TITLE: "SAFETY TESTING OF SEED AND VACCINES FOR DENGUE VIRUSES IN MICE, GUINEA PIGS, RABBITS AND BACTERIAL AND MYCOPLASMA CULTURE MEDIA"

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Frederick, Maryland 21702-5012

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**Title:** "Safety Testing of Seed and Vaccines for Dengue Viruses in Mice, Guinea Pigs, Rabbits and Bacterial and Mycoplasma Culture Media".

**Author:** Potash, Louis

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**Abstract:**
Preclinical safety testing of dengue virus production seeds consisted of inoculation in: 1) 5 different cell culture lines; 2) rabbits, mice (adult & newborn sucklings) and guinea pigs; and 3) bacterial fungal and mycoplasma culture media. Inocula for these tests were the crude, unclarified harvests of both control and virus fluids. For dengue-1, 3 different PDK passage levels grown in FRH-2 cells and the same for dengue-4. All tests carried out following guidelines established by the FDA for live and inactivated virus vaccines as found in 21 CFR, Part 600 and were performed in accordance with GLP regulations. A dengue-1 vaccine lot was subjected to 20 additional serial passages (total 30) in dog kidney cell cultures in attempts at further attenuation. Problems with lack of high-titered immune serum resulted in unsatisfactory findings in tissue culture purity (AGMK cells) for both viruses and in suckling mice tests with dengue-4 viruses.
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I. INTRODUCTION

The Virus Vaccine Production Laboratory of Flock Laboratories, Inc. had been awarded a contract by USAMRDC to conduct preclinical testing services for Walter Reed Army Institute of Research (WRAIR) to evaluate dengue vaccines and seeds for purity, safety and potency in accordance with the technical proposal entitled "Safety Testing of Seed and Vaccines for Dengue Viruses in Mice, Guinea Pigs, Rabbits and Bacterial and Mycoplasma Culture Media". Effective Jan 1, 1990, this research, production and testing Laboratory was purchased by Program Resources, Inc. (PRI) and this contractual effort continued under an approved subcontract with PRI.

The preclinical testing services consisted of the inoculation of submitted test articles in: 1) five different cell culture lines; 2) mice (adult and new-born sucklings), guinea pigs and rabbits; and 3) bacterial, fungal and mycoplasma culture media. The inocula for these tests were crude, unclarified harvests of both control and virus fluids plus specific immune serum. All tests were carried out following guidelines established by the FDA for live and inactivated virus vaccines as found in 21 CFR, Parts 310.11, 610.12, 610.30, 630.10 - 630.19, 630.30 - 630.37, 630.40, 630.50 - 630.57 and 630.66 - 630.67 and were performed in accordance with Good Laboratory Practices (CLP) regulations for preclinical testing of biologics (21 CFR, Part 58).

Although initially designed as a three (3) year contract, this effort has been brought to a close within an 13 month period with the completion of the preclinical safety testing of the following fluids: production seed pools of 3 different FDK passage levels grown in FRhL-2 cells of both dengue-1 (#45425) and dengue-4 (CARIB #341750) viruses.

In addition, in an effort to attenuate a dengue-1 vaccine lot which had previously undergone 10 serial passages in dog kidney (DK) cell cultures, the virus was serially passed 20 more times (total 30 passages) in DK cell cultures with aliquots of each passage level submitted to the CCR, as directed. It was 3 of these 30 DK passage level harvests that served as the inocula for the above dengue-1 production seeds in FRhL-2 cell cultures.
II. PRECLINICAL SAFETY TESTING

Over the 13 month period, a total of 8 test articles, composed of 6 crude, unclarified virus fluids and 2 related, crude, unclarified control fluids, was safety tested. As specified in the contract workscope, these fluids were tested for:

a) microbial sterility (bacterial, fungal and mycoplasmal);

b) purity (safety) in tissue cultures (four tissue culture systems - AGMK, PHA, PRK and Flow 5000 plus the cell system in which the virus was grown - FRhL-2);

c) animal safety in rabbits, mice (adult and newborn sucklings) and guinea pigs.

The test articles consisted of 3 dengue-1 virus production seeds plus a control fluid and 3 dengue-4 virus production seeds plus a control fluid. For the dengue-1 seeds grown in FRhL-2 cell cultures, the 10th, 20th and 27th dog kidney passage levels served as the specific inoculum. For the dengue-4 seeds grown in FRhL-2 cell cultures, the 6th, 10th and 15th dog kidney passage levels served as the specific inoculum.

DENGUE-1 VIRUS (#45A25)

Production Seed: PDK-16, FRhL-2/c7 of 15 Feb 90
PDK-20, FRhL-2/c7 of 15 Feb 90
PDK-27, FRhL-2/c7 of 15 Feb 90
Control Fluid .................... of 15 Feb 90

All fluids satisfactorily passed the microbial sterility tests. The results of the tissue culture purity (safety) tests were unsatisfactory only in the AGMK cell culture system with all 3 virus pools and were attributed to the failure of the supplied antiserum to completely neutralize the dengue-1 viruses. Based on a previous test with a dengue-1 virus (strain Western Pacific 1974), difficulties with the AGMK purity test were anticipated; however, pre-treatment of the primary flask cultures with immune serum 24 hours prior to inoculation with neutralized virus did not prevent dengue virus-attributed cytopathology from occurring both in the primary flasks and in the secondary tube subcultures. As expected, the tube subcultures were completely resistant to challenge with the Coxsackie A-9 virus. All virus fluids satisfactorily passed the prescribed animal safety tests. Because the AGMK purity test was unsatisfactory, these fluids were not considered to have passed all the above prescribed preclinical tests. A Phase Report detailing all of the above test results is being submitted together with this Final Report.
DENGUE-4 VIRUS (CARIB #341750)

Production Seed: PZ-K-6, FRhL-2/dA7 of 9 Mar 90
PCK-10, FRhL-2/dA7 of 9 Mar 90
FKL-13, FRhL-2/dA7 of 9 Mar 90
Control Fluids ..................... of 9 Mar 90

All fluids satisfactorily passed the microbial sterility tests. The results of the tissue culture purity (safety) tests were unsatisfactory only in the AGMK cell culture system with all 3 virus pools and were attributed to the failure of the supplied antiserum to completely neutralize the dengue-4 viruses. Based on previous tissue culture purity tests with dengue viruses, difficulties with the AGMK purity test were anticipated. Dengue virus-attributed cytopathology was detected in the primary flasks as non-descript morphological changes and in the secondary tube subcultures as lytic changes. As expected, the tube subcultures were completely resistant to challenge with the Coxsackie A-9 virus. All virus fluids satisfactorily passed the prescribed animal safety tests in rabbits, adult mice and guinea pigs but results were inconclusive in suckling mice. The difficulties in the suckling mice tests, where many of the sucklings were found either dead, moribund or lethargic, were attributed to the failure of the supplied antiserum to completely neutralize the dengue-4 viruses. Because the AGMK purity test was unsatisfactory and the suckling mice tests were considered inconclusive, these fluids were not considered to have passed all the above prescribed preclinical tests. A Phase Report detailing all of the above test results is being submitted together with this Final Report.

III. SERIAL PASSAGES

Dengue Virus Type 1, Strain #45AZ5: Live-Attenuated Vaccine, Lot No. 1-82, Run 2. As an adjunct to these Proroclinical Safety Tests, this laboratory continued the serial passages of this virus in dog kidney cell cultures in an effort to achieve further attenuation. Pre-screened, frozen ampules of primary dog kidney (PDK) cells (LOT 222) had been supplied by the COR. All studies were carried out in accordance with protocols submitted by the COR and included passages control cultures. Commencing with the 10th serial passage harvest fluid produced during the previous contract, the laboratory successfully completed 20 additional serial passages using both 1st and 2nd passage DK cell cultures. Multiple 2 ml vials of each passage level (day 7 harvests of both virus infected and control cultures) were submitted to the COR.
IV. CONCLUSIONS

The preclinical safety testing of dengue virus production seeds in accordance with the specified scope were completed. The test articles consisted of crude, unclarified harvest fluids of 3 different PCK passage levels of both dengue-1 and dengue-4 viruses grown in the FRhL-2 cell system. Due to the lack of high-titered, specific immune sera, unsatisfactory results were reported for the AGMK tissue culture purity (safety) tests on all 6 dengue virus fluids assayed. In addition, the inconclusive results obtained in the suckling mice test with the dengue-4 fluids - many of the sucklings were found either dead, moribund or lethargic - are attributed to this same lack of immune serum. It is imperative that, for any future preclinical safety testing of dengue virus fluids whether production seeds or vaccine fluids, high-titered, specific immune sera be made available so as to ensure the satisfactory completion of all the prescribed tests.
December 17, 1990

Dr. Louis Potash  
Program Resources, Inc.  
Biomedical Services Division  
7655 Old Springhouse Road  
McLean, VA  22101

Dear Dr. Potash,

Microbiological Associates, Inc. is an AAALAC accredited animal facility, and all studies are performed in accordance with the "Guide for the Care and Use of Laboratory Animals", U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH Publication No. 86-23.

Sincerely,

Mary D. Whiteman  
Study Director, In Vivo Assays  
Biotechnology Division
May 14, 1991

TO: Mr. Donald Holzworth, Vice President
    Dr. Louis Potash, Study Director

FROM: James R. Plautz
    Sr. QA Advisor

RE: GLP Compliance Audit of Final Reports for Safety Testing of Dengue Virus Type 1 and Type 4

On April 14, 1991 a complete audit for GLP compliance (21 CFR, Part 58) was conducted for the subject final reports and their respective raw data.

Our complete findings indicate that the studies were conducted under the guidance of the referenced Standard Operating Procedures (SOPs), the variations from the SOPs had no apparent effect on study outcome, and that the final report for each study is substantiated by the raw data.

Animal safety testing was conducted and reported separately from these final reports.

[Signature]
May 14, 1991
APPENDIX I

Dengue-1 Virus Strain #45AZ5
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 85-23, Revised 1985) - (see Attachment A).

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

[Signature]

PI Signature 12-20-80

Date
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I. INTRODUCTION

The accompanying protocol is a description of the safety testing of 3 crude harvest lots of dengue virus type 1 designated as:

Dengue Virus Type 1 (45AZ5):
- PDK-10, FRhL-2/d7: PDK-20, FRhL-2/d7
- PDK-27, FRhL-2/d7 of 16 Feb 1990

Utilizing the testing procedures herein described, this fluid is considered to have not passed satisfactorily all tests for safety including purity. The detailed record with respect to passage history, pool production, and subsequent safety testing may be found in the laboratory notebooks located at:

The Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, DC 20307-5100 - (Dr. Ken Eckels)

The Experimental Virus Vaccine Production Laboratory - Suite #500 - (Flow Laboratories, Inc.) Program Resources, Inc. (PRI), McLean, VA - (Dr. Louis Potash)

All procedures performed at PRI followed Good Laboratory Practices (GLP) regulations (21 CFR, Part 58) and were carried out in accordance with the guidelines established by the FDA for live and inactivated vaccines as found in 21 CFR, Parts 610.11, 610.12, 610.30, 630.10 - 630.17, etc. of April 1989. These procedures are detailed in the following SOPs and recorded on the indicated VVPL Forms:

SCP No.: 500.001 - Issued 29 Oct 1980, Revised 13 Feb 1986
- 500.008 - " 13 Jan 1981, " 3 Mar 1986

VVPL FORM #008 - Issued 29 Oct 1980, Revised 3 May 1984
- 017 - " 16 Jan 1981, " 13 Jan 1986
- 019 - " 8 Oct 1984
II. SYNOPSIS

A. Crude Virus Harvests:

Dengue Virus Type 1 (45AZ5)
- PDK-10, FRhL-2/d7 of 16 Feb 90
- PDK-20, FRhL-2/d7 of 16 Feb 90
- PDK-27, FRhL-2/d7 of 16 Feb 90

B. Safety Tests on Crude Harvest Fluids:

1. Sterility: Fluid Thioglycollate (FTG), Tryptone Soya Broth (TSB), Mycoplasma
   a. PDK-10 Virus Fluid (47 ml) No Growth
   b. PDK-20 Virus Fluid (47 ml) No Growth
   c. PDK-27 Virus Fluid (47 ml) No Growth
   d. Control Fluid (TCF) (47 ml) No Growth

   a. PDK-10 Virus Fluid (25 ml) Unsatisfactory*
   b. PDK-20 Virus Fluid (25 ml) Unsatisfactory*
   c. PDK-27 Virus Fluid (25 ml) Unsatisfactory*
   d. Control Fluid (TCF) (25 ml) Satisfactory

3. Animal Safety:
   a. Rabbits: I.D. & S.C. - (Appendix - B)
      (1) PDK-10 Virus Fluid (30 ml) Satisfactory
      (2) PDK-20 Virus Fluid (30 ml) Satisfactory
      (3) PDK-27 Virus Fluid (30 ml) Satisfactory
   b. Adult Mice: I.C. & I.P - (Appendix - C)
      (1) PDK-10 Virus Fluid (10.6 ml) Satisfactory
      (2) PDK-20 Virus Fluid (10.6 ml) Satisfactory
      (3) PDK-27 Virus Fluid (10.6 ml) Satisfactory

* Test unsatisfactory only in the AGMK test system. Non-descript morphological changes observed in primary AGMK flask cultures, particularly after films were stained. All AGMK tube subcultures exhibited 2-3+ cytopathology. Both flask and tube subcultures were negative for hemadsorption. All tube subcultures completely inhibited the Coxsackie A-9 challenge virus.
3. Animal Safety (continued):

c. Suckling Mice: I.C. & I.P. - (Appendix - C)
(1) PDK-10 Virus Fluid* (2.2 ml) Satisfactory
(2) PDK-20 Virus Fluid* (2.2 ml) Satisfactory
(3) PDK-27 Virus Fluid* (2.2 ml) Satisfactory

d. Guinea Pigs: I.C. & I.P. - (Appendix - D)
(1) PDK-6 Virus Fluid (15.3 ml) Satisfactory
(2) PDK-10 Virus Fluid (15.3 ml) Satisfactory
(3) PDK-15 Virus Fluid (15.3 ml) Satisfactory

* Virus fluid was mixed with equal parts of a 1:5 dilution of the immune serum and incubated at 37°C for 90 minutes prior to inoculation.
III. DETAILED SUMMARY RELATING TO THE SAFETY TESTING OF THREE (3) DIFFERENT PASSAGE LEVELS OF DENGUE VIRUS TYPE 1 (#45AZ5) PRODUCTION SEEDS: PROPAGATED IN DBS-FRH2-2 CELL CULTURES

A. Inocula

In May 1990, the following frozen materials were obtained for testing from Dr. K. Eckels, Contracting Officer's Representative, Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, D.C.:

1. Dengue-1 (#45AZ5) crude, unclarified harvest fluids of 16 Feb 1990:
   a. PDK-10, FRH2-2 (day 7 harvest) ........... 20 x 10 ml vials
   b. PDK-20, FRH2-2 (day 7 harvest) ........... 20 x 10 ml vials
   c. PDK-27, FRH2-2 (day 7 harvest) ........... 20 x 10 ml vials
   d. Control Fluids ............................ 4 x 25 ml vials

2. Dengue-1 Antiserum: Jamaica HPAF of 4/17/79 ... 1 x 8 ml

On arrival in this laboratory, the virus and control fluids were stored at -70°C, or below, and the antiserum at -20°C, or below.

