Canyon (JC), and trivittatus (TVT). After 24 hours of incubation, cells were fixed in 4% paraformaldehyde and stored in 70% ETOH. For hybridization, slides were removed from ethanol, rehydrated in PBS, and permeabilized by incubating for 10 min. at room temperature in 50 ug/ml of nuclease free protease (Pronase, Calbiochem). Viral RNA was denatured by incubation of the slides in 2xSSC-50% formamide at 65C for 10 min., then cells were prehybridized for one hour at room temperature in hybridization buffer without probe. This buffer consisted of 45% formamide, 2xSSC, 10% dextran sulfate, 200 ug/ml herring sperm DNA, 10mM VRC, 1X Denhardts (0.02% w/v each Ficoll, PVP, and BSA), and 2ug/ml yeast tRNA. Slides were hybridized in the same buffer plus 1ug/ml ^{35}S labeled probe and 100mM DTT. DTT is necessary to prevent nonspecific adherence of the ^{35}S to the cells or the slide. The specific activity of the probe was 1-2 x 10^6 cpm/ug. Hybridization was at 37C for 16 hours. After hybridization, slides were washed in 2xSSC-45% formamide twice for 10 min. each at 37C, once in 2xSSC at 37C for 10 min, and once in 2xSSC for 10 min. at room temperature. Slides were dehydrated through an alcohol series, and hybridized probe was detected by autoradiography. For autoradiography, Kodak NTB-2 nuclear track emulsion was melted at 45C, slides dipped in the liquid emulsion, and allowed to dry at room temperature for 1 hour. Exposure was at 4C for 3 days. Silver grains were developed by placing the slides in Kodak Dektol developer diluted 1:1 with water, for 2 min; stopping in water for 10 seconds, fixing in Kodak fixer for 5 min, and rinsing in water for 5 min. All of these reagents were at 15C. Slides were air dried overnight; then counterstained with Mayers hematoxylin. Hybridized probe is seen by the presence of black grains over the cells.

The probe strongly hybridized to LAC infected cells but not uninfected cells (Figure 1). The probe also hybridized to all of the tested California group viruses (Figure 2), including TVT virus which is only distantly related serologically. This was not unexpected because the S RNA is considered to be the most conserved portion of the bunyavirus genome.

2. Hybridization - specificity of ^{35}S labeled SSH M RNA cDNA

Studies were subsequently conducted to determine the specificity of a cDNA of a portion of the M RNA segment. The rationale was that since the M RNA codes for the envelope glycoproteins it would be more likely to be type specific. The probe (SSH M60-78) was a cDNA copy of a 2161 bp portion of the SSH M RNA segment cloned into the PstI site of pBR322 (14). The construct was labeled with ^{35}S by nick translation. BHK-21 cells were infected with LAC or SSH viruses. Cells were harvested, processed, and hybridized as related above. Autoradiography protocols were also identical. Studies are in progress to determine if the SSH M60-78 probe can differentiate between SSH and alternate California group viruses.

Interestingly, hybridization signal was only detected in cells infected with SSH virus; cells infected with LAC virus were negative (Figure 3). Thus the middle RNA probe is type specific.

3. Blot Hybridization - LAC RNA probes:

The 569-bp Pst I fragment of the cDNA of LAC virus small RNA segment was cloned into the Pst I site of the plasmid vector pSP65 (Promega Biotec). This plasmid contains the bacteriophage SP6 promoter (9), a multiple cloning region, and an ampicillin resistance gene. E. coli HB101 were transformed with the recombinant plasmid and selected for ampicillin resistance.

Plasmid was purified from large-scale cultures of transformed E. coli by a standard alkaline lysis procedure, and linearized with the restriction enzyme Hind III. Linearized SP65-LAC plasmid was used as template for the in vitro transcription of RNA by SP6 RNA polymerase. Labeled RNA was prepared by incorporating either ^{32}P or ^{35}S UTP. DNA template was removed by treatment with DNase, and labeled RNA recovered by ETOH precipitation. This labeled RNA was used to detect LAC virus RNA which was blotted onto nitrocellulose.

RNA was purified from LAC virus infected cells by phenol-chloroform extraction. RNA was blotted onto nitrocellulose (10) in dilutions and fixed at 80C in vacuo for 2 hours. Blots were prehybridized in a solution consisting of 50% formamide, 5xSSC, 25 mM NaPO4, 5X Denhardts solution, 250 ug/ml sonicated salmon sperm DNA, 0.1% SDS, and 200 ug/ml yeast tRNA, for 4 hours at 42C. Blots were hybridized in the same solution plus 5×10^6 CPM ^{32}P labeled RNA or 3×10^6 CPM ^{35}S labeled RNA, overnight at 42 C. After hybridization, blots were washed extensively, dried, and exposed to X-ray film. ^{32}P blots were exposed for 3 days, and ^{35}S blots were exposed for 5 days. Both the ^{32}P and ^{35}S labeled probes were equivalent in sensitivity (Figures 4 and 5).

B. Dengue virus: development of techniques to detect NA species:

1. Inefficiency of dengue RNA immobilization on nitrocellulose:

To determine the efficiency of RNA attachment to nitrocellulose, ^{32}P labeled dengue RNA was transcribed from the KO65-1200 DNA template (see below). One microliter of the labeled transcript was mixed with 1 ug of denatured salmon sperm DNA and applied to nitrocellulose in high salt (10xSSC) using a dot/blot apparatus (10). Counts per minute were determined before and after application of samples to nitrocellulose. Counts were also assessed after a mock hybridization with subsequent washes to determine RNA finally retained on the filter. Results are summarized in Table 1. Briefly, For all samples, more than 80% of the RNA eluted from the nitrocellulose.

2. Dengue sandwich hybridization

Sandwich hybridization methodologies have been reported to be sensitive and specific techniques for detecting nucleic acids (11,12). One important advantage of sandwich hybridization over conventional dot/blot hybridization schemes is that there is apparently no need to extract the target NA species. If true, such a technique would greatly facilitate detection of arbovirus NA species in mosquito pools. Accordingly, we have expended considerable effort in developing a sandwich hybridization technique for dengue viruses. To accomplish this goal, we have generated dengue-2

specific single stranded (SS) RNA probes and SS DNA probes by subcloning the 1.95Kb cDNA insert from pVV-17 obtained from Dr. R. Padmanabhan. The probes were made by subcloning all or part of the cDNA into an appropriate vector. Many of the constructs took advantage of a unique EcoRI restriction endonuclease site within the 1.95 Kb cDNA to produce fragments containing dengue-2 cDNAs of approximately 1200bp and 750 bp. Clone K064-750 was produced by subcloning the 750 bp fragment into the PstI and EcoRI sites in pSP64. Clone K065-1200 was constructed by subcloning the 1200 bp cDNA into identical restriction sites in pSP65. The two in vitro transcription plasmids, pSP64 and pSP65, differ only in orientation of their unique cloning sites with repect to the transcription promoter derived from the SP6 bacteriophage. This ensures that each cDNA is ligated into the correct orientation to produce anti-sense RNA complementary to the positive (+) sense genomic RNA of dengue virus. Anti-sense RNA is made by transcribing the DNA template in the presence of a specific SP6-phage encoded RNA polymerase. Clones K065-1950A and K065-1950B were constructed by subcloning the entire 1.95 Kb cDNA into the PstI site of pSP65. The two clones represent opposite orientations of the insert. K065-1950A DNA can be used to transcribe antisense RNA probes and K065-1950B DNA is used to transcribe positive (+) sense RNA which can be used to simulate viral RNA in optimizing hybridization conditions.

The M13 recombinant phages were produced by isolating double stranded DNA replicative form (RF) from infected E. coli (13). Clone K01318-750 and K01319-750 were constructed by subcloning the 750 bp cDNA into the EcoRI-Pst I sites of the RF DNA. Clone K01318-750 and K01319-750 were derived from M-13 vectors M13mp18 and M13mp19 respectively. The two vectors differed only in the orientation of their unique cloning sites. Clone K01318-1200 and K01319-1200 were constructed similarly by subcloning the 1200 bp cDNA into each M-13 RF. Phage produced by transforming E. coli with the two constructs, K01319-750 and K01318-1200, contain SS DNA having dengue specific sequences complementary to viral RNA. Figure 6a shows the original plasmid pVV constructions, and Figure 6b shows the relative locations of homologous cDNAs on the yellow fever virus genome. The in vitro transcription plasmids and M13 replicative form (RF) constructs which have been developed to date are shown in Figures 7 and 8. Similar constructs are being prepared using the pVV-1 and pVV-9 plasmid inserts.

We have started to evaluate the efficacy of these probes for the detection of dengue-2 RNA sequences using sandwich hybridization. Briefly, the technique utilizes a nitrocellulose-bound M13 derived SS DNA catcher probe containing the dengue-2 sequences which hybridize to dengue-2 RNA from the nonstructural genomic region. Simultaneously, an SP6 derived SS RNA detector probe hybridizes to the genomic RNA at sequences adjacent to those recognized by the catcher probe. A schematic diagram of the technique is shown in Figure 9. Sandwich hybridization has several advantages in that it does not require the binding of RNA to nitrocellulose, which appears to be an inefficient process even in the presence of high salt. A second advantage is that relatively crude extracts of samples can be used during hybridization. Preliminary tests using purified genomic dengue-2 RNA and dengue RNA of (+) polarity produced in vitro are encouraging. Figures 10 and 11 are autoradiographs of sandwich hybridizations in which 100 ng of viral RNA or transcribed RNA were detected. We have optimized the amount of immobilized catcher probe necessary to anneal dengue viral RNA and form a sandwich hybridization complex. Multiple trials, using serially diluted

(1:2) single-stranded catcher probe (M13 recombinant phage KO1319-750) have shown that 0.2 to 0.5 ug of catcher is required for the best signal. Signal strength was assessed from autoradiographs both visually and by densitometry. We are currently binding catcher probe to a modified nylon membrane (Nytran) by UV-crosslinking. The advantage of this approach is that catcher probe DNA is stably bound to the nylon membrane principally by UV-light activated thymine bases interacting with the primary amine groups of the nylon matrix. This procedure permits highly stringent assay conditions without a loss of the substrate molecule. Figure 12 shows the results of a hybridization between UV cross-linked SS DNA (clone KO1318-750) and a complementary labeled RNA probe (recombinant transcription plasmid KO64-750). Sensitivity levels were comparable to heat fixing DNA on nitrocellulose thus providing a less fragile membrane solid support with equal or better sensitivity than nitrocellulose.

We are also attempting to minimize background in this technique by treating blots post-hybridization with RNase A to remove detector probe which has nonspecifically bound to nitrocellulose. Before and after treatment of blots with 100ug/ml of RNase A are shown in Figure 13.

We have intrathoracically inoculated mosquitoes (Aedes albopictus) with dengue-2 virus and are presently using the sandwich hybridization technique to detect dengue specific RNA sequences in mosquitoes. We are using both radioactive and biotinylated detector probes in these assays. First attempts, using crude mosquito extracts have failed to distinguish pools of infected mosquitoes from pools of uninfected mosquitoes (Figure 14). Several plasmid or phage (KO65-1200 and KO1318-1200 constructs) used in the procedure were not used in the previously reported sandwich hybridization protocol (see previous progress report). These are being recharacterized to assure that the probes are the correct polarity and size.

C. Publications/Manuscripts

The following manuscripts that detail much of the preceding information are in preparation for submission.

1. Chandler, L.J., Beaty, B.J., Bishop, D.H.L., and Ward, D.C. 1987. Dynamics and cellular distribution of LaCrosse virus S RNA synthesis in BHK-21 cells determined by in situ hybridization using a biotinylated cDNA probe. J gen Virol., ms prepared for submission.

2. Olson, K., Beaty, B.J., Blair, C.D., and Padmanabhan, R. 1987. Detection of Dengue RNA in cells and in mosquitoes by hybridization. Am J Trop Med Hyg., in preparation for submission.

VI. Discussion

We previously developed an in situ hybridization procedure to detect LAC virus RNA in cells (15). The probe used was a cDNA of the S RNA segment of LAC virus. Although the probe was sensitive for detection of analyte RNA, it was not specific. The S RNA sequences of SSH, TVT, TAH, JC, and CE all hybridized with the probe (Figure 2). Thus this probe construct is group specific, detecting all tested members of the California group. This probe would be most useful in a screening test. In contrast, the SSH M RNA cDNA probe hybridized to SSH virus infected cells but not to LAC infected

cells. Thus this probe is apparently type specific, permitting differentiation between LAC and SSH virus. Both group and type specific probes have their relative advantages in diagnosis. Application of hybridization techniques using such probes should provide group and type specific diagnosis of an etiologic agent in a clinically relevant time frame.

Studies have also been initiated to develop RNA probe systems for diagnosis. The RNA transcript probes were demonstrated to be specific (Figures 4 and 5), but the preliminary results do not suggest that sensitivity is signficantly greater than that attained with cDNA probes. This would suggest that the major determinant of sensitivity in the blot hybridization protocols that are used is the efficiency of RNA analyte binding to nitrocellulose (Table 1). Development of RNA probes will be continued because they are relatively easily prepared and used. In addition, strand specific probes can be prepared, adding a more sophisticated system for determination of viral replication mechanisms and dynamics.

Considerable effort was devoted in this granting period to the development of catcher and detector probe systems. A variety of dengue specific SS DNA and RNA probes were constructed and have been used to detect dengue RNA by sandwich hybridization. So far nanogram quantities of purified RNA have been detected, but we are confident that the sensitivity of the technique can be improved significantly. Additional advantages include minimizing sample manipulation steps and precluding binding of analyte RNA directly onto the nitrocellulose. We are currently determining the efficacy of the technique for detection of dengue RNA in infected mosquitoes and mosquito pools.

V. Cost Analysis (curve) of budgetary expenditures:

Projected and actual budget expenditures are noted for this past year of the contract and projected expenditures are noted for the period Oct 1, 1986 to Sept 30, 1987 (Figure 15). No significant budgetary deficits or balances are anticipated.