B. Safety Testing Procedures and Results on the Crude, Unclarified Harvest Fluids

(SOP No.: 500.008)

1. Microbial Sterility - (VVPL FORM #011)

   Aliquots of the bulk frozen fluids were thawed and tested for microbial sterility as follows:

   a. Fluid Thioglycollate Medium - FTM - (LOT VVPL #030): Each of 10 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluids and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 culture tubes were included as un inoculated controls. All cultures were vortex mixed and incubated at 32°C (+ 2°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 50 culture tubes.

   b. Tryptone Soya Broth - TSB - (LOT VVPL #030): Each of 10 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluids and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 22°C (+ 2°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 50 culture tubes.

The results of the above described Microbial Sterility Assays are summarized in Table I.
c. Mycoplasma Sterility: These assays were performed by PRI's Mycoplasma Testing Laboratory and included both the routine PPL3 agar and broth assays and the specific test for the detection of M. hyorhinis. Samples (1 x 2 ml and 1 x 25 ml) of the 3 crude virus fluids and of the 1 control fluid were submitted for testing. All samples were reported to be negative for mycoplasmas. A copy of this report is appended to this Protocol - (Appendix A - 1, 2, 3 & 4).

2. Identity in Tissue Culture (Serum-Neutralization)

No attempt was made to identify the crude virus pools in tissue cultures.

3. Purity (Safety) in Tissue Cultures - (VVPL FORM #016)

a. Tissue Cultures: All flask and roller tube cell cultures were prepared by contract personnel. Cultures were maintained on Medium MEM containing 5 to 10% fetal bovine serum (heat-inactivated) plus antibiotics: gentamicin @ 100 mcg/ml; neomycin @ 50 mcg/ml; and amphotericin B (I.V.) @ 2.5 mcg/ml. Cultures were inoculated, refed and subpassaged as indicated below. The following tissue culture systems were utilized:

(1) Tertiary African Green Monkey Kidney (AGMK) ....... MEM + 5% serum
(2) Primary Human Amnion (PHA) ......................... MEM + 10% serum
(3) Fetal Rhesus Lung (FRL-2) ............................... MEM + 5% serum
(4) Primary Rabbit Kidney (PRK) ............................. MEM + 5% serum
(5) Whole Human Embryo Fibroblast (Flow 5000) ......... MEM + 5% serum

b. General Testing Procedures

(1) Crude Virus Fluids

(a) Primary Flask Cultures: Equal 5 ml volumes of the bulk crude virus fluids and of a 1:5 dilution of the rabbit immune serum (Dn-l, Jamaica H'A') were well mixed and incubated at 37°C (water bath) for 90 minutes. Due to the small volume of antiserum available, only 5 ml of each of the virus fluids were tested per tissue culture system wherein 1 x 75 cm² flask per tissue culture system was inoculated with 10 ml of these serum-virus mixtures. These flasks were pre-treated 24 hours earlier with 0.5 ml of undiluted immune serum and overlayed with 25 ml of maintenance medium. Cultures were incubated at 35°C (37°C for PHA) for 14 days with periodic microscopic examination for any signs of CPE and/or cellular degradation. When necessary to maintain the integrity of the cell films, cultures were refed with 35 ml of fresh medium.

(b) Secondary Tube Subcultures: On the 14th day of incubation, the primary cultures were re-examined microscopically and the fluids harvested individually and treated with the specific immune serum - 0.1 ml per harvest. In addition, to each individual harvest was added: 0.1 ml gentamicin (50 mg/ml); 1 ml penicillin-streptomycin solution (5000 units/ml and 5000 mcg/ml, respectively); and 10% of 10X SPG* (v/v).

* 10X SPG: sucrose, 2.13 M; KH₂PO₄, 0.038 M; K₂HPO₄, 0.072 M; monosodium glutamate, 0.049 M.
Following mixing, the fluids were incubated at room temperature for 60 min. and then subpassed into homologous roller tube cultures - 0.5 ml of each harvest into each of 20 tubes. The remainder of the harvest fluids was saved and stored at -75°C, or below. All primary cultures were tested for hemadsorption by the addition of 0.1% guinea pig RBC (in PBS) and incubation at 4°C for a minimum of 30 minutes. All cultures were negative for hemadsorption.

Tube cultures (refed with 2 ml of maintenance medium prior to inoculation) were incubated at 35°C (37°C for PHA) for 13-14 additional days. When necessary to maintain the integrity of the cell films, cultures were refed with 2 ml of fresh medium. Cultures were examined microscopically at periodic intervals and at the end of the incubation period for any signs of CPE. After final examination, tubes were divided - depending on the specific cell system - for additional testing:

AGMK, PHA, FRhL-2 and Flow 5000 Tube Cultures: These were divided into 3 groups as follows:

1/4th tested for the presence of hemadsorbing agents,
1/4th fixed and stained with a solution of 5% glutaraldehyde + 1:10 giemsa stain and examined microscopically for any CPE,
1/2 challenged with Coxsackie A-9 virus (0.2 ml per tube at dilutions noted in the tables) for the detection of non-CPE producing agents and/or latent agents via the interference phenomenon.

PRK Tube Cultures: These were equally divided into 2 groups:

1/2 tested for the presence of hemadsorbing agents,
1/2 fixed and stained with the glutaraldehyde-giemsa stain solution and examined microscopically for any CPE.

No challenge studies were carried out with the Coxsackie A-9 virus since this virus does not produce any discernible CPE in this tissue culture system.

(2) Crude Control Fluid

A single 75 cm² flask per tissue culture system was inoculated with 10 ml of crude control fluid. Cultures were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included.

(3) Uninoculated Cell Lot Controls

Two x 75 cm² flasks per tissue culture system were included as uninoculated cell lot controls and were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included. In addition, an appropriate number of uninoculated roller tube cultures were included as cell lot controls for the secondary tube subcultures.

In all challenge studies, 1 to 4 culture tubes per set were left unchallenged to serve as controls to the challenge virus.

The results of these in vitro Tissue Culture Purity (Safety) tests are summarized in Tables II-A through -E.
4. Animal Safety Tests

Due to the dismantling of Flow's Animal Facility during December 1989, all animal studies were performed by Microbiological Associates, Inc. The inocula for these animal studies were the three crude virus suspensions:

a. **Adult Rabbits** - MBA Studies #ZA356.005101, #ZA357.005101 and #ZA358.005101 - these tests were reported to be satisfactory and copies of these Final Reports may be found in Appendix - B.

b. **Adult and Suckling Mice** - MBA Studies #ZA356.005100, #ZA357.005100 and #ZA358.005100 - all three tests in both adult mice and in suckling mice were reported to be satisfactory and copies of these Final Reports may be found in Appendix - C.

c. **Adult Guinea Pigs** - MBA Studies #ZA356.005102, #ZA357.005102 and #ZA358.005102 - these tests were reported to be satisfactory and copies of these Final Reports may be found in Appendix - D.
### Table I. Microbial Sterility Test Results on the Crude Dengue-1 Virus (#45A25) Production Seed Pools

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<th>Temperature</th>
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<th>Off Test</th>
<th>Results</th>
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<td>Fluid Thioglycollate</td>
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<tr>
<td>(PDM) LOT VVPL-#030</td>
<td>10</td>
<td>32°C (+2°C)</td>
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<td>PDK-10 Virus Fluid</td>
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<td>(TSB) LOT VVPL #030</td>
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<td>22°C (+2°C)</td>
<td>11/12/90</td>
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<td>1.0</td>
<td></td>
<td></td>
<td>No Growth</td>
</tr>
<tr>
<td>PDK-27 Virus Fluid</td>
<td>10</td>
<td>1.0</td>
<td></td>
<td></td>
<td>No Growth</td>
</tr>
<tr>
<td>Control Fluid</td>
<td>10</td>
<td>1.0</td>
<td>11/12/90</td>
<td>12/03/90</td>
<td>No Growth</td>
</tr>
</tbody>
</table>
Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-1 Virus (#45A25) Production Seed Pools

A. Tertiary African Green Monkey Kidney (AGMK)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>CPE **</th>
<th>Hads</th>
<th>Stain **</th>
<th>CPE ***</th>
<th>Hads</th>
<th>Stain ***</th>
<th>10^-3</th>
<th>10^-4</th>
<th>10^-5</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>1/1</td>
<td>0/1</td>
<td>1/1</td>
<td>20/20</td>
<td>0/5</td>
<td>5/5</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-20 Virus/ Serum Mixture</td>
<td>1/1</td>
<td>0/1</td>
<td>1/1</td>
<td>20/20</td>
<td>0/5</td>
<td>5/5</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-27 Virus/ Serum Mixture</td>
<td>1/1</td>
<td>0/1</td>
<td>1/1</td>
<td>20/20</td>
<td>0/5</td>
<td>5/5</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control Fluid (TCP)</td>
<td>0/1</td>
<td>0/1</td>
<td>0/0</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Coxsackie A-9 Challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td>40/10</td>
</tr>
<tr>
<td>60/12</td>
</tr>
</tbody>
</table>

* Coxsackie A-9 Challenge Results based on a 6-day incubation at 35°C. Prior to challenge, all tubes refed with 2 ml of fresh medium. Complete inhibition of Coxsackie A-9 challenge virus by virus/serum mixture series.

** Non-descriptive cytopathology initially observed on day 10 and confirmed on staining on day 14 for all 3 virus/serum inoculated flasks only.

*** On day 20 (days 14 + 6), all tubes inoculated with harvests from vi-5a/serum inoculated flasks exhibited cytopathology which progressed to 3-4+ by day 28 (days 14 + 14). This cytopathology, confirmed on staining, was attributed to dengue virus breakthroughs. Islands of cells remained which proved to be resistant to the Coxsackie A-9 challenge virus.
### Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-1 Virus (454.5) Production Seed Tissue

#### B. Primary Human Amnion (PHA)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>10⁻³</th>
<th>10⁻⁴</th>
<th>10⁻⁵</th>
<th>10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-20 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-?7 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control Fluid (TCF)</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2</td>
<td>ND</td>
<td>0/40</td>
<td>0/10</td>
<td>0/10</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>2/4</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/60</td>
<td>0/12</td>
<td>ND</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
<td>8/8</td>
<td>8/8</td>
<td>7/8</td>
<td>1/8</td>
</tr>
</tbody>
</table>

* Coxsackie A-9 Challenge Results based on a 4-day incubation at 37°C. Prior to challenge, all tubes refed with 2 ml of fresh medium.

** On day 7, all flasks were refed with 35 ml of fresh medium.

ND = Not done
Table II. Tissue Culture Purity (Safety) Test Results on the Crude Leuvs-1 Virus (45A25) Production Seed Pools

C. Fetal Rhesus Lung (FRHL-2)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>Initial Flasks</th>
<th></th>
<th>0.5 ml per tube</th>
<th></th>
<th>0.5 ml per tube</th>
<th></th>
<th>Coxsackie A-9 Challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lot # 1610 p21</td>
<td>Lot # 1687 p24</td>
<td>Lot # 1610 p21</td>
<td>Lot # 1687 p24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 14</td>
<td>Day 14 + 14 = 28</td>
<td>Day 14</td>
<td>Day 14 + 14 = 28</td>
<td>Coxsackie A-9 Challenge*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PK-10 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>PK-20 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/19</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>PK-27 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>Control Fluid (TCP)</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2</td>
<td>NE</td>
<td>0/40</td>
<td>0/10</td>
<td>0/10</td>
<td>4/4</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/2</td>
<td>0/2</td>
<td>NE</td>
<td>0/40</td>
<td>0/10</td>
<td>0/10</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* Coxsackie A-9 Challenge Results based on a 3-day incubation at 35°C. Prior to challenge, all tubes refed with 2 ml of fresh medium.

ND = Not done
**Table II.** Tissue Culture Purity (Safety) Test Results on the Crude Dengue-1 Virus (#45A25) Production Seed Pools

**D. Primary Rabbit Kidney (PRK)**

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>PDK-20 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>PDK-27 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Control Fluid (TCF)</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2</td>
<td>ND</td>
<td>0/40</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/24</td>
<td>0/12</td>
<td>ND</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>

0.5 ml per tube

<table>
<thead>
<tr>
<th>Initial Flasks</th>
<th>Passage #1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot # 1650</td>
<td>Lot # 1693</td>
</tr>
<tr>
<td>Day: 14</td>
<td>Day: 14 + 14 = 28</td>
</tr>
</tbody>
</table>

**ND = Not done**
Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-1 Virus (#45AZ5) Production Seed Pools  

E. Human Embryo Fibroblasts (Flow 5000)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>Control - (1)</th>
<th>Control - (2)</th>
<th>Coxsackie A-9 Challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/2</td>
<td>0/60</td>
<td>8/8  8/8  5/8  2/8</td>
</tr>
<tr>
<td>PDK-20 Virus/ Serum Mixture</td>
<td>0/2</td>
<td>0/10</td>
<td>4/4  4/4  4/4  1/4</td>
</tr>
<tr>
<td>PDK-27 Virus/ Serum Mixture</td>
<td>0/2</td>
<td>0/12</td>
<td>2/2  2/2  2/2  2/2</td>
</tr>
<tr>
<td>Control Fluid (TCP)</td>
<td>0/2</td>
<td>0/2</td>
<td>2/2  2/2  1/2  1/2</td>
</tr>
</tbody>
</table>

* Coxsackie A-9 Challenge Results based on a 5-day incubation at 35°C. Prior to challenge, all tubes were refed with 2 ml of fresh medium.

ND = Not done
8 August, 1990.

To: Dr. Louis Potash.

From: Jim Quartey.