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Table 1

RNA binding efficiencies to nitrocellulose

Sample[1]	CPM Initial	CPM Prehybridization	CPM Posthybridization	RNA (%)[2] Bound
1.	391,445	131,974	49,319	12.6
2.	186,225	72,208	20,567	11.0
3.	103,575	38,843	12,667	12.2
4.	46,590	19,065	6,206	13.3
5.	26,255	10,677	3,274	12.5
6.	12,735	5,024	2,369	18.6

[1] Counts given per sample is the mean of three replicate applications of the sample to nitrocellulose. Samples 2 through 6 represent sequential two-fold dilutions of sample 1.

[2] % RNA bound was determined by the formula:

$$\frac{\text{CPM posthybridization}}{\text{CPM initial}} \times 100$$

Table 2

Dengue probe constructs

Clone designation	Vector	Insert Data	Probe Type	Probe Sense
1. KOBR-1	pBR322	1.4 kb, pVV-1, PstI	DS-DNA	+/-
2. KOBR-17	pBR322	1.95kb, pVV-17, PstI	DS-DNA	+/-
3. KOBR-750	pBR322	750 bp, pVV-17, EcoRI-PstI	DS-DNA	+/-
4. KOBR-1200	pBR322	1200bp, pVV-17, EcoRI-PstI	DS-DNA	+/-
5. KOTZ-750	PTZr18	750 bp, pVV-17, EcoRI-PstI	SS-RNA	+
6. KOTZ-1200	PTZr18	1200bp, pVV-17, EcoRI-PstI	SS-RNA	-
7. KO64-750	pSP64	750 bp, pVV-17, EcoRI-PstI	SS-RNA	-
8. KO65-1200	pSP65	1200bp, pVV-17, EcoRI-PstI	SS-RNA	-
9. KO65-1	pSP65	1.4 kb, pVV-1, PstI	SS-RNA	+or-[a]
10. KO65-17	pSP65	1.95kb, pVV-17, PstI	SS-RNA	+or-
11. KO65-9	pSP65	1.6 kb, pVV-9, PstI	SS-RNA	+or-
12. KO1318-750	M13mp18	750 bp, pVV-17, EcoRI-PstI	SS-DNA	+
13. KO1319-750	M13mp19	750 bp, pVV-17, EcoRI-PstI	SS-DNA	-
14. KO1318-1200	M13mp18	1200bp, pVV-17, EcoRI-PstI	SS-DNA	-
15. KO1319-1200	M13mp19	1200 bp, pVV-17, Ecor1-Pst1	SS-DNA	+

[a]sense not yet determined

Figure 1

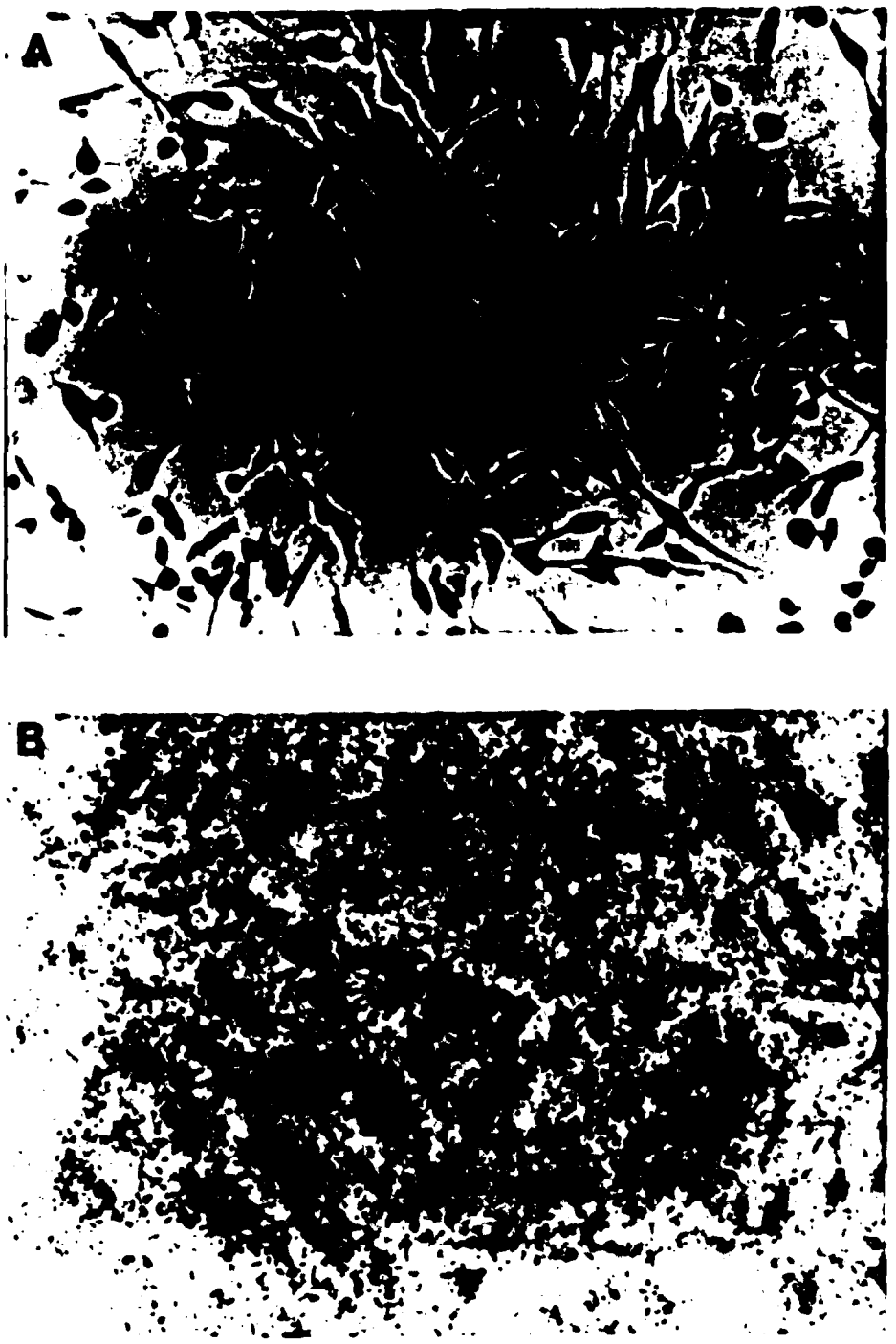


Figure 1. BHK-21 cells hybridized with ^{35}S labeled LAC S RNA cDNA probe
A. Uninfected cells.
B. LAC infected cells.