Subject: Mycoplasma Testing. (Charge # 807)

This letter is to inform you that, the eight (8) samples listed below which you had submitted for the detection of Mycoplasma hyorhinis using the direct immunofluorescence staining and for the detection of Mycoplasma in general using the DNA Hoechst stain and Agar testing were found to be negative.

a. Dengue-1 (#45AZ5) Production Seed of 16 Feb 90:

1. PDK-10, FRhL-2/d7.
2. PDK-20, FRhL-2/d7.
4. Control Fluid.

b. Dengue-4 (#341750) Production Seed of 9 Mar 90:

1. PDK-6, FRhL-2/d7
2. PDK-10, FRhL-2/d7
3. PDK-15, FRhL-2/d7
4. Control Fluid.
<table>
<thead>
<tr>
<th>Identification</th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Final Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dengue-1 (545A25)</td>
<td>0 0 0 0</td>
<td>PDK-10, FRAL-2/17 197 0 0 0 0</td>
<td>1 0 0 0 0</td>
</tr>
<tr>
<td>PDK-20, FRAL-2/17 198 0 0 0 0</td>
<td>PDK-27, FRAL-2/17 199 0 0 0 0</td>
<td>Control Fluid 200 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>Dengue-4 (4.24750)</td>
<td>0 0 0 0</td>
<td>PDK-6, FRAL-2/17 201 0 0 0 0</td>
<td>1 0 0 0 0</td>
</tr>
<tr>
<td>PDK-10, FRAL-2/17 202 0 0 0 0</td>
<td>PDK-15, FRAL-2/17 203 0 0 0 0</td>
<td>Control Fluid 204 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>
**MYCOPLASMA TEST RECORD SHEET**

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>LOT #</th>
<th>Aerobic</th>
<th>Anaerobic</th>
<th>On Test</th>
<th>Off Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPLO Agar</td>
<td>900523</td>
<td>2</td>
<td>2</td>
<td>7/9/90</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>PPLO Broth</td>
<td>900503</td>
<td>25.0</td>
<td>25.0</td>
<td>7/16/90</td>
<td>7/31/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D5 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/19/90</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
<td></td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/19/90</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
<td></td>
</tr>
<tr>
<td>D10 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
<td></td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/24/90</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
<td></td>
</tr>
<tr>
<td>D15 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
<td></td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/24/90</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
<td></td>
</tr>
</tbody>
</table>

Positive Control (+): *M. arginini*  Negative Control (-): *FB 29101 C070*

Date: 7/18/90  Signed: [Signature]
**Mycoplasma Test Record Sheet**

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>LOT #</th>
<th>No. ml Tested</th>
<th>Date</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENGUE-1 Virus Fluid</td>
<td>PK27 FRL-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPLO Agar</td>
<td>900523</td>
<td>2</td>
<td>7/9/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>PPLO Broth</td>
<td>900503</td>
<td>25.0</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D5 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/14/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/14/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D10 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/19/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D15 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>8/8/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>DENGUE-1 Control Fluid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPLO Agar</td>
<td>2</td>
<td>2</td>
<td>7/9/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>PPLO Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D5 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/14/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/14/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D10 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/19/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D15 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>8/8/90</td>
<td>NEGATIVE</td>
</tr>
</tbody>
</table>

Positive Control (+): *M. arginense*  
Negative Control (-): *FB 29101 C070*

Date: 7/9/90  
Signed: [Signature]
APPENDIX

B

ANIMAL SAFETY TEST IN ADULT RABBITS

Study NO.: ZA356.005101
Dengue-1 Prod Seed: PDK-10, FRhL-2/d7 ........ pages 21 - 31

Study NO.: ZA357.005101
Dengue-1 Prod Seed: PDK-20, FRhL-2/d7 ........ pages 32 - 42

Study NO.: ZA358.005101
Dengue-1 Prod Seed: PDK-27, FRhL-2/d7 ........ pages 43 - 53
ANIMAL SAFETY TEST IN ADULT RABBITS

Study No.: ZA356.005101

Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7

Final Report
For

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9000 Blackwell Road
Rockville, Maryland 20850
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
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<td>28</td>
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<td>VII. Quality Assurance Statement</td>
<td>31</td>
</tr>
</tbody>
</table>
SUMMARY

The purpose of this assay is to detect the presence of adventitious agent(s) in the test article pre-clarified bulk live virus vaccine and/or fluids, other than the specific virus in the product. The test article was inoculated into adult rabbits.

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It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilized inoculation of adult rabbits. The test is performed as described in CFR Title 21, Section 630.16.

Adult rabbits are utilized in this assay to detect possible contamination of the test article with B-virus or other adventitious agent(s) including other Simian agents, adenovirus(es), etc. which might be present in the test article. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Rabbits

B. Study Number: ZA356.005101

C. Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7 was received at Microbiological Associates, Inc. 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Rabbits, four females, SPF NZW, 1.5 - 2.5 Kg.
   Source: Buckshire Corp.
   P.O. Box 155
   Perkasie, PA 18944

G. Sponsor: Program Resources, Inc.
   Biomedical Services Division
   7655 Old Springhouse Road
   McLean, VA 22102

   Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:

1. Study Director: Mary D. Whiteman

2. Associate
   Study Director: Janet Luczak, M.T. (ASCP), M.G.A.

J. Schedule:

1. Study Initiation Date: 07/16/90

2. Lab Initiation Date: 08/03/90

3. Lab Completion Date: 08/31/90

4. Study Completion Date: See Study Director’s
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All raw data, records, protocol and all report copies
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Associates, Inc., 9900 Blackwell Road, Rockville,
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The study objective is to detect inapparent
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Each rabbit was housed individually. Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Each rabbit was identified by a unique number tattooed on its ear. The rabbit's number was recorded on the cage card.

The rabbits were randomized according to SOP #OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1.

Note: In error, animals were inoculated by intraocular route. This deviation from the protocol did not affect the outcome of this assay.

The rabbits were observed for 28 days for clinical signs of illness or distress.

3. Animal Husbandry

a. Rabbits were fed certified rabbit chow ad libitum and water was supplied via water bottles, ad libitum.

b. Rabbit's cages were changed weekly.

c. Animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All rabbits inoculated with the test article and the uninoculated control rabbit remained normal and healthy for the 28 day observation period.

See Tables 2 and 3 for a summary of the data.
V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article, Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration’s Good Laboratory Practice Regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director

9/27/90

Date
TABLE 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 S.Q.</td>
<td>1.0 ml</td>
<td>Test Observe</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 I.O.</td>
<td>0.03 ml</td>
<td>Article Illness</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1 SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>3</td>
<td>1 SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>4</td>
<td>1 None</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above  
I.D. = Intradermal Inoculation  
S.Q. = Subcutaneous Inoculation  
I.O. = Intraocular Inoculation
### TABLE 2
Survival Summary for Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7

<table>
<thead>
<tr>
<th></th>
<th>Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Article</td>
<td>3/3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>1/1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of surviving, healthy animals after 28 days/Number of animals inoculated.

<sup>b</sup> Number of surviving, healthy animals after 28 days/Number of animals on lab initiation date.
**TABLE 3**
Summary of Daily Observations for Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Test</td>
<td>1</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Article</td>
<td>2</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>Control</td>
<td>4</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT RABBITS

Study Number: ZA356.005101

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/03/90 - 08/03/90, TO STUDY DIR 08/03/90, TO MGMT 08/06/90
PHASES: ADMINISTRATION OF TEST ARTICLE TO TEST SYSTEM

INSPECT ON 09/21/90 - 09/24/90, TO STUDY DIR 09/24/90, TO MGMT 09/28/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Ed Warburton Date
Quality Assurance Unit

- 31 - Microbiological Associates Inc.
ANIMAL SAFETY TEST IN ADULT RABBITS

Study No.: ZA357.005101

Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

Final Report
For

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By

Microbiological Associates, Inc.
Life Sciences Center
9000 Blackwell Road
Rockville, Maryland 20850
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<table>
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<tr>
<th>Section</th>
<th>Page</th>
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<td>II. Study Information</td>
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<td>IV. Results</td>
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</tr>
<tr>
<td>V. Conclusions</td>
<td>37</td>
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<tr>
<td>VI. Approvals</td>
<td>38</td>
</tr>
<tr>
<td>VII. Quality Assurance Statement</td>
<td>42</td>
</tr>
</tbody>
</table>
SUMMARY

The purpose of this assay is to detect the presence of adventitious agent(s) in the test article pre-clarified bulk live virus vaccine and/or fluids, other than the specific virus in the product. The test article was inoculated into adult rabbits.

No evidence of contamination with adventitious agent(s) was observed due to the test article Dengue-1 (#45A25) Prod Seed, PDK-20, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilized inoculation of adult rabbits. The test is performed as described in CFR Title 21, Section 630.16.

Adult rabbits are utilized in this assay to detect possible contamination of the test article with B-virus or other adventitious agent(s) including other Simian agents, adenovirus(es), etc. which might be present in the test article. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Rabbits

B. Study Number: ZA357.005101

C. Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7 was received at Microbiological Associates, Inc. 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Rabbits, four females, SPF NZW, 1.5 - 2.5 kg.
   Source: Buckshire Corp.
   P.O. Box 155
   Perkasie, PA 18944

G. Sponsor: Program Resources, Inc.
   Biomedical Services Division
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   Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department
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Life Sciences Center
9900 Blackwell Road
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Animal Facility: Microbiological Associates, Inc.
5221 River Road
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I. Personnel:
1. Study Director: Mary D. Whiteman
2. Associate Study Director: Janet Luczak, M.T. (ASCP), M.G.A.

J. Schedule:
1. Study Initiation Date: 07/16/90
2. Lab Initiation Date: 08/03/90
3. Lab Completion Date: 08/31/90
4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:
All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:
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III. PROCEDURES
A. Objective:
The study objective is to detect inapparent adventitious agent(s) which might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.
B. Methods:

1. Test System Identification and Randomization

   Each rabbit was housed individually. Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Each rabbit was identified by a unique number tattooed on its ear. The rabbit's number was recorded on the cage card.

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2. Animal Inoculation with Test Article

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3. Animal Husbandry

   a. Rabbits were fed certified rabbit chow ad libitum and water was supplied via water bottles, ad libitum.

   b. Rabbit's cages were changed weekly.

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IV. RESULTS

All rabbits inoculated with the test article and the uninoculated control rabbit remained normal and healthy for the 28 day observation period.

See Tables 2 and 3 for a summary of the data.

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No evidence of contamination with adventitious agent(s) due to the test article, Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7, was observed.
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Mary D. Whiteman  
Study Director  
9/27/90  
Date
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<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I.D.</td>
<td>1.0 ml</td>
<td>Test Article Observe for Illness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.Q.</td>
<td>9.0 ml</td>
<td>SAA</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above  
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S.Q. = Subcutaneous Inoculation
TABLE 2
Survival Summary
for Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

RABBITS

<table>
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<th>Test Article</th>
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for Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

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<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Test</td>
<td></td>
<td>5</td>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td>Article</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>7</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>4</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT RABBITS

Study Number: ZA357.005101

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Ed Warburton
Quality Assurance Unit

9-29-90

Microbiological Associates Inc.
ANIMAL SAFETY TEST IN ADULT RABBITS

Study No.: ZA358.005101

Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

Final Report
For
Program Resources, Inc.
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By
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Mary D. Whiteman
Study Director

9/27/90
Date
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<th>Volume of Inoculum</th>
<th>Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I.D.</td>
<td>1.0 ml</td>
<td>S.Q.</td>
<td>Test Article for Illness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.Q.</td>
<td>9.0 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>S AA</td>
<td>S AA</td>
<td>S AA</td>
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<td>None</td>
<td>S AA</td>
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</tbody>
</table>

SAA = Same As Above  
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S.Q. = Subcutaneous Inoculation
TABLE 2
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for Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

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<tr>
<th>RABBITS</th>
<th></th>
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<tr>
<td>Test Article</td>
<td>3/3(^a)</td>
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<tr>
<td>Uninoculated Control</td>
<td>1/1(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Number of surviving, healthy animals after 28 days/Number of animals inoculated.

\(^b\) Number of surviving, healthy animals after 28 days/Number of animals on lab initiation date.
TABLE 3
Summary of Daily Observations for Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
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</thead>
<tbody>
<tr>
<td>Rabbit</td>
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<tr>
<td>Uninoculated</td>
<td>Control</td>
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<td>Normal</td>
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</table>
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT RABBITS

Study Number: ZA358.005101

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/23/90 - 07/23/90, TO STUDY DIR 07/23/90, TO MGMT 07/23/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/16/90 - 08/16/90, TO STUDY DIR 08/16/90, TO MGMT 08/21/90
PHASES: ANIMAL OBSERVATIONS

INSPECT ON 09/21/90 - 09/24/90, TO STUDY DIR 09/24/90, TO MGMT 09/28/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Ed Warburton
Quality Assurance Unit

Date

- 53 - Microbiological Associates Inc.
APPENDIX C

ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study NO.: ZA356.005100
Dengue-1 Prod Seed: PDK-10, FRhL-2/d7 .......... pages 55 - 67

Study NO.: ZA357.005100
Dengue-1 Prod Seed: PDK-20, FRhL-2/d7 .......... pages 68 - 80

Study NO.: ZA358.005100
Dengue-1 Prod Seed: PDK-27, FRhL-2/d7 .......... pages 81 - 93
ANIMAL SAFETY TEST IN
ADULT MICE AND SUCKLING MICE

Study No.: ZA356.005100
Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7

Final Report
For
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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<th>Section</th>
<th>Page</th>
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<td>VII. Quality Assurance Statement</td>
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</table>
The purpose of this assay is to attempt to detect the presence of adventitious agent(s) in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product. The test article was inoculated into adult mice and suckling mice. The suckling mouse portion of the assay included a subpassage of homogenized tissue, after 14 days, into a new group of suckling mice, followed by an additional 14 day observation period.

No evidence of viral contamination due to the test article Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7 was observed.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent virus(es). The experimental design utilizes inoculations of adult and suckling mice. The test is performed as described in CFR Title 21, Section 630.35 (a)(1)(2).

Adult mice are included in the assay to detect possible contamination of the test article with neurotropic or other viruses such as lymphocytic choriomeningitis virus. Suckling mice are used to detect Coxsackie or other viruses. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Mice and Suckling Mice

B. Study Number: ZA356.005100

C. Test Article: Dengue-1 (§45AZ5) Prod Seed, PDK=10, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. The test article was received frozen. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Mice

Suckling litters (Primary Inoculation): Tac:(SW)FBR, three adult females each with ten <24 hour old suckling pups, Source: Taconic Farms
Germantown, New York
Suckling litters (Blind Passage): Tac:(SW)FBR, four adult females each with ten <24 hour old suckling pups,
Source: Taconic Farm,
Germantown, New York

Adult - HSD:ICR, Fifteen males and fifteen females,
Body Weight range: 15-20 grams.
Source: Harlan Sprague Dawley, Frederick, Maryland

G. Sponsor: Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

Authorized Representative: Dr. Louis Potash

H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: 5221 River Road
Bethesda, Maryland 20816

I. Personnel:

1. Study Director: Mary D. Whiteman
2. Associate
   Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:

1. Study Initiation Date: 07/16/90
2. Lab Initiation Date: 07/24/90
3. Lab Completion Date: 08/23/90
4. Study Completion Date: See Study Director’s
   Signature Date, in the
   "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.
L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

With approval of the sponsor, a previously thawed sample which was frozen back and stored at -70°C was utilized in the inoculation of the suckling mouse portion of the assay on 07/24/90. In addition, at the request of the sponsor, in the suckling mouse portion of the assay, 1.3 ml of the test article was combined with 1.3 ml of sponsor supplied antisera Den-l Jamaica HMAF, 4-17-78 and heated at 37°C for 90 minutes, prior to inoculation of the suckling mice. The remaining untreated sample was again frozen back and was utilized along with a previously unthawed aliquot of the test article to inoculate the adult mouse portion of the assay on 07/26/90.

A. Objective:

The study objective is to detect inapparent virus(es) that might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.

B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Adult mice were ear-tagged but housed in groups according to inoculum type and sex. Suckling mice were not individually identified.

Suckling and adult mice were randomized according to SOP #OPET0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. The adult mice were observed every working day for 28 days for clinical signs. In the suckling mouse portion of the assay, the animals were inoculated
according to Table 1 and were then observed every working day for 14 days for clinical signs. Fourteen days post-inoculation, all surviving suckling mice from each group were euthanized using cervical dislocation. Following euthanasia their skin and gastrointestinal were removed, the carcasses cut into pieces and placed in a sterile pre-weighed bowl. After determining the weight of the entire group of mice from a cage, enough Hank’s Balanced Salt Solution (HBSS) with gentamicin was added to make a 20% w/v suspension. The entire content of the bowl was then homogenized in a sterile blender, clarified by centrifugation, diluted 1:2 in HBSS and subsequently inoculated into a new group of suckling mice by the same routes and in the same volumes as the original group. These newly inoculated mice were observed for a period of fourteen days. Ten suckling mice were held as uninoculated controls and observed for fourteen days.

3. Animal Husbandry

a. All animals were fed the following diet ad libitum:

   Mice - autoclavable chow.

b. Water was supplied ad libitum via fresh apples (disinfected).

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All adult mice inoculated with the test article and all uninoculated control adult mice remained normal and healthy for the twenty-eight day observation period.

All uninoculated control suckling mice and all test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. The surviving mice of each group were homogenized and the homogenate of each group was passaged into a new group of suckling mice. The remainder of the homogenates was frozen at -70°C.
In the blind passage, all of the uninoculated control suckling mice, all of the suckling mice inoculated with the homogenate of the uninoculated control suckling mice and all of the suckling mice inoculated with the homogenate of the test article inoculated suckling mice appeared normal and healthy for the 14 day observation period.

See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

No evidence of viral contamination due to the test article, Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration’s Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman  8/31/90
Mary D. Whiteman  Date
Study Director
<table>
<thead>
<tr>
<th>Group #</th>
<th>Number of Animals</th>
<th>Sex</th>
<th>Species</th>
<th>Route(s) of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH 1</td>
<td>1</td>
<td>female mouse (lactating)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10 various Mouse (suckling)</td>
<td>i.p.</td>
<td>0.1 ml test article</td>
<td></td>
<td></td>
<td>Observed for illness after 14 days passage a single pool of emulsified tissue (minus skin and gastrointestinal) of all surviving mice onto at least 10 additional suckling mice. Use same routes and volumes as original.</td>
</tr>
<tr>
<td>SH 2</td>
<td>SAA*</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>SH 3</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
<td>SAA</td>
</tr>
<tr>
<td>AM 1</td>
<td>5</td>
<td>male mouse</td>
<td>i.p.</td>
<td>0.5 ml test article</td>
<td>i.c.</td>
<td>0.05 ml test article</td>
<td>Observe for illness</td>
</tr>
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<td>AM 2</td>
<td>5</td>
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<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
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<td>AM 5</td>
<td>5</td>
<td>male</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
<td>SAA</td>
</tr>
<tr>
<td>AM 6</td>
<td>5</td>
<td>female</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

*SAA = Same as above
i.c. = Intracranial
i.p. = Intraperitoneal
### TABLE 2
Survival Summary for Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7

<table>
<thead>
<tr>
<th></th>
<th>Adult Mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primary Inoculation</th>
<th>Blind Passage</th>
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</thead>
<tbody>
<tr>
<td>Test Article</td>
<td>20/20</td>
<td>20/20</td>
<td>20/20</td>
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<tr>
<td>Uninoculated Control</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Uninoculated Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>10/10</td>
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</tbody>
</table>

<sup>a</sup> Number of surviving, healthy animals after 28 days/Number of animals inoculated.

<sup>b</sup> In the suckling mice portion of the assay, animals are inoculated and observed for 14 days. On day 14 post-inoculation a homogenate is prepared from the surviving sucklings from each group. This homogenate was used to inoculate another group of suckling mice which were observed for an additional 14 days. The number of surviving sucklings/number of animals inoculated is presented.

<sup>c</sup> In the blind passage of the suckling mouse portion of the assay an uninoculated control group was held with the blind passage animals inoculated with the homogenate of the test article and the homogenate of the uninoculated control group from the primary inoculation.
TABLE 3

Summary of Daily Observations for Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Sacrifice (Post-inoc.)</th>
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<td>Animal Species</td>
<td>Inoculum</td>
<td>Number/ Cage^a</td>
<td>Clinical Signs</td>
<td>Day of Onset (Post-Inoc.)</td>
<td>Day of Sacrifice (Post-Inoc.)</td>
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<td>Suckling Mice (Primary Inoculation)</td>
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<td>SM1 (10)</td>
<td>Normal</td>
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<tr>
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<td>Article</td>
<td>SM2 (10)</td>
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<td>Uninoculated Control</td>
<td>SM3 (10)</td>
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<tr>
<td>(Blind Passage)</td>
<td>Test</td>
<td>Homo-</td>
<td>SM1 (10)</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Article</td>
<td>genate</td>
<td>SM2 (10)</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Uninoculated Control</td>
<td></td>
<td>SM3 (10)</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

^a Ten suckling mice inoculated per cage.

^b Surviving suckling mice from primary inoculation were sacrificed on day 14 for preparation of blind passage tissue homogenate.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study Number: ZA356.005100

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/23/90
PHASES: OBSERVATION OF ANIMALS FOR CLINICAL SIGNS

INSPECT ON 08/30/90 - 08/30/90, TO STUDY DIR 08/30/90, TO MGMT 08/31/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan
Quality Assurance Unit

Date: 8/31/90
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VII. Quality Assurance Statement ................. 80
SUMMARY

The purpose of this assay is to attempt to detect the presence of adventitious agent(s) in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product. The test article was inoculated into adult mice and suckling mice. The suckling mouse portion of the assay included a subpassage of homogenized tissue, after 14 days, into a new group of suckling mice, followed by an additional 14 day observation period.

No evidence of viral contamination due to the test article Dengue-1 (#45A25) Prod Seed, PDK-20, FRhL-2/d7 was observed.
I. INTRODUCTION

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Adult mice are included in the assay to detect possible contamination of the test article with neurotropic or other viruses such as lymphocytic choriomeningitis virus. Suckling mice are used to detect Coxsackie or other viruses. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Mice and Suckling Mice

B. Study Number: ZA357.005100

C. Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. The test article was received frozen. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Mice

Suckling litters (Primary Inoculation): Tac:(SW)FBR, three adult females each with ten <24 hour old suckling pups,
Source: Taconic Farms
Germantown, New York
Suckling litters (Blind Passage): Tac: (SW) FBR, four adult females each with ten <24 hour old suckling pups,  
Source: Taconic Farms  
Germantown, New York  

Adult - HSD: ICR, Fifteen males and fifteen females,  
Body Weight range: 15-20 grams.  
Source: Harlan Sprague Dawley, Frederick, Maryland  

G. Sponsor: Program Resources, Inc.  
Biomedical Services Division  
7655 Old Springhouse Road  
McLean, VA 22102  

Authorized Representative: Dr. Louis Potash  

H. Testing Facility: Biotechnology Services Department  
Microbiological Associates, Inc.  
Life Sciences Center  
9900 Blackwell Road  
Rockville, Maryland 20850  

Animal Facility: 5221 River Road  
Bethesda, Maryland 20816  

I. Personnel:  
1. Study Director: Mary D. Whiteman  
2. Associate  
   Study Director: Janet Luczak, M.T. (ASCP), M.G.A.  

J. Schedule:  
1. Study Initiation Date: 07/16/90  
2. Lab Initiation Date: 07/24/90  
3. Lab Completion Date: 08/23/90  
4. Study Completion Date: See Study Director’s Signature Date, in the "Approvals" Section.  

K. Raw Data, Records and Test Article Samples:  
All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.
III. PROCEDURES

With approval of the sponsor, a previously thawed sample which was frozen back and stored at -70°C was utilized in the inoculation of the suckling mouse portion of the assay on 07/24/90. In addition, at the request of the sponsor, in the suckling mouse portion of the assay, 1.3 ml of the test article was combined with 1.3 ml of sponsor supplied antisera Den-1 Jamaica HMAF, 4-17-78 and heated at 37°C for 90 minutes, prior to inoculation of the suckling mice. The remaining untreated sample was again frozen back and was utilized along with a previously unthawed aliquot of the test article to inoculate the adult mouse portion of the assay on 07/26/90.

A. Objective:

The study objective is to detect inapparent virus(es) that might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.

B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Adult mice were ear-tagged but housed in groups according to inoculum type and sex. Suckling mice were not individually identified.

Suckling and adult mice were randomized according to SOP #OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. The adult mice were observed every working day for 28 days for clinical signs. In the suckling mouse portion of the assay, the animals were inoculated according to Table 1 and were then observed every
working day for 14 days for clinical signs. Fourteen days post-inoculation, all surviving suckling mice from each group were euthanized using cervical dislocation. Following euthanasia their skin and gastrointestinal were removed, the carcasses cut into pieces and placed in a sterile pre-weighed bowl. After determining the weight of the entire group of mice from a cage, enough Hank's Balanced Salt Solution (HBSS) with gentamicin was added to make a 20% w/v suspension. The entire content of the bowl was then homogenized in a sterile blender, clarified by centrifugation, diluted 1:2 in HBSS and subsequently inoculated into a new group of suckling mice by the same routes and in the same volumes as the original group. These newly inoculated mice were observed for a period of fourteen days. Ten suckling mice were held as uninoculated controls and observed for fourteen days.

3. Animal Husbandry

a. All animals were fed the following diet ad libitum:

   Mice - autoclavable chow.

b. Water was supplied ad libitum via fresh apples (disinfected).

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All adult mice inoculated with the test article and all uninoculated control adult mice remained normal and healthy for the twenty-eight day observation period.

All uninoculated control suckling mice and test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. The surviving mice of each group were homogenized and the homogenate of each group was passaged into a new group of suckling mice. The remainder of the homogenates was frozen at -70°C.
In the blind passage all of the uninoculated control suckling mice, all of the suckling mice inoculated with the homogenate of the uninoculated control suckling mice, and nineteen of the twenty suckling mice inoculated with the homogenate of the test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. One of the test article homogenate inoculated suckling mice was missing and presumed cannibalized day 3 post-inoculation.

See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

No evidence of viral contamination due to the test article, Dengue-1 (#45AZ5) Prod Seed, FDK-20, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director

8/31/90

Date
<table>
<thead>
<tr>
<th>Group #</th>
<th>Number of Animals</th>
<th>Sex</th>
<th>Species</th>
<th>Route(s) of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM 1</td>
<td>1</td>
<td>female</td>
<td>mouse (lactating)</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>various Mouse (suckling)</td>
<td>i.p.</td>
<td>0.1 ml</td>
<td>test article</td>
<td>Observed for illness after 14 days passage a single pool of emulsified tissue (minus skin and gastrointestinal) of all surviving mice onto at least 10 additional suckling mice. Use same routes and volumes as original.</td>
</tr>
<tr>
<td>SM 2</td>
<td>SAA*</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>SM 3</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
</tr>
<tr>
<td>AM 1</td>
<td>5</td>
<td>male</td>
<td>mouse</td>
<td>i.p.</td>
<td>0.5 ml</td>
<td>test article</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>i.c.</td>
<td>0.03 ml</td>
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<td>male</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
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<td>male</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
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<tr>
<td>AM 6</td>
<td>5</td>
<td>female</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
</tr>
</tbody>
</table>

*SAA = Same as above
i.c. = Intracranial
i.p. = Intraperitoneal

MICROBIOLOGICAL ASSOCIATES, INC.
TABLE 2
Survival Summary for Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

<table>
<thead>
<tr>
<th>Suckling Mice&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Adult Mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primary Inoculation</th>
<th>Blind Passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Article</td>
<td>20/20</td>
<td>20/20</td>
<td>19/20</td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Uninoculated Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of surviving, healthy animals after 28 days/Number of animals inoculated.

<sup>b</sup> In the suckling mice portion of the assay, animals are inoculated and observed for 14 days. On day 14 post-inoculation a homogenate is prepared from the surviving sucklings from each group. This homogenate was used to inoculate another group of suckling mice which were observed for an additional 14 days. The number of surviving sucklings/number of animals inoculated is presented.