Figure 2

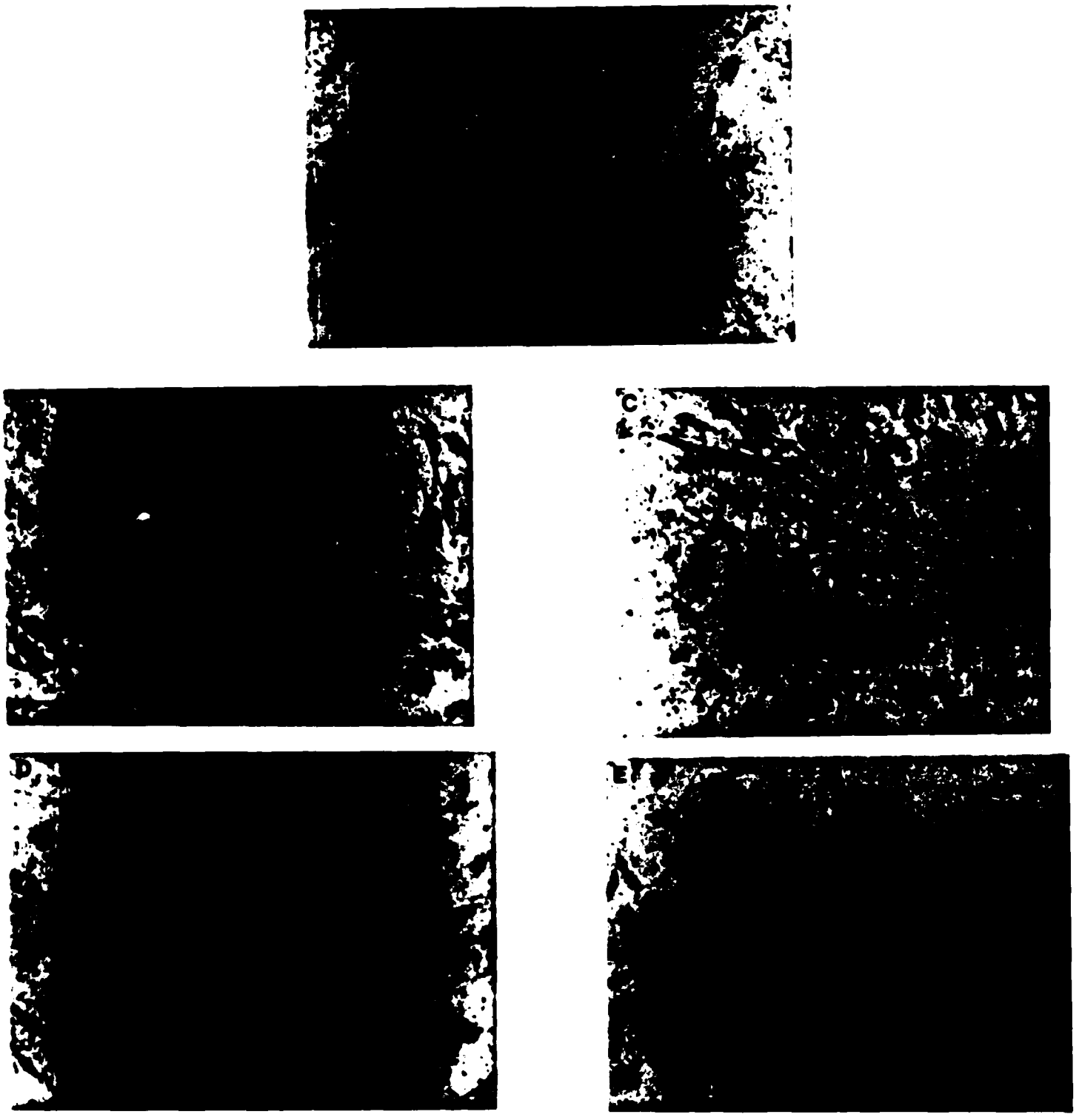


Figure 2. Specificity of ^{35}S labeled LAC S RNA cDNA probe.
A. SSH infected cells. B. CE infected cells.
C. TVT infected cells. D. TAH infected cells.
E. JC infected cells.

Figure 3

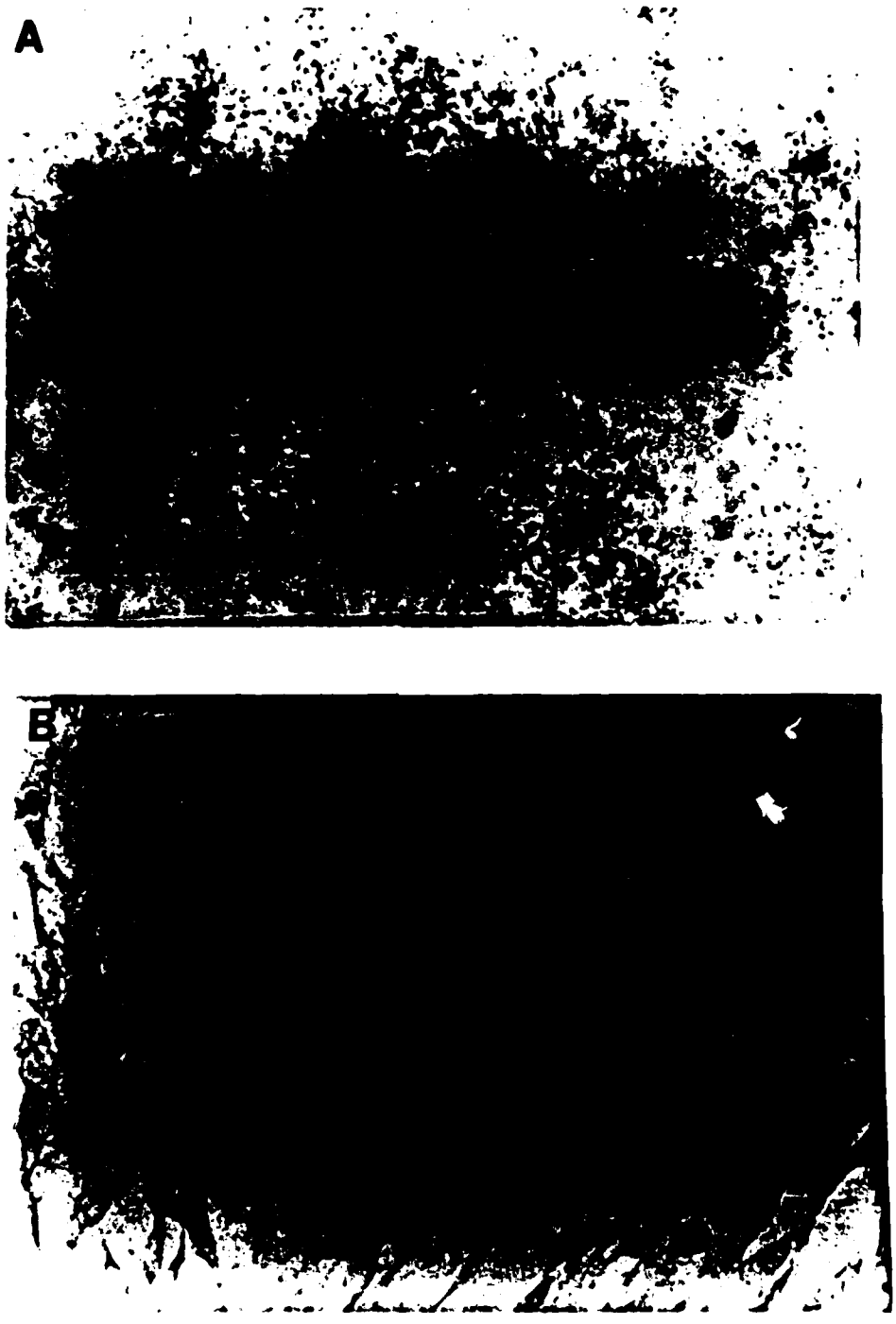


Figure 3. Specificity of ^{35}S labeled SSH M RNA cDNA. Cells hybridized with ^{35}S labeled SSH M60-78 cDNA probe.
A. SSH infected cells. B. LAC infected cells.