<sup>c</sup> In the blind passage of the suckling mouse portion of the assay an uninoculated control group was held with the blind passage animals inoculated with the homogenate of the test article and the homogenate of the uninoculated control group from the primary inoculation.
# TABLE 3

Summary of Daily Observations for Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset</th>
<th>Day of Death/Sacrifice</th>
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</thead>
<tbody>
<tr>
<td>Adult</td>
<td>Test</td>
<td>10531</td>
<td>Normal</td>
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<td>Mice</td>
<td>Article</td>
<td>10532</td>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>10533</td>
<td>Normal</td>
<td></td>
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<td></td>
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<td>Uninoculated</td>
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<td>10530</td>
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</table>
TABLE 3 (Cont.)
Summary of Daily Observations for Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Incubum</th>
<th>Number/ Cage (^a)</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-Inoc.)</th>
<th>Day of Death/ Sacrifice (Post-Inoc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling (^b)</td>
<td>Test</td>
<td>SM1 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mice (Primary</td>
<td>Article</td>
<td>SM2 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Uninoculated Control</td>
<td>SM3 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Blind Passage)</td>
<td>Test</td>
<td>Article SM1 (10)</td>
<td>Normal</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Homo-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>genate</td>
<td>SM2 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Uninoculated Control Homo-</td>
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<tr>
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<td>genate</td>
<td>SM3 (10)</td>
<td>Normal</td>
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<tr>
<td></td>
<td>Uninoculated Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Ten suckling mice inoculated per cage.
\(^b\) Surviving suckling mice from primary inoculation were sacrificed on day 14 for preparation of blind passage tissue homogenate.
\(^c\) One suckling mouse missing and presumed cannibalized.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study Number: ZA357.005100

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/23/90
PHASES: OBSERVATION OF ANIMALS FOR CLINICAL SIGNS

INSPECT ON 08/30/90 - 08/30/90, TO STUDY DIR 08/30/90, TO MGMT 08/31/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan Date
Quality Assurance Unit
ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study No.: ZA358.005100

Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

Final Report
For
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

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</tr>
</tbody>
</table>
SUMMARY

The purpose of this assay is to attempt to detect the presence of adventitious agent(s) in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product. The test article was inoculated into adult mice and suckling mice. The suckling mouse portion of the assay included a subpassage of homogenized tissue, after 14 days, into a new group of suckling mice, followed by an additional 14 day observation period.

No evidence of viral contamination due to the test article Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7 was observed.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent virus(es). The experimental design utilizes inoculations of adult and suckling mice. The test is performed as described in CFR Title 21, Section 630.35 (a)(1)(2).

Adult mice are included in the assay to detect possible contamination of the test article with neurotropic or other viruses such as lymphocytic choriomeningitis virus. Suckling mice are used to detect Coxsackie or other viruses. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Mice and Suckling Mice

B. Study Number: ZA358.005100

C. Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. The test article was received frozen. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Mice

Suckling litters (Primary Inoculation): Tac:(SW)FBR, three adult females each with ten <24 hour old suckling pups,
Source: Taconic Farms
   Germantown, New York
Suckling litters (Blind Passage): Tac:(SW)FBR, four adult females each with ten <24 hour old suckling pups,
Source: Taconic Farms
Germantown, New York

Adult - HSD:ICR, Fifteen males and fifteen females,
Body Weight range: 15-20 grams.
Source: Harlan Sprague Dawley, Frederick, Maryland

G. Sponsor: Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

Authorized Representative: Dr. Louis Potash

H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: 5221 River Road
Bethesda, Maryland 20816

I. Personnel:
1. Study Director: Mary D. Whiteman
2. Associate
   Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:
1. Study Initiation Date: 07/16/90
2. Lab Initiation Date: 07/24/90
3. Lab Completion Date: 08/23/90
4. Study Completion Date: See Study Director’s Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.
L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

With approval of the sponsor, a previously thawed sample which was frozen back and stored at \(-70^\circ\text{C}\) was utilized in the inoculation of the suckling mouse portion of the assay on 07/24/90. In addition, at the request of the sponsor, in the suckling mouse portion of the assay, 1.3 ml of the test article was combined with 1.3 ml of sponsor supplied antisera Den-l Jamaica HMAF, 4-17-78 and heated at 37\(^\circ\text{C}\) for 90 minutes, prior to inoculation of the suckling mice. The remaining untreated sample was again frozen back and was utilized along with a previously unthawed aliquot of the test article to inoculate the adult mouse portion of the assay on 07/26/90.

A. Objective:

The study objective is to detect inapparent virus(es) that might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.

B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Adult mice were ear-tagged but housed in groups according to inoculum type and sex. Suckling mice were not individually identified.

Suckling and adult mice were randomized according to SOP \#OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. The adult mice were observed every working day for 28 days for clinical signs. In the suckling mouse portion of the assay, the animals were inoculated according to Table 1 and were then observed every working day for 14 days for clinical signs.
Fourteen days post-inoculation, all surviving suckling mice from each group were euthanized using cervical dislocation. Following euthanasia their skin and gastrointestinal were removed, the carcasses cut into pieces and placed in a sterile pre-weighed bowl. After determining the weight of the entire group of mice from a cage, enough Hank's Balanced Salt Solution (HBSS) with gentamicin was added to make a 20% w/v suspension. The entire content of the bowl was then homogenized in a sterile blender, clarified by centrifugation, diluted 1:2 in HBSS and subsequently inoculated into a new group of suckling mice by the same routes and in the same volumes as the original group. These newly inoculated mice were observed for a period of fourteen days. Ten suckling mice were held as uninoculated controls and observed for fourteen days.

3. Animal Husbandry

   a. All animals were fed the following diet ad libitum:
      
      Mice - autoclavable chow.

   b. Water was supplied ad libitum via fresh apples (disinfected).

   c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

   d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All adult mice inoculated with the test article and all uninoculated control adult mice remained normal and healthy for the twenty-eight day observation period.

All uninoculated control suckling mice and all test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. The surviving mice of each group were homogenized and the homogenate of each group was passaged into a new group of suckling mice. The remainder of the homogenates was frozen at -70°C.
In the blind passage, all of the uninoculated control suckling mice, all of the suckling mice inoculated with the homogenate of the uninoculated control suckling mice and all of the suckling mice inoculated with the homogenate of the test article inoculated suckling mice appeared normal and healthy for the 14 day observation period.

See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

No evidence of viral contamination due to the test article, Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration’s Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director
**TABLE 1**

<table>
<thead>
<tr>
<th>Group #</th>
<th>Number of Animals</th>
<th>Sex</th>
<th>Species</th>
<th>Route(s) of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM 1</td>
<td>1</td>
<td>female mouse (lactating)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10</td>
<td>various Mouse (suckling)</td>
<td>i.p.</td>
<td>0.1 ml</td>
<td>test article</td>
<td>Observed for illness after 14 days passage a single pool of emulsified tissue (minus skin and gastrointestinal) of all surviving mice onto at least 10 additional suckling mice. Use same routes and volumes as original.</td>
</tr>
<tr>
<td>SM 2</td>
<td>SAA*</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>test article</td>
<td>SAA</td>
</tr>
<tr>
<td>SM 3</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
<td>SAA</td>
</tr>
<tr>
<td>AM 1</td>
<td>5</td>
<td>male mouse</td>
<td>i.p.</td>
<td>0.5 ml</td>
<td>test article</td>
<td>Observe for illness</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>i.c.</td>
<td>0.03 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM 2</td>
<td>5</td>
<td>male</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td></td>
</tr>
<tr>
<td>AM 3</td>
<td>5</td>
<td>female</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td></td>
</tr>
<tr>
<td>AM 4</td>
<td>5</td>
<td>female</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td></td>
</tr>
<tr>
<td>AM 5</td>
<td>5</td>
<td>male</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
<td>SAA</td>
</tr>
<tr>
<td>AM 6</td>
<td>5</td>
<td>female</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

*SAA = Same as above
i.c. = Intracranial
i.p. = Intraperitoneal
TABLE 2

Survival Summary
for Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

<table>
<thead>
<tr>
<th></th>
<th>Suckling Mice&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult Mice&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Test Article</td>
<td>20/20</td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>10/10</td>
</tr>
<tr>
<td>Uninoculated Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10/10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of surviving, healthy animals after 28 days/Number of animals inoculated.

<sup>b</sup> In the suckling mice portion of the assay, animals are inoculated and observed for 14 days. On day 14 post-inoculation a homogenate is prepared from the surviving sucklings from each group. This homogenate was used to inoculate another group of suckling mice which were observed for an additional 14 days. The number of surviving sucklings/number of animals inoculated is presented.

<sup>c</sup> In the blind passage of the suckling mouse portion of the assay, an uninoculated control group was held with the blind passage animals inoculated with the homogenate of the test article and the homogenate of the uninoculated control group from the primary inoculation.
### TABLE 3

Summary of Daily Observations for Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Mice</td>
<td>Test</td>
<td>10551</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Article</td>
<td>10552</td>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>10553</td>
<td>Normal</td>
<td></td>
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<td></td>
<td></td>
<td>10554</td>
<td>Normal</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>10555</td>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>10556</td>
<td>Normal</td>
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<td></td>
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<td>10560</td>
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<td></td>
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<td>10561</td>
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<td>10569</td>
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</tr>
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<td></td>
<td></td>
<td>10570</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td></td>
<td>10521</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10522</td>
<td>Normal</td>
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<td></td>
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<td>10523</td>
<td>Normal</td>
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<td></td>
<td></td>
<td>10524</td>
<td>Normal</td>
<td></td>
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<td></td>
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<td>10525</td>
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<td>10526</td>
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<td></td>
<td></td>
<td>10527</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10528</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10529</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10530</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 3 (Cont.)

Summary of Daily Observations for Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Number/Cage</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-Inoc.)</th>
<th>Day of Death/Sacrifice (Post-Inoc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling Mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Primary Inoculation)</td>
<td>Test</td>
<td>SM1 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Article</td>
<td>SM2 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>SM3 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Blind Passage)</td>
<td>Test</td>
<td>SM1 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Article</td>
<td>SM2 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>Homogenate</td>
<td>SM3 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>Homogenate</td>
<td>SM4 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

a Ten suckling mice inoculated per cage.
b Surviving suckling mice from primary inoculation were sacrificed on day 14 for preparation of blind passage tissue homogenate.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study Number: ZA358.005100

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/21/90
PHASES: OBSERVATION OF ANIMALS FOR CLINICAL SIGNS

INSPECT ON 08/30/90 - 08/30/90, TO STUDY DIR 08/30/90, TO MGMT 08/31/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan
Quality Assurance Unit

Date

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APPENDIX

D

ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study NO.: ZA356.005102
Dengue-1 Prod Seed: PDK-10, FRhL-2/d7 ........... 95 - 108

Study NO.: ZA357.005102
Dengue-1 Prod Seed: PDK-20, FRhL-2/d7 ........... 109 - 122

Study NO.: ZA358.005102
Dengue-1 Prod Seed: PDK-27, FRhL-2/d7 ........... 123 - 136
ANIMAL SAFETY TEST IN
ADULT GUINEA PIGS

Study No.: ZA356.005102
Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7

Final Report
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
TABLE OF CONTENTS

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VIII. Appendix ....................................................... 105
The purpose of this test is to detect the presence of adventitious agent(s) which might be present in the test article, preclarified bulk live virus and/or fluids, other than the specific virus in the product, by the inoculation and observation of guinea pigs.

No evidence of contamination with adventitious agents was observed due to the test article Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilizes the inoculation of adult guinea pigs. The test is performed as described in CFR Title 21, Section 630.16(a)(4).

Adult guinea pigs are used in this assay to detect possible contamination of the test article with M. tuberculosis or other adventitious agent(s). Animals are examined for signs of illness, and any that become sick or show any abnormalities are examined in an attempt to determine the cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Guinea Pigs

B. Study Number: ZA356.005102

C. Test Article: Dengue-1 (945AZ5) Prod Seed, PDK=10, FRHL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Guinea Pigs
   Hartley Albino
   6 adult females,
   Body weight range: 350-400 g
   Source: Hazleton Research Animals
   Denver, Pennsylvania

G. Sponsor: Program Resources, Inc.
   Biomedical Services Division
   7655 Old Springhouse Road
   McLean, VA 22102

   Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:
1. Study Director: Mary D. Whiteman
2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:
1. Study Initiation Date: 07/11/90
2. Lab Initiation Date: 07/12/90
3. Lab Completion Date: 09/06/90
4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

A. Objective:

The study objective is to detect adventitious agent(s) that might be present in the test article.
B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Animals were housed separately and were identified by ear tags.

The guinea pigs were randomized according to SOP OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. Three were held as uninoculated controls. All animals were observed every working day of the 42 day test period for death or clinical signs of illness or distress. Beginning day 21 post inoculation, rectal temperatures were recorded through day 42 post-inoculation. All remaining guinea pigs were sacrificed. Gross pathological examinations were performed on all guinea pigs.

3. Animal Husbandry

a. Animals were fed Ralston Purina Certified Guinea Pig Chow.

b. Water was supplied ad libitum via water bottles. Water was disinfected with 7 ppm chlorine.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.
<table>
<thead>
<tr>
<th>Group #</th>
<th>Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I.C. 0.1 ml</td>
<td>Test Article</td>
<td>Observe for Illness, Record Rectal Temp. On Days 21-42 Post-Inoculation</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>SAA SAA SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>SAA SAA SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>None None SAA</td>
<td>Uninoc Control</td>
<td>SAA</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>SAA SAA SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>SAA SAA SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above  
I.C. = Intracranial  
I.P. = Intraperitoneal
IV. RESULTS

All of the uninoculated control guinea pigs and two of the three test article inoculated guinea pigs appeared normal and healthy throughout the 42 day observation period. One of the test article inoculated guinea pigs (#10401) was noted to have a noticeably decreased amount of feces production on day 15 post-inoculation, but appeared otherwise normal. By day 19 post-inoculation, feces production appeared normal and the animal remained normal and healthy for the duration of the 42 day observation period. See Table 2 for a summary of the data.

None of the uninoculated control guinea pigs and none of the test article inoculated guinea pigs had significant temperature rises indicative of either viral or bacterial infections during the 21 day recording period from day 21 through 42 post-inoculation. See Table 2 for a summary of the data.

At examination on day 42 for gross pathology, no lesions were found in the control or test article guinea pigs. (See Pathology report in Appendix.)

V. CONCLUSIONS

No evidence of contamination with adventitious agents due to the test article, Dengue-1 (#45AZ5) Prod Seed, PDK=10, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman  
Study Director

Date 9/12/90
### TABLE 2

Summary of Daily Observations for Dengue-1 (#45825) Prod Seed, PRK=10, PRH1-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
<th>Range of Body Temp in °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea Test</td>
<td>10401</td>
<td>Normal</td>
<td>15</td>
<td>38.0 - 38.5</td>
<td>37.9 - 38.7</td>
<td>D-21 to D-42</td>
</tr>
<tr>
<td>Pig Article</td>
<td>10402</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10403</td>
<td>Normal</td>
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<tr>
<td>Uninoculated</td>
<td>10404</td>
<td>Normal</td>
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<td></td>
<td></td>
<td>38.0 - 38.5</td>
</tr>
<tr>
<td>Control</td>
<td>10405</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td>38.2 - 39.0</td>
</tr>
<tr>
<td></td>
<td>10406</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td>38.0 - 38.9</td>
</tr>
</tbody>
</table>

\(^a\) Animal was noted to have reduced feces production, but appeared otherwise normal. Feces production was normal by day 19 and appeared normal for the duration of the 42 day observation period.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study Number: ZA356.005102

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/16/90 - 07/16/90, TO STUDY DIR 07/16/90, TO MGMT 07/16/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/23/90
PHASES: ANIMAL OBSERVATIONS

INSPECT ON 09/12/90 - 09/12/90, TO STUDY DIR 09/12/90, TO MGMT 09/14/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan
Quality Assurance Unit

Date 9/14/90
VIII. APPENDIX
PATHOLOGY REPORT

CAHS-2190

SOURCE: Biotech Services

ZA356.005102 ZA357.005102
ZA358.005102 ZA359.005102
ZA360.005102 ZA361.005102

DATE RECORDED: 09/06/90

SPECIES: Guinea Pig

Results of gross examination according to SOP #865.201.