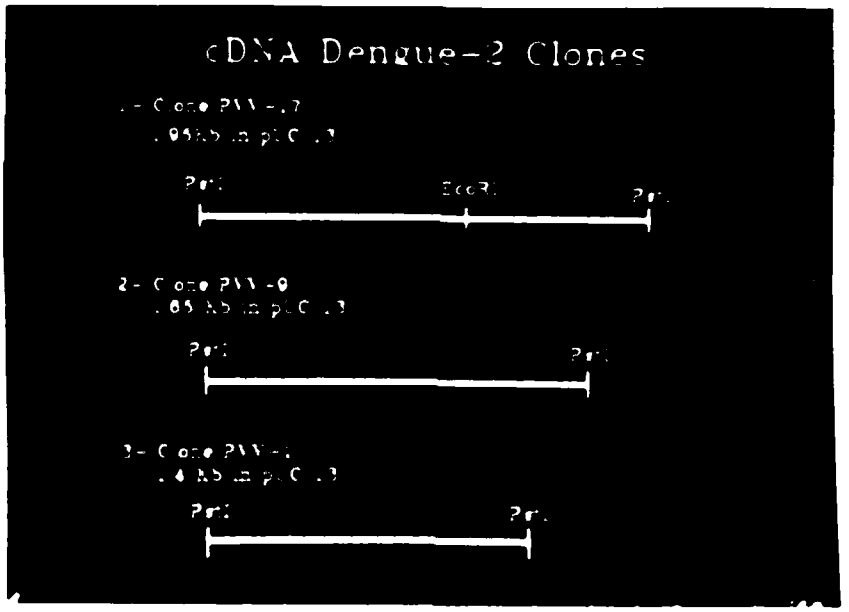


Figure 6a. pVV recombinant plasmid constructs containing the dengue-2 cDNAs of 1.95kb, 1.65kb, and 1.4kb. A unique EcoRI site has been identified in the 1.95kb cDNA and was utilized in constuction of the probes forthe sandwich hybridization.

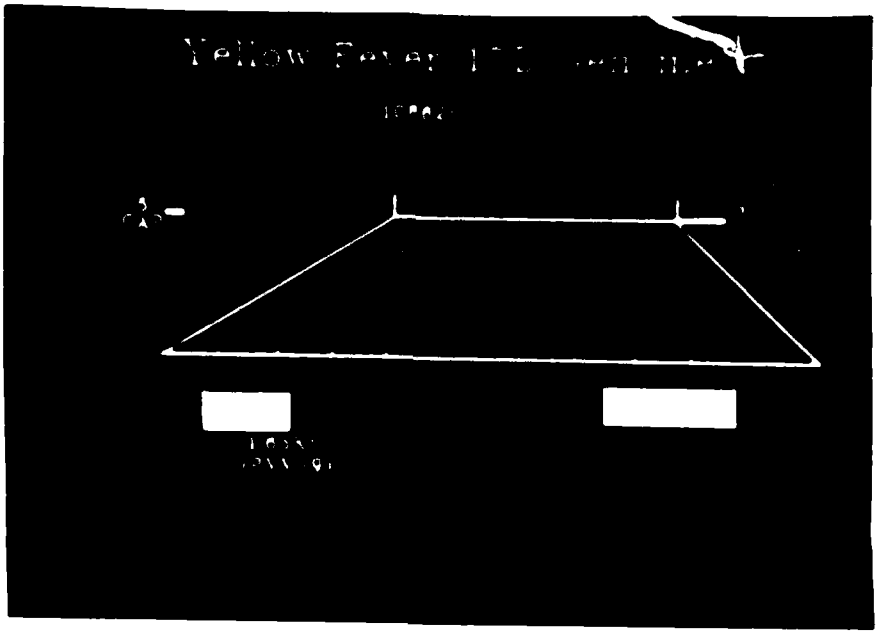


Figure 6b. Relative locations of the dengue cDNAs on the yellow fever genome.

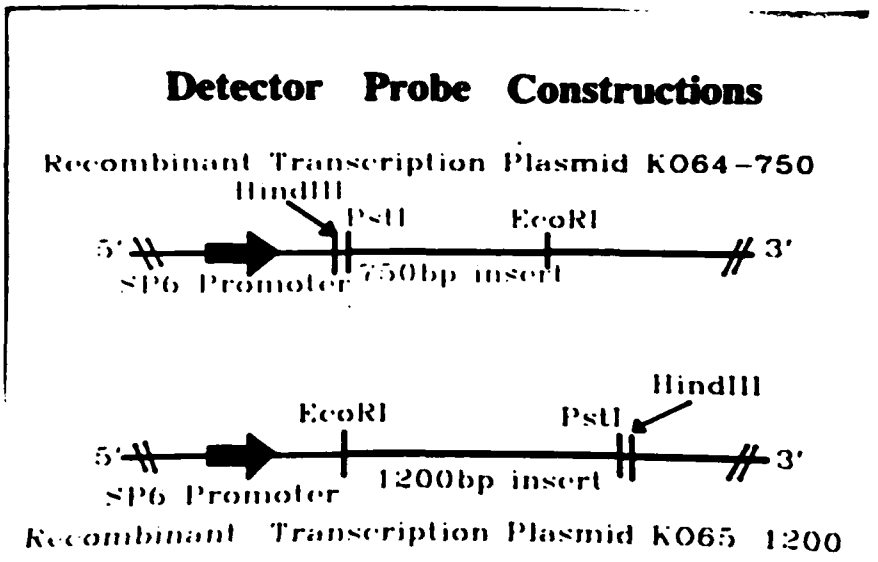


Figure 7. Detector probe constructs. K064-750 and K065-1200 detector probes were produced by inserting the 750 bp and 1200 bp cDNAs into pSP-64 and pSP-65 RNA transcription plasmids. Inserted DNAs were isolated from PstI-EcoRI digests of construct pVV-17. Both constructs generate (-) sense RNA complementary to the dengue genomic RNA.

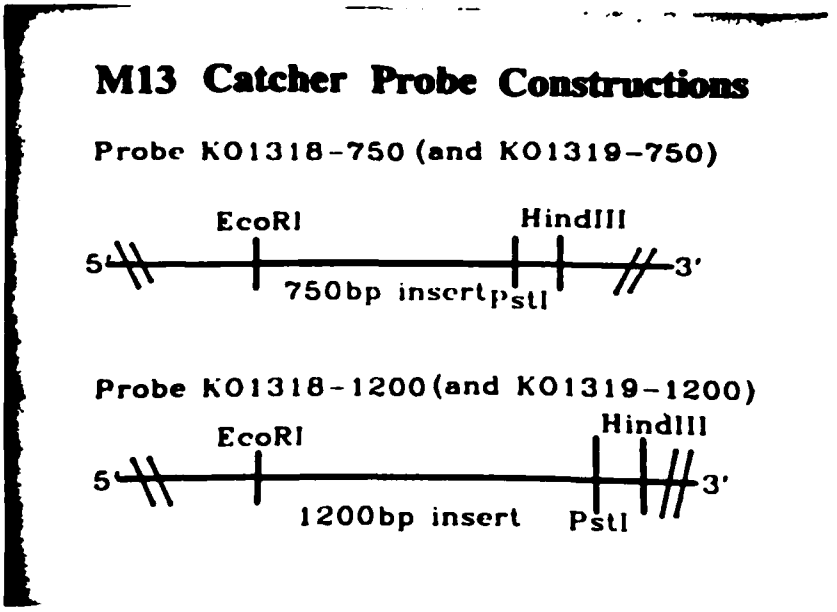


Figure 8. Catcher probe constructs. K01318-750, K01319-750, K01318-1200, and K01319-1200 catcher probes were produced by inserting the 750 bp and 1200 bp dengue cDNAs into M13mp18 and M13mp19 phage vectors. Clones K01318-750 and K01319-1200 produce (+) sense ss dengue DNA and K01319-750 and K01318-1200 produce (-) sense ss DNA. The (-) sense ss DNAs are complementary to dengue genomic RNA.

FIGURE 9

Sandwich Hybridization Schematic

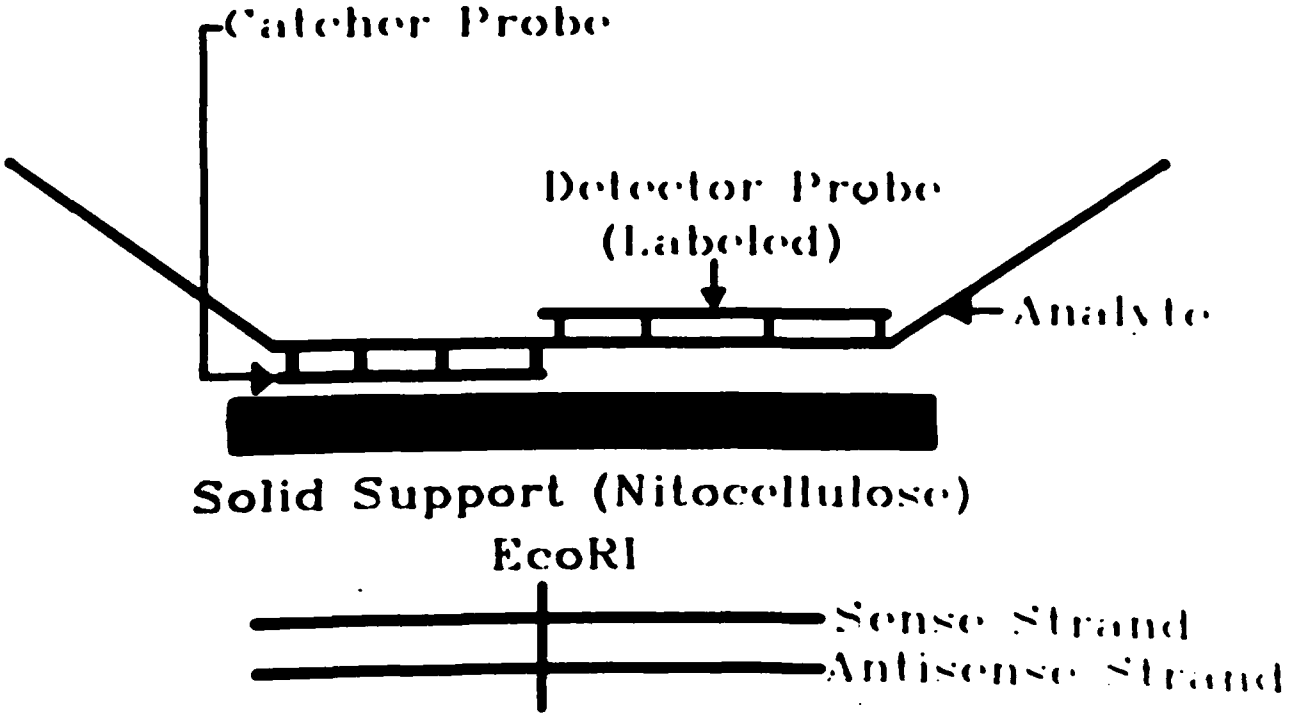
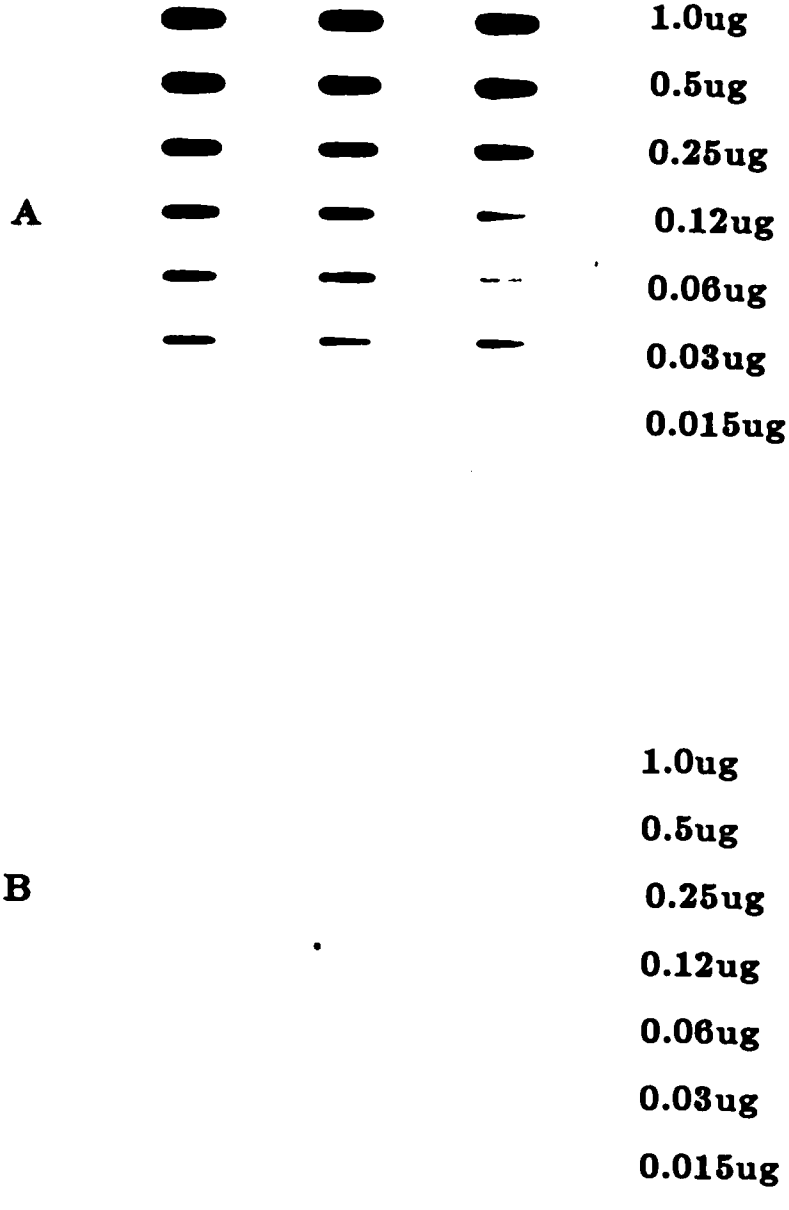