ZA356.005102

2190-1 (10401) Test Article 563.9 g
Gross: No lesions found

2190-2 (10402) Test Article 684.2 g
Gross: No lesions found

2190-3 (10403) Test Article 614.8 g
Gross: No lesions found

2190-4 (10404) Control 614.4 g
Gross: No lesions found

2190-5 (10405) Control 673.3 g
Gross: No lesions found

2190-6 (10406) Control 646.2 g
Gross: No lesions found

ZA357.005102

2190-7 (10407) Test Article 629.0 g
Gross: No lesions found

2190-8 (10408) Test Article 583.6 g
Gross: No lesions found

2190-9 (10409) Test Article 528.1 g
Gross: No lesions found
**PATHOLOGY REPORT**  
CAHS-2190  
SOURCE: Biotech Services  
PAGE 2

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
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<th>Gross Remarks</th>
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<td>Test Article</td>
<td>636.3</td>
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</tr>
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<td>2190-11</td>
<td>Test Article</td>
<td>490.5</td>
<td>Retroperitoneal abscess (4x6 cm) and peritonitis</td>
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<td>595.5</td>
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<td>Test Article</td>
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<td>Test Article</td>
<td>547.4</td>
<td>No lesions found</td>
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<td>Control</td>
<td>651.5</td>
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<td>Control</td>
<td>623.6</td>
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<td>2190-18</td>
<td>Control</td>
<td>615.9</td>
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</tr>
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<td>Test Article</td>
<td>561.8</td>
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</tr>
<tr>
<td>2190-20</td>
<td>Test Article</td>
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<td>Test Article</td>
<td>Gross:</td>
<td>Weight</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>2190-22</td>
<td>No lesions found</td>
<td>559.8 g</td>
<td></td>
</tr>
<tr>
<td>2190-23</td>
<td>No lesions found</td>
<td>547.9 g</td>
<td></td>
</tr>
<tr>
<td>2190-24</td>
<td>No lesions found</td>
<td>624.8 g</td>
<td></td>
</tr>
</tbody>
</table>

COMMENT: The retroperitoneal abscess in guinea pig #2190-11 is believed to be due to a rectal perforation (no longer visible) caused by insertion of a temperature measuring probe. This is a common lesion in guinea pigs in which the probe is inserted on multiple occasions.

Anton M. Allen, DVM, Ph.D.
Director of Veterinary Services
ANIMAL SAFETY TEST IN
ADULT GUINEA PIGS

Study No.: ZA357.005102
Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

Final Report
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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VII. Quality Assurance Statement ............... 118
VIII. Appendix .................................. 119
SUMMARY

The purpose of this test is to detect the presence of adventitious agent(s) which might be present, in the test article, preclarified bulk live virus and/or fluids, other than the specific virus in the product, by the inoculation and observation of guinea pigs.

No evidence of contamination with adventitious agents was observed, due to the test article Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilizes the inoculation of adult guinea pigs. The test is performed as described in CFR Title 21, Section 630.16(a)(4).

Adult guinea pigs are used in this assay to detect possible contamination of the test article with M. tuberculosis or other adventitious agent(s). Animals are examined for signs of illness, and any that become sick or show any abnormalities are examined in an attempt to determine the cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Guinea Pigs

B. Study Number: ZA357.005102

C. Test Article: Dengue-1 (§45AZ5) Prod Seed, PDK-20, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Guinea Pigs
   Hartley Albino
   6 adult females,
   Body weight range: 350-400 g
   Source: Hazleton Research Animals
   Denver, Pennsylvania

G. Sponsor: Program Resources, Inc.
   Biomedical Services Division
   7655 Old Springhouse Road
   McLean, VA 22102

   Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:

1. Study Director: Mary D. Whiteman

2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:

1. Study Initiation Date: 07/11/90

2. Lab Initiation Date: 07/12/90

3. Lab Completion Date: 09/06/90

4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

A. Objective:

The study objective is to detect adventitious agent(s) that might be present in the test article.
B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Animals were housed separately and were identified by ear tags.

The guinea pigs were randomized according to SOP OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. Three were held as uninoculated controls. All animals were observed every working day of the 42 day test period for death or clinical signs of illness or distress. Beginning day 21 post inoculation, rectal temperatures were recorded through day 42 post-inoculation. All remaining guinea pigs were sacrificed. Gross pathological examinations were performed on all guinea pigs.

3. Animal Husbandry

a. Animals were fed Ralston Purina Certified Guinea Pig Chow.

b. Water was supplied ad libitum via water bottles. Water was disinfected with 7 ppm chlorine.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.
<table>
<thead>
<tr>
<th>Group #</th>
<th>No. of Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I.C.</td>
<td>0.1 ml</td>
<td>Test</td>
<td>Observe for Illness, Record Rectal Temp. On Days 21-42 Post-Inoculation</td>
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<td></td>
<td></td>
<td>I.P.</td>
<td>5.0 ml</td>
<td>Article</td>
<td></td>
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<tr>
<td>2</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
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<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above
I.C. = Intracranial
I.P. = Intraperitoneal
IV. RESULTS

All of the uninoculated control guinea pigs and all of the test article inoculated guinea pigs appeared normal and healthy throughout the 42 day observation period. See Table 2 for a summary of the data.

None of the uninoculated control guinea pigs and none of the test article inoculated guinea pigs had significant temperature rises indicative of either viral or bacterial infections during the 21 day recording period from day 21 through day 42 post-inoculation.

At examination, on day 42, for gross pathology, no lesions were found in the uninoculated control or test article guinea pigs. (See Pathology Report in Appendix.)

V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article, Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration’s Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whitteman
Study Director

9/12/90
**TABLE 2**

**Summary of Daily Observations**
for Dengue-1 (#45AZ5) Prod Seed, PDK-20, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset Sacrifice (Post- inoc.)</th>
<th>Range of Body Temp in °C</th>
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<tr>
<td>Guinea Test</td>
<td></td>
<td>10407</td>
<td>Normal</td>
<td>38.0 - 38.5</td>
<td></td>
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<tr>
<td>Pig</td>
<td>Article</td>
<td>10408 Normal</td>
<td>Normal</td>
<td>38.1 - 38.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10409 Normal</td>
<td>Normal</td>
<td>37.9 - 38.7</td>
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<tr>
<td>Uninoculated</td>
<td></td>
<td>10404 Normal</td>
<td>Normal</td>
<td>38.0 - 38.5</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>10405 Normal</td>
<td>Normal</td>
<td>38.2 - 39.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10406 Normal</td>
<td>Normal</td>
<td>38.0 - 38.9</td>
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</table>
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study Number: ZA357.005102
Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/16/90 - 07/16/90, TO STUDY DIR 07/16/90, TO MGMT 07/16/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/24/90
PHASES: EXAM. OF ABDOMINAL AND THORACIC VISCERA AT DAY 42 POST-INOCULATION FOR OBVIOUS OR SUGGESTIVE ABNORMALITIES

INSPECT ON 09/12/90 - 09/12/90, TO STUDY DIR 09/12/90, TO MGMT 09/14/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan
Quality Assurance Unit

9/14/90
Date
PATHOLOGY REPORT

CAHS-2190

SOURCE: Biotech Services
ZA356.005102 ZA357.005102
ZA358.005102 ZA359.005102
ZA360.005102 ZA361.005102

DATE RECEIVED: 08/23/90
DATE NECROPSIED: 08/23/90
DATE REPORTED: 09/06/90

SPECIES: Guinea Pig
Results of gross examination according to SOP #865.201.

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<tr>
<td>2190-3 (10403)</td>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>2190-4 (10404)</td>
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</tr>
<tr>
<td>Gross: No lesions found</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2190-5 (10405)</td>
<td>Control</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>2190-6 (10406)</td>
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<tr>
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<td>Test Article</td>
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<td>2190-8 (10408)</td>
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<td>2190-9 (10409)</td>
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<tr>
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PATHOLOGY REPORT
CAHS-2190
SOURCE: Biotech Services
AGE 2

ZA358.005102

2190-10 (10410) Test Article 636.3 g
Gross: No lesions found

2190-11 (10411) Test Article 490.5 g
Gross: Retroperitoneal abscess (4x6 cm) and peritonitis

2190-12 (10412) Test Article 595.5 g
Gross: No lesions found

ZA359.005102

2190-13 (10421) Test Article 605.3 g
Gross: No lesions found

2190-14 (10422) Test Article 631.8 g
Gross: No lesions found

2190-15 (10423) Test Article 547.4 g
Gross: No lesions found

2190-16 (10424) Control 651.5 g
Gross: No lesions found

2190-17 (10425) Control 623.6 g
Gross: No lesions found

2190-18 (10426) Control 615.9 g
Gross: No lesions found

ZA360.005102

2190-19 (10427) Test Article 561.8 g
Gross: No lesions found

2190-20 (10428) Test Article 594.6 g
Gross: No lesions found

2190-21 (10429) Test Article 598.4 g
Gross: No lesions found
COMMENT: The retroperitoneal abscess in guinea pig #2190-11 is believed to be due to a rectal perforation (no longer visible) caused by insertion of a temperature measuring probe. This is a common lesion in guinea pigs in which the probe is inserted on multiple occasions.

Anton M. Allen, DVM, Ph.D.
Director of Veterinary Services
ANIMAL SAFETY TEST IN
ADULT GUINEA PIGS

Study No.: ZA358.005102
Test Article: Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7

Final Report
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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<td>II. Study Information</td>
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<td>VI. Approvals</td>
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<td>VII. Quality Assurance Statement</td>
<td>132</td>
</tr>
<tr>
<td>VIII. Appendix</td>
<td>133</td>
</tr>
</tbody>
</table>
SUMMARY

The purpose of this test is to detect the presence of adventitious agent(s) which might be present, in the test article, preclarified bulk live virus and/or fluids, other than the specific virus in the product, by the inoculation and observation of guinea pigs.

No evidence of contamination with adventitious agents was observed, due to the test article Dengue-1 (#45AZ5) Prod Seed, PDK-27, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilizes the inoculation of adult guinea pigs. The test is performed as described in CFR Title 21, Section 630.16(a)(4).

Adult guinea pigs are used in this assay to detect possible contamination of the test article with *M. tuberculosis* or other adventitious agent(s). Animals are examined for signs of illness, and any that become sick or show any abnormalities are examined in an attempt to determine the cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Guinea Pigs

B. Study Number: ZA358.005102

C. Test Article: Dengue-1 (§45A25) Prod Seed, PDK-27, FRHL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:

1. Positive Control: none

2. Negative Control: none

3. Vehicle Control: none

F. Test System: Guinea Pigs

Hartley Albino

6 adult females,

Body weight range: 350-400 g

Source: Hazleton Research Animals
Denver, Pennsylvania

G. Sponsor: Program Resources, Inc.

Biomedical Services Division

7655 Old Springhouse Road

McLean, VA 22102

Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:
1. Study Director: Mary D. Whiteman
2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:
1. Study Initiation Date: 07/11/90
2. Lab Initiation Date: 07/12/90
3. Lab Completion Date: 09/06/90
4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:
All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:
Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES
A. Objective:
The study objective is to detect adventitious agent(s) that might be present in the test article.
B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Animals were housed separately and were identified by ear tags.

The guinea pigs were randomized according to SOP OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. Three were held as uninoculated controls. All animals were observed every working day of the 42 day test period for death or clinical signs of illness or distress. Beginning day 21 post inoculation, rectal temperatures were recorded through day 42 post-inoculation. All remaining guinea pigs were sacrificed. Gross pathological examinations were performed on all guinea pigs.

3. Animal Husbandry

a. Animals were fed Ralston Purina Certified Guinea Pig Chow.

b. Water was supplied ad libitum via water bottles. Water was disinfected with 7 ppm chlorine.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.
TABLE 1

<table>
<thead>
<tr>
<th>Group #</th>
<th>No. of Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I.C. 0.1 ml</td>
<td>Test Record Rectal Temp. On Days 21-42 Post-Inoculation</td>
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</tr>
<tr>
<td></td>
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<td>I.P. 5.0 ml</td>
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<td>1</td>
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SAA = Same As Above
I.C. = Intracranial
I.P. = Intraperitoneal
IV. RESULTS

All of the uninoculated control guinea pigs and two of the three test article inoculated guinea pigs appeared normal and healthy throughout the 42 day observation period. One of the test article inoculated guinea pigs (10411) was noted to appear lethargic with a rough hair coat and decreased fluid intake on day 35 post-inoculation. By day 38 post-inoculation, the animal appeared normal. See Table 2 for a summary of the data.

None of the uninoculated control guinea pigs and none of the test article inoculated guinea pigs had significant temperature rises indicative of either viral or bacterial infection during the 21 day recording period from day 21 through day 42. See Table 2 for a summary of the data.

At examination on day 42 for gross pathology, no lesions were found in any of the uninoculated control animals and no lesions were found in two of the three test article inoculated animals. One of the test article inoculated animals (10411) was found to have a (4 x 6 cm) retroperitoneal abscess and peritonitis. The abscess was attributed to a rectal perforation caused by insertion of a temperature measuring probe and was considered to be a common lesion in guinea pigs in which a probe is inserted on multiple occasions. (See Pathology Report in Appendix.)

V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article, Dengue-1 (#45A25) Prod Seed, PDK-27, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration’s Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director

9/13/90

Date
### TABLE 2


<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
<th>Range of Body Temp in °C</th>
<th>Day of Death/Sacrifice</th>
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<tr>
<td>Guinea Test</td>
<td>10410</td>
<td>Normal</td>
<td>37.8 - 38.8</td>
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<tr>
<td>Pig Article</td>
<td>10411</td>
<td>Normal</td>
<td>38.0 - 38.8</td>
<td>D-21 to D-42</td>
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<td></td>
<td>10412</td>
<td>Normal</td>
<td>37.9 - 38.6</td>
<td>D-21 to D-42</td>
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<tr>
<td>Uninoculated</td>
<td>10404</td>
<td>Normal</td>
<td>38.0 - 38.5</td>
<td>D-21 to D-42</td>
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<tr>
<td>Control</td>
<td>10405</td>
<td>Normal</td>
<td>38.2 - 39.0</td>
<td>D-21 to D-42</td>
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<td></td>
<td>10406</td>
<td>Normal</td>
<td>38.0 - 38.9</td>
<td>D-21 to D-42</td>
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</table>

*a* Animal had a rough hair coat and appeared lethargic with a decreased fluid intake day 35 post-inoculation. Animal appeared normal by day 38 post-inoculation.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study Number: ZA358.005102

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/16/90 - 07/16/90, TO STUDY DIR 07/16/90, TO MGMT 07/16/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/23/90
PHASES: RECTAL TEMPERATURE DETERMINATION

INSPECT ON 09/12/90 - 09/12/90, TO STUDY DIR 09/12/90, TO MGMT 09/14/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan Date

Quality Assurance Unit

- 132 -
VIII. APPENDIX
PATHOLOGY REPORT

CAHS-2190

SOURCE: Biotech Services

<table>
<thead>
<tr>
<th>SPECIES: Guinea Pig</th>
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<td>Results of gross examination according to SOP #865.201.</td>
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<table>
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<tr>
<td>2190-1 (10401) Test Article 563.9 g</td>
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<tr>
<td>Gross: No lesions found</td>
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<td>2190-2 (10402) Test Article 684.2 g</td>
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<td>Gross: No lesions found</td>
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<td>2190-3 (10403) Test Article 614.8 g</td>
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<td>Gross: No lesions found</td>
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<td>2190-4 (10404) Control 614.4 g</td>
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<td>Gross: No lesions found</td>
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<tr>
<td>2190-5 (10405) Control 673.3 g</td>
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<tr>
<td>2190-6 (10406) Control 646.2 g</td>
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<table>
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<tbody>
<tr>
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</tr>
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<td>Gross: No lesions found</td>
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<td>2190-8 (10408) Test Article 583.6 g</td>
</tr>
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<td>Gross: No lesions found</td>
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<tr>
<td>2190-9 (10409) Test Article 528.1 g</td>
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</table>
2190-10 (10410) Test Article 636.3 g
Gross: No lesions found

2190-11 (10411) Test Article 490.5 g
Gross: Retroperitoneal abscess (4x6 cm) and peritonitis

2190-12 (10412) Test Article 595.5 g
Gross: No lesions found

2190-13 (10421) Test Article 605.3 g
Gross: No lesions found

2190-14 (10422) Test Article 631.8 g
Gross: No lesions found

2190-15 (10423) Test Article 547.4 g
Gross: No lesions found

2190-16 (10424) Control 651.5 g
Gross: No lesions found

2190-17 (10425) Control 623.6 g
Gross: No lesions found

2190-18 (10426) Control 615.9 g
Gross: No lesions found

2190-19 (10427) Test Article 561.8 g
Gross: No lesions found

2190-20 (10428) Test Article 594.6 g
Gross: No lesions found

2190-21 (10429) Test Article 598.4 g
Gross: No lesions found
2190-22 (10430) Test Article 559.8 g
Gross: No lesions found

2190-23 (10431) Test Article 547.9 g
Gross: No lesions found

2190-24 (10432) Test Article 624.8 g
Gross: No lesions found

COMMENT: The retroperitoneal abscess in guinea pig #2190-11 is believed to be due to a rectal perforation (no longer visible) caused by insertion of a temperature measuring probe. This is a common lesion in guinea pigs in which the probe is inserted on multiple occasions.

Anton M. Allen, DVM, Ph.D.
Director of Veterinary Services
December 17, 1990

Dr. Louis Potash
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22101

Dear Dr. Potash,

Microbiological Associates, Inc. is an AAALAC accredited animal facility, and all studies are performed in accordance with the "Guide for the Care and Use of Laboratory Animals", U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH Publication No. 86-23.

Sincerely,

Mary D. Whiteman
Study Director, In Vivo Assays
Biotechnology Division
May 14, 1991

TO: Mr. Donald Holzworth, Vice President
    Dr. Louis Potash, Study Director

FROM: James R. Plautz
    Sr. QA Advisor

RE: GLP Compliance Audit of Final Reports for Safety Testing of Dengue Virus Type 1 and Type 4

On April 14, 1991 a complete audit for GLP compliance (21 CFR, Part 58) was conducted for the subject final reports and their respective raw data.

Our complete findings indicate that the studies were conducted under the guidance of the referenced Standard Operating Procedures (SOPs), the variations from the SOPs had no apparent effect on study outcome, and that the final report for each study is substantiated by the raw data.

Animal safety testing was conducted and reported separately from these final reports.
APPENDIX II

Dengue-4 Virus Strain Carib 341750
FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 85-23, Revised 1985) - (see Attachment A).

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

[Signature]

PI Signature 12-20-80

Date
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<td>Attachment: B Quality Assurance Statement from Program Resources, Inc.</td>
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I. INTRODUCTION

The accompanying protocol is a description of the safety testing of 3 crude harvest lots of dengue virus type 4 designated as:

Dengue Virus Type 4 (Carib 341750):
PDK-6, FRH-2/d7: PDK-10, FRH-2/d7
and PDK-15, FRH-2/d7 of 9 March 1990

Utilizing the testing procedures herein described, this fluid is considered to have not passed satisfactorily all tests for safety including purity. The detailed records with respect to passage history, pool production, and subsequent safety testing may be found in the laboratory notebooks located at:

The Walter Reed Army Institute of Research (WRAIR), Eldg. 501, Washington, DC 20307-5100 - (Dr. Ken Eckels)

The Experimental Virus Vaccine Production Laboratory - Suite #500 - (Flow Laboratories, Inc.) Program Resources, Inc. [PRI], McLean, VA - (Dr. Louis Potash)

All procedures performed at PRI followed Good Laboratory Practices (GLP) regulations (21 CFR, Part 50) and were carried out in accordance with the guidelines established by the FDA for live and inactivated vaccines as found in 21 CFR, Parts 610.11, 610.12, 610.30, 630.10 - 630.17, etc. of April 1989. These procedures are detailed in the following SOPs and recorded on the indicated VVPL Forms:

SOP No.: 500.001 - Issued 29 Oct 1980, Revised 13 Feb 1986
500.008 - " 13 Jan 1981, " 3 Mar 1986

VVPL FORM #008 - Issued 29 Oct 1980, Revised 3 May 1984
016 - " 15 Jan 1981, " 13 July 1984
017 - " 16 Jan 1981, " 13 Jan 1986
019 - " 8 Oct 1984
II. SYNOPSIS

A. Crude Virus Harvests: Dengue Virus Type 4 (Carib 341750)
PDK-6, FRhL-2/d7 of 9 Mar 90
PDK-10, FRhL-2/d7 of 9 Mar 90
PDK-15, FRhL-2/d7 of 9 Mar 90

B. Safety Tests on Crude Harvest Fluids:

1. Sterility: Fluid Thioglycollate (FTN),
   Tryptone Soya Broth (TSB), Mycoplasma
   a. PDK-6 Virus Fluid (47 ml) No Growth
   b. PDK-10 Virus Fluid (47 ml) No Growth
   c. PDK-15 Virus Fluid (47 ml) No Growth
   d. Control Fluid (TCF) (47 ml) No Growth

2. Tissue Culture Identity and Purity
   (Safety): AGK, PHA, FRhL-2, PRK, and Flow 5000.
   a. PDK-6 Virus Fluid (25 ml) Unsatisfactory*
   b. PDK-10 Virus Fluid (25 ml) Unsatisfactory*
   c. PDK-15 Virus Fluid (25 ml) Unsatisfactory*
   d. Control Fluid (TCF) (25 ml) Satisfactory

3. Animal Safety:
   a. Rabbits: I.D. & S.Q. - (Appendix - B)
      (1) PDK-6 Virus Fluid (30 ml) Satisfactory
      (2) PDK-10 Virus Fluid (30 ml) Satisfactory
      (3) PDK-15 Virus Fluid (30 ml) Satisfactory
   b. Adult Mice: I.C. & I.P - (Appendix - C)
      (1) PDK-6 Virus Fluid (10.6 ml) Satisfactory
      (2) PDK-10 Virus Fluid (10.6 ml) Satisfactory
      (3) PDK-15 Virus Fluid (10.6 ml) Satisfactory

* Test unsatisfactory only in the AGK test system. Non-descript morphological changes observed in primary AGK flask cultures, particularly after films were stained. All AGK tube subcultures exhibited varying degrees of cytopathology ranging from 1-3+. Both flask and tube subcultures were negative for hemadsorption. All tube subcultures completely inhibited the Coxsackie A-9 challenge virus.
3. Animal Safety (continued):

c. Suckling Mice: I.C. & I.P. – (Appendix - C)
   (1) PDK-6 Virus Fluid* (2.2 ml) Satisfactory
   (2) PDK-10 Virus Fluid* (2.2 ml) Inconclusive**
   (3) PDK-15 Virus Fluid* (2.2 ml) Inconclusive***

d. Guinea Pigs: I.C. & I.P. – (Appendix - D)
   (1) PDK -6 Virus Fluid (15.3 ml) Satisfactory
   (2) PDK-10 Virus Fluid (15.3 ml) Satisfactory
   (3) PDK-15 Virus Fluid (15.3 ml) Satisfactory

* Virus fluid was mixed with equal parts of a 1:5 dilution of the immune serum and incubated at 37°C for 90 minutes prior to inoculation.

** Although all of the 20 sucklings appeared normal and survived the initial 14-day incubation period, only 9 of the 20 sucklings inoculated with their emulsified tissue survived the final 14-day blind passage with 2 lethargic and with hunched postures.

*** Only 6 of 16 inoculated sucklings appeared normal at the end of the initial 14-day incubation period and were emulsified for blind passage. Of the other 10 original sucklings, 1 was apparently cannibalized, 4 were found dead (marked autolysis on necroscopy & histopathology) and 5 were found moribund and were homogenized for subpassage. None of the 20 sucklings inoculated with the 'normal' homogenate survived the final 14-day blind passage and, of the 20 sucklings inoculated with the moribund homogenate, 10 survived but of these 2 were moribund and 1 was lethargic and runted.
III. DETAILED SUMMARY RELATING TO THE SAFETY TESTING OF THREE (3) DIFFERENT PASSAGE LEVELS OF DENGUE VIRUS TYPE 4 (CARIB 341750) PRODUCTION SEEDS: PROPAGATED IN DBS-FRH-L-2 CELL CULTURES

A. Inocula

In May 1990, the following frozen materials were obtained for testing from Dr. K. Eckels, Contracting Officer's Representative, Walter Reed Army Institute of Research (WRAIR), Bldg. 501, Washington, D.C.:

1. Dengue-4 (Carib 341750) crude, unclarified harvest fluids of 9 Mar 90:
   a. PDK-6, FRhL2-2 (day 7 harvest)........... 20 x 10 ml vials
   b. PDK-10, FRhL2-2 (day 7 harvest)........... 20 x 10 ml vials
   c. PDK-15, FRhL2-2 (day 7 harvest)........... 20 x 10 ml vials
   d. Control Fluids ......................... 4 x 25 ml vials

2. Dengue-4 Antiserum: 814699 CAREC, SEM-5 OF 11/21/82 ... 1 x 8 ml

On arrival in this laboratory, the virus and control fluids were stored at -70°C, or below, and the antiserum at -20°C, or below.

B. Safety Testing Procedures and Results on the Crude, Unclarified Harvest Fluids (SOP No.: 500.008)

1. Microbial Sterility - (VVPL FORM #011)

   Aliquots of the bulk frozen fluids were thawed and tested for microbial sterility as follows:

   a. Fluid Thioglycollate Medium - FTM - (LOT VVPL #030): Each of 10 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluids and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 culture tubes were included as uninoculated controls. All cultures were vortex mixed and incubated at 32°C (+ 2°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 50 culture tubes.

   b. Tryptone Soya Broth - TSB - (LOT VVPL #030): Each of 10 culture tubes (9-10 ml medium per tube) was inoculated with 1 ml volumes of the crude virus fluids and each of 10 culture tubes was inoculated with 1 ml volumes of the crude control fluid. An additional 10 cultures were included as uninoculated controls. All cultures were vortex mixed and incubated at 22°C (+ 2°C) for 21 days with periodic examination for evidence of growth. No growth was observed in any of the 50 culture tubes.

   The results of the above described Microbial Sterility Assays are summarized in Table I.
c. Mycoplasma Sterility: These assays were performed by PRI's Mycoplasma Testing Laboratory and included both the routine PFLO agar and broth assays and the specific test for the detection of *M. hyorhinis*. Samples (1 x 2 ml and 1 x 25 ml) of the 3 crude virus fluids and of the 1 control fluid were submitted for testing. All samples were reported to be negative for mycoplasmas. A copy of this report is appended to this Protocol - (Appendix A - 1, 2, 3 & 4).

2. Identity in Tissue Culture (Serum-Neutralization) -

No attempt was made to identity the crude virus pools in tissue cultures.

3. Purity (Safety) in Tissue Cultures - (VVP1 FORM #016)

a. Tissue Cultures: All flask and roller tube cell cultures were prepared by contract personnel. Cultures were maintained on Medium MEM containing 5 to 10% fetal bovine serum (heat-inactivated) plus antibiotics: gentamicin @ 100 mcg/ml; neomycin @ 50 mcg/ml; and amphotericin B (I.V.) @ 2.5 mcg/ml. Cultures were inoculated, refed and subpassaged as indicated below. The following tissue culture systems were utilized:

1. Tertiary African Green Monkey Kidney (AGMK) .... MEM + 5% serum
2. Primary Human Amnion (PHA) ................................ MEM + 10% serum
3. Fetal Rhesus Lung (FRHL-2) ................................ MEM + 5% serum
4. Primary Rabbit Kidney (PRK) ............................ MEM + 5% serum
5. Whole Human Embryo Fibroblast (Flow 5000) ........ MEM + 5% serum

b. General Testing Procedures

1. Crude Virus Fluids

   a. Primary Flask Cultures: Equal 5 ml volumes of the bulk crude virus fluids and of a 1:10 dilution of the rabbit immune serum (Den-4, CAREC 814669, smb 5) were well mixed and incubated at 37°C (water bath) for 90 minutes. Due to the small volume of antiserum available, only 5 ml of each crude virus fluids were tested per tissue culture system wherein 1 x 75 cm² flask per tissue culture system was inoculated with 10 ml of these serum-virus mixtures. Flasks contained approximately 25 ml of maintenance medium at the time of inoculation. Cultures were incubated at 35°C (37°C for PHA) for 14 days with periodic microscopic examination for any signs of CPE and/or cellular degradation. When necessary to maintain the integrity of the cell films, cultures were refed with 35 ml of fresh medium.

   b. Secondary Tube Subcultures: On the 14th day of incubation, the primary cultures were re-examined microscopically and the fluids harvested individually and treated with the specific immune serum - 0.1 ml per harvest. In addition, to each individual harvest was added: 0.1 ml gentamicin (50 mcg/ml); 1 ml penicillin-streptomycin solution (5000 units/ml and 5000 mcg/ml, respectively); and 10% of 10X SPG* (v/v).