FIGURE 10

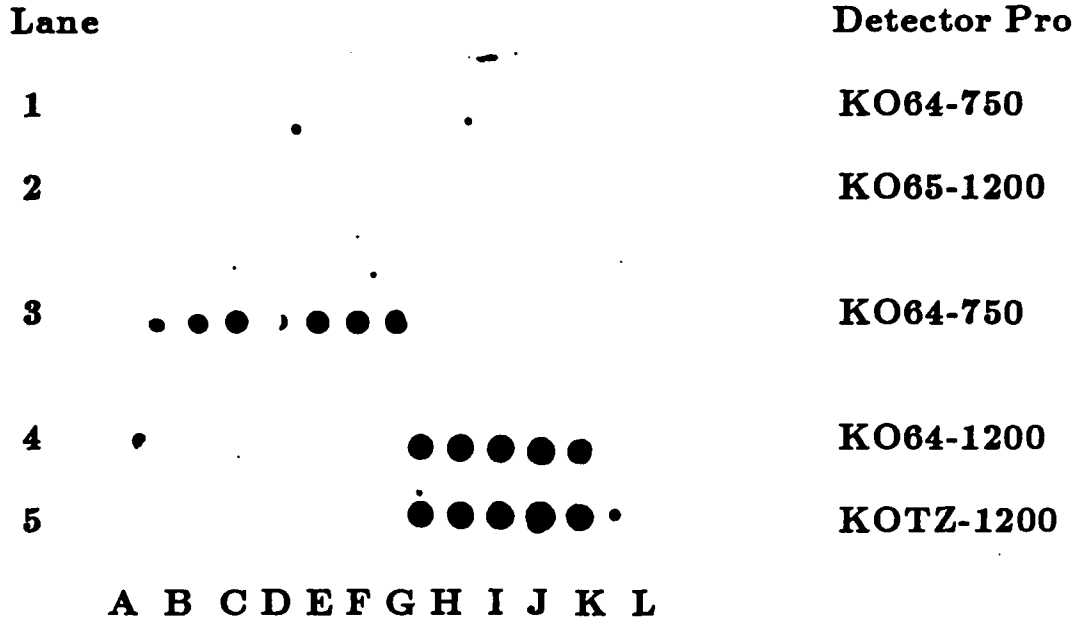
Detection of Dengue Virus RNA by Sandwich Hybridization



Sandwich hybridization. A) Purified dengue-2 (Jamaica) RNA was the analyte; 100ng of the viral RNA was used in hybridization; 0.25 to 0.5ug of the carcher probe is adequate to detect viral RNA. B) No viral RNA was added.

FIGURE 11

Sandwich Hybridization Using Different Detector and Catcher Probe



Analyte for lanes 1 and 2 was a (-) strand full length transcribed RNA from construct KO65-1950A. For lanes 3,4, and 5 the analytes were (+) strand transcribed from the construct KO65-1950B. The catcher probes were as follows: rows A,B-M13mp18 RF; rows C - G KO1318-1200 SS DNA; rows H - L KO1319-750 SS DNA. All capture probes were applied at 1.0ug/spot. 100ng of analyte was used to hybridize to each of 5 test strips. Blots were treated with 100ug/ml of RNase A.

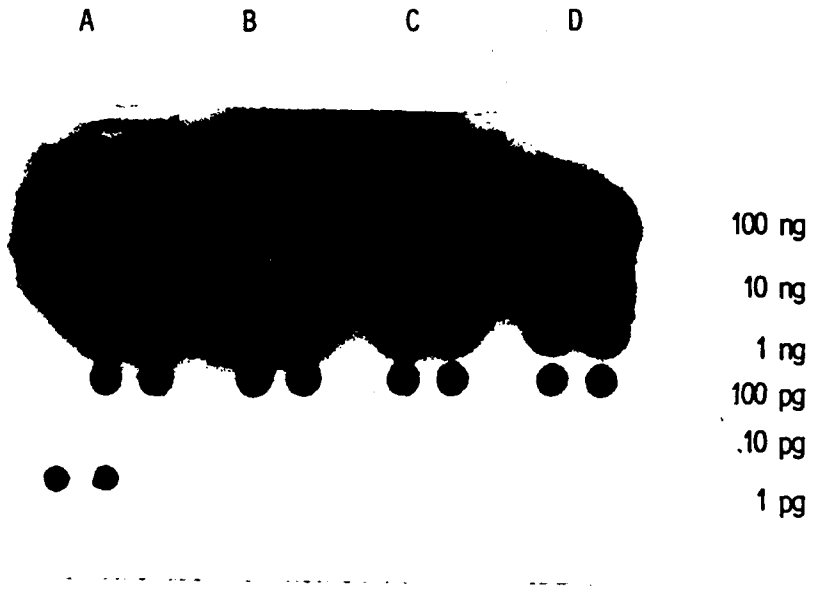
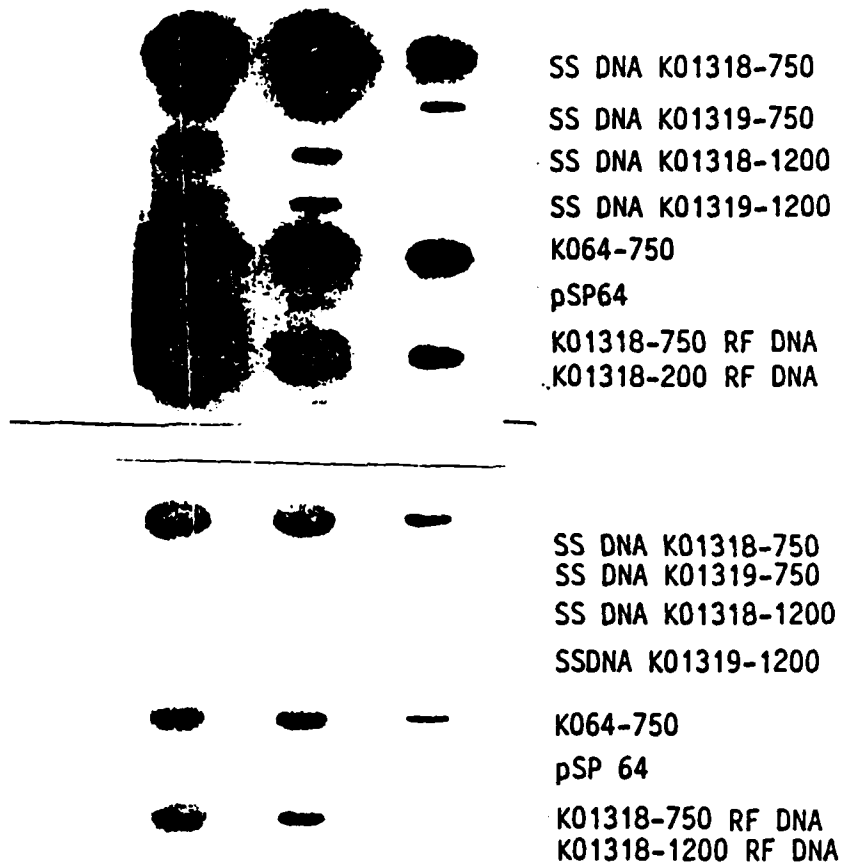


Figure 12. Results of ultraviolet light crosslinking of DNA to a nylon filter (Nytran). Samples A-D contain identical DNAs crosslinked to the filter with each group composed of a negative control (ss DNA of M13mp18) followed by duplicate ss DNAs generated from the recombinant phage K01318-750. DNAs of groups A-D are crosslinked by exposure to a UV source for 3 min, 4 min, 5 min, and 6 min respectively. The probe is an α-^{32}P CTP labeled transcript generated from clone K064-750 and complementary to the K01318-750 insert DNA.