   * 10X SPG: sucrose, 2.18 M; KH₂PO₄, 0.038 M; K₂HPO₄, 0.072 M; monosodium glutamate, 0.049 M.
Following mixing, the fluids were incubated at room temperature for 50 min. and then subassed into homologous roller tube cultures - 0.5 ml of each harvest into each of 20 tubes. The remainder of the harvest fluids was saved and stored at -75°C, or below. All primary cultures were tested for hemadsorption by the addition of 0.1% guinea pig RBC (in PBS) and incubation at 4°C for a minimum of 30 minutes. All cultures were negative for hemadsorption.

Tube cultures (refed with 2 ml of maintenance medium prior to inoculation) were incubated at 35°C (37°C for PHA) for 13-14 additional days. When necessary to maintain the integrity of the cell films, cultures were refed with 2 ml of fresh medium. Cultures were examined microscopically at periodic intervals and at the end of the incubation period for any signs of CPE. After final examination, tubes were divided - depending on the specific cell system - for additional testing:

**AGMK, PHA, FRhL-2 and Flow 5000 Tube Cultures:** These were divided into 3 groups as follows:

- 1/4th tested for the presence of hemadsorbing agents,
- 1/4th fixed and stained with a solution of 5% glutaraldehyde + 1:10 giemsa stain and examined microscopically for any CPE,
- 1/2 challenged with Coxsackie A-9 virus (0.2 ml per tube at dilutions noted in the tables) for the detection of non-CPE producing agents and/or latent agents via the interference phenomenon.

**PRK Tube Cultures:** These were equally divided into 2 groups:

- 1/2 tested for the presence of hemadsorbing agents,
- 1/2 fixed and stained with the glutaraldehyde-giemsa stain solution and examined microscopically for any CPE.

No challenge studies were carried out with the Coxsackie A-9 virus since this virus does not produce any discernible CPE in this tissue culture system.

(2) **Crude Control Fluid**

A single 75 cm² flask per tissue culture system was inoculated with 10 ml of crude control fluid. Cultures were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included.

(3) **Uninoculated Cell Lot Controls**

Two x 75 cm² flasks per tissue culture system were included as uninoculated cell lot controls and were handled in a manner similar to that described above for the crude virus fluid except that immune serum was not included. In addition, an appropriate number of uninoculated roller tube cultures were included as cell lot controls for the secondary tube subcultures.

In all challenge studies, 1 to 4 culture tubes per set were left unchallenged to serve as controls to the challenge virus.

The results of these in vitro Tissue Culture Purity (Safety) tests are summarized in Tables II-A through -E.
4. **Animal Safety Tests**

Due to the dismantling of Flow's Animal Facility during December 1989, all animal studies were performed by Microbiological Associates, Inc. The inocula for these animal studies were the three crude virus suspensions:

a. **Adult Rabbits** - MBA Studies #ZA359.005101, #ZA360.005101, and #ZA361.005101 - these tests were reported to be satisfactory and copies of these Final Reports may be found in Appendix - E.

b. **Adult and Suckling Mice** - MBA Studies #ZA359.005100, #ZA360.005100 and #ZA361.005100 - all three tests in adult mice were reported to be satisfactory. However, of the three sucking mice tests, only Study #ZA359.005100 (PDK-6, PRH-2/37 inoculum) was reported to be satisfactory, whereas the tests in the other 2 studies (#ZA360.005100 and #ZA361.005100 - PDK-10 & PDK-15) were reported to be inconclusive due to the lethal effect of the test articles. Copies of these Final Reports may be found in Appendix - C.

c. **Adult Guinea Pigs** - MBA Studies #ZA359.005102, #ZA360.005102 and #ZA361.005102 - these tests were reported to be satisfactory and copies of these Final Reports may be found in Appendix - D.
Table I. Microbial Sterility Test Results on the Crude Dengue-4 Virus (Carib 341750) Production Seed Pools

<table>
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<tr>
<th>Culture Medium</th>
<th>Vol. per culture (ml)</th>
<th>Temperature</th>
<th>On Test</th>
<th>Off Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluid Thioglycollate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(PTH) LOT VVPL-#030</td>
<td>10 ----</td>
<td>32°C (+2°C)</td>
<td>11/12/90</td>
<td>12/03/90</td>
<td>No Growth</td>
</tr>
<tr>
<td>PDK- 6 Virus Fluid</td>
<td>10 1.0</td>
<td></td>
<td></td>
<td></td>
<td>No Growth</td>
</tr>
<tr>
<td>PDK-10 Virus Fluid</td>
<td>10 1.0</td>
<td></td>
<td></td>
<td></td>
<td>No Growth</td>
</tr>
<tr>
<td>PDK-15 Virus Fluid</td>
<td>10 1.0</td>
<td></td>
<td></td>
<td></td>
<td>No Growth</td>
</tr>
<tr>
<td>Control Fluid</td>
<td>10 1.0</td>
<td>11/12/90</td>
<td>12/03/90</td>
<td></td>
<td>No Growth</td>
</tr>
</tbody>
</table>

| **Tryptone Soya Broth** | | | | | |
| (TSB) LOT VVPL #030 | 10 ---- | 22°C (+2°C) | 11/12/90 | 12/03/90 | No Growth |
| PDK- 6 Virus Fluid | 10 1.0 | | | | No Growth |
| PDK-10 Virus Fluid | 10 1.0 | | | | No Growth |
| PDK-15 Virus Fluid | 10 1.0 | | | | No Growth |
| Control Fluid | 10 1.0 | 11/12/90 | 12/03/90 | | No Growth |
Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Production Seed Pools

A. Tertiary African Green Monkey Kidney (AGMK)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>10^-3</th>
<th>10^-4</th>
<th>10^-5</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK-6 Virus/Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>20/20</td>
<td>0/5</td>
<td>5/5</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-10 Virus/Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>20/20</td>
<td>0/5</td>
<td>5/5</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-15 Virus/Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>20/20</td>
<td>0/5</td>
<td>5/5</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control Fluid (TCF)</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>1/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/40</td>
<td>0/10</td>
<td>0/10</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>2/4</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/50</td>
<td>0/12</td>
<td>0/12</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>4/8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Coxsackie A-9 Challenge Results based on a 6-day incubation at 35°C.
  Prior to challenge, all tubes refed with 2 ml of fresh medium
  Complete inhibition of Coxsackie A-9 challenge virus by virus-serum mixture series.

** Stained flasks revealed non-descript CPE in virus-serum inoculated flasks only.

### All tubes inoculated with harvests from virus-serum inoculated flasks exhibited
cytology on day 20 (days 14 + 6) which was attributed to dengue virus breakthroughs.

*** Staining of tubes confirmed the varying degrees of cytology: PDK-6 = PDK-15 < PDK-10.
Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Production Seed Pools

0. Primary Human Amnion (PHA)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>10^-3</th>
<th>10^-4</th>
<th>10^-5</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDK-6 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>1/2</td>
</tr>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>1/2</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-15 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control Fluid (TCF)</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2</td>
<td>ND</td>
<td>0/40</td>
<td>0/10</td>
<td>0/10</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>2/4</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/60</td>
<td>0/12</td>
<td>0/12</td>
<td>8/8</td>
<td>8/8</td>
<td>7/8</td>
<td>1/8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Coxsackie A-9 Challenge*

0.5 ml per tube
Passage #1
Lot # 1541 Lot # 1639
Day: 14 Day: 14 + 14 = 23

** Coxsackie A-9 Challenge Results based on a 4-day incubation at 37°C.
Prior to challenge, all tubes refed with 2 ml of fresh medium.

** On day 7, all flasks were refed with 35 ml of fresh medium.

ND = Not done
Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Production Seed Pools

C. Fetal Rhesus Lung (FRhL-2)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>Initial Flasks</th>
<th>0.5 ml per tube</th>
<th>Coxsackie A-9 Challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5 ml per tube</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Passage #1</td>
<td></td>
</tr>
<tr>
<td>Lot # 1610 p21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDK-6 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1 ND</td>
<td>0/20</td>
</tr>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1 ND</td>
<td>0/20</td>
</tr>
<tr>
<td>PDK-15 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1 ND</td>
<td>0/20</td>
</tr>
<tr>
<td>Control Fluid (TCF)</td>
<td>0/1</td>
<td>0/1 ND</td>
<td>0/20</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2 ND</td>
<td>0/40</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/60</td>
<td>0/12</td>
<td>0/12</td>
</tr>
</tbody>
</table>

* Coxsackie A-9 Challenge Results based on a 3-day incubation at 35°C. Prior to challenge, all tubes refed with 2 ml of fresh medium.

**ND = Not done**
Table II. Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Production Seed Foils

D. Primary Rabbit Kidney (PRK)

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>Initial Flasks</th>
<th>Passage #1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 ml per tube</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lot # 1650</td>
<td>Lot # 1693</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day: 14</td>
<td>Day: 14 + 14 = 28</td>
<td></td>
</tr>
<tr>
<td>PDK-6 Virus/ Serum Mixture</td>
<td>0/1*</td>
<td>0/1</td>
<td>ND</td>
</tr>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/1*</td>
<td>0/1</td>
<td>ND</td>
</tr>
<tr>
<td>PDK-15 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
</tr>
<tr>
<td>Control Fluid (TCF)</td>
<td>0/1</td>
<td>0/1</td>
<td>ND</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2</td>
<td>ND</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/24</td>
<td>0/12</td>
<td></td>
</tr>
</tbody>
</table>

* Commencing on day 10, focal area of vacuolation observed in these flasks only. Whatever caused these morphological changes was not subpassaged into the tube subcultures.

ND = Not done
## Table II.
Tissue Culture Purity (Safety) Test Results on the Crude Dengue-4 Virus (Carib 341750) Production Seed Pools

*Whole Human Embryo Fibroblasts (Flow 5000)*

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>Initial Flasks</th>
<th>Coxsackie A-9 Challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lot # 1630 p10</td>
<td>Day 14</td>
</tr>
<tr>
<td></td>
<td>Lot # 1669 p20</td>
<td>Day 14 + 1d = 28</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDK-6 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>PDK-15 Virus/ Serum Mixture</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>Control Fluid (TCP)</td>
<td>0/1</td>
<td>0/2</td>
</tr>
<tr>
<td>Control - (1)</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Control - (2)</td>
<td>0/60</td>
<td>0/12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>CPE</th>
<th>Hads</th>
<th>Stain</th>
<th>Coxsackie A-9 Challenge*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10^-2</td>
</tr>
<tr>
<td>PDK-6 Virus/ Serum Mixture</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>PDK-10 Virus/ Serum Mixture</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>PDK-15 Virus/ Serum Mixture</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>Control Fluid (TCP)</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>0/20</td>
<td>0/5</td>
<td>0/5</td>
<td>2/2</td>
</tr>
<tr>
<td>Control - (1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4/4</td>
</tr>
<tr>
<td>Control - (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8/8</td>
</tr>
</tbody>
</table>

* Coxsackie A-9 Challenge Results based on a 5-day incubation at 35°C. Prior to challenge, all tubes refed with 2 ml of fresh medium.

ND = Not done
To: Dr. Louis Potas'.
From: Jim Quartey.
Subject: Mycoplasma Testing. (Charge # 807)

This letter is to inform you that, the eight (8) samples listed below which you had submitted for the detection of Mycoplasma hyorhinis using the direct immunofluorescence staining and for the detection of Mycoplasma in general using the DNA Hoechst stain and Agar testing were found to be negative.

a. Dengue-1 (#45AZ5) Production Seed cf 16 Feb 90:
   1. PDK-10, FRhL-2/B7.
   2. PDK-20, FRhL-2/d7.
   4. Control Fluid.

b. Dengue-4 (#341750) Production Seed of 9 Mar 90:
   1. PDK-6, FRhL-2/d7
   2. PDK-10, FRhL-2/d7
   3. PDK-15, FRhL-2/d7
   4. Control Fluid.
<table>
<thead>
<tr>
<th>Identification</th>
<th>#</th>
<th>Preliminary Reading</th>
<th>Final</th>
<th>Sub</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Agar</td>
<td>B75-7</td>
<td>Hoechst</td>
</tr>
<tr>
<td>Negative Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Positive Control</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dengue-1 (5452)</td>
<td>197</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PDK-10, FRAL-2/47</td>
<td>198</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PDK-20, FRAL-2/47</td>
<td>199</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PDK-27, FRAL-2/47</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control Fluid</td>
<td>201</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dengue-4 (34750)</td>
<td>202</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PDK-10, FRAL-2/47</td>
<td>203</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PDK-15, FRAL-2/47</td>
<td>204</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Read by: xxxxxxxx
Date: 7/16 25 7/31 7/31

KEY: 
* = Positive
- = Negative
? = Questionable
BC = Bacterial contamination

Plate Lot No. 900523
Hoechst Lot No. 944487

Planted by Chuck

---

Page 1 of 1
## APPENDIX A - 3

### MYCOPLASMA TEST RECORD SHEET

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>LOT #</th>
<th>Aerobic</th>
<th>Anaerobic</th>
<th>On Test</th>
<th>Off Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DENGUE-4(#341750)</strong> Virus Fluid - LOT #: PDK-6, FRI2-2/17. Myc#: 201</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPLO Agar</td>
<td>900523</td>
<td>2</td>
<td>2</td>
<td>7/9/90</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>PPLO Broth</td>
<td>900503</td>
<td>25.0</td>
<td>25.0</td>
<td>7/16/90</td>
<td>7/31/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D5 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/16/90</td>
<td>7/31/90</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/19/90</td>
<td>8/3/90</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D10 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/19/90</td>
<td>8/3/90</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/24/90</td>
<td>8/8/90</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D15 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>8/8/90</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**DENGUE-4(#341750)** Virus Fluid