FIGURE 13

Effect of RNase treatment on background signal in autoradiograms



Results of RNase A treatment of blots to reduce background. A. Blots with no RNase A treatment compared to the same blot, B. treated with 100ug/ml RNase A for 15 minutes at room temperature. Probe used in hybridizations of blots A and B was an RNA probe transcribed from clone K064-750.

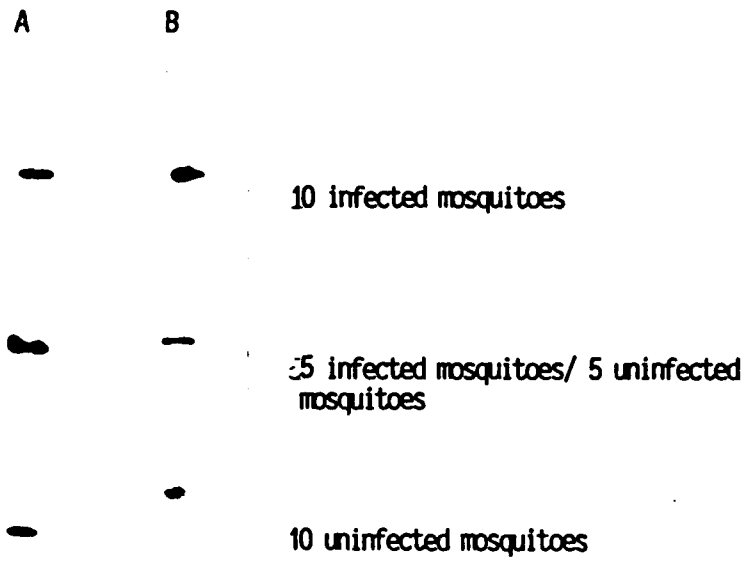
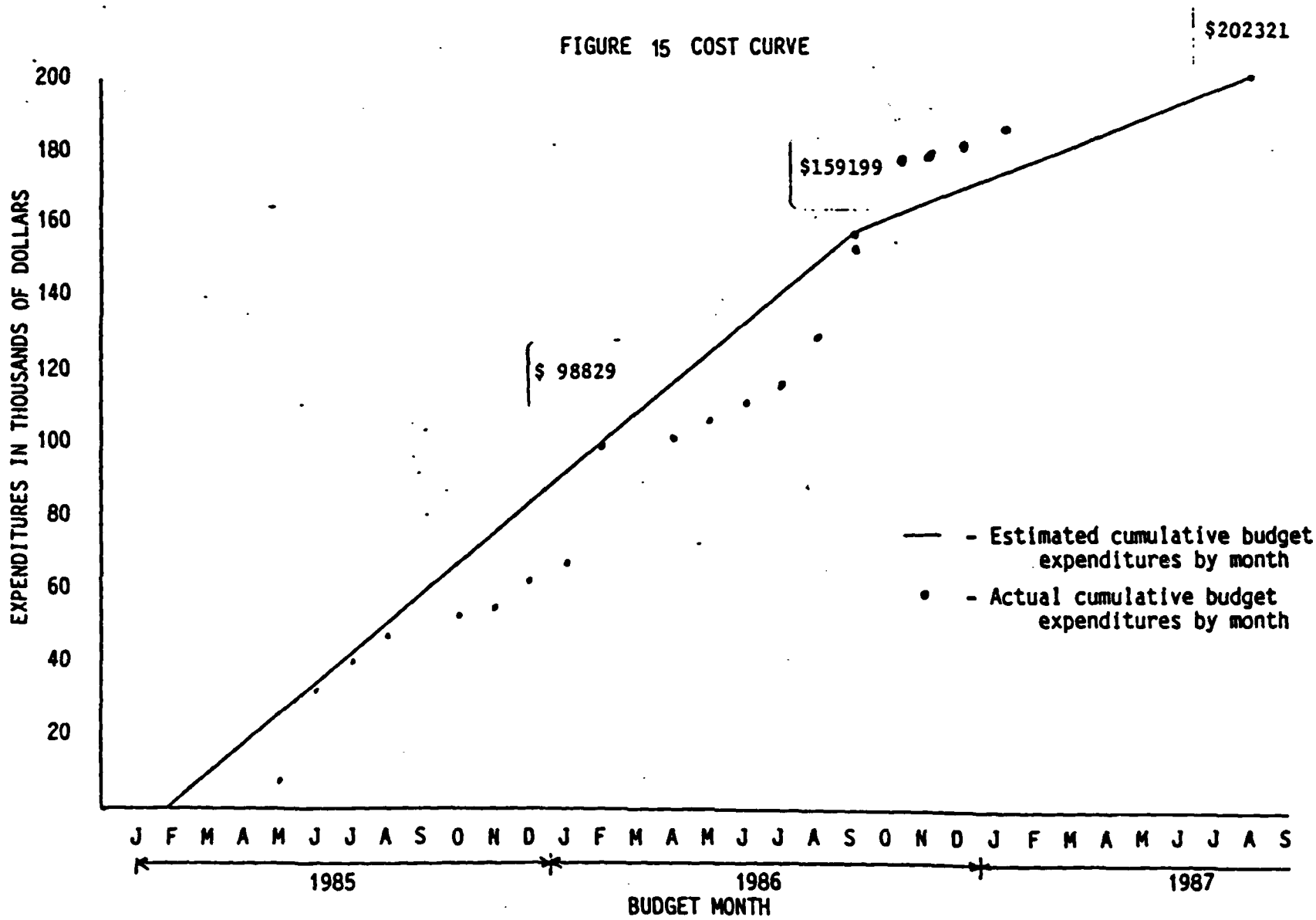


Figure 14. Results of analyzing mosquito pools for presence of dengue-2 RNA sequences by the sandwich hybridization (SH) technique. Aedes albopictus were inoculated intrathoracically with virus and RNA was extracted 14 days later. A) SH technique using K01318-1200 ss DNA as catcher probe and labeled K064-750 RNA as detector probe. B) SH technique using K0BR-1200 as catcher probe and labeled K064-750 RNA as detector probe.

FIGURE 15 COST CURVE



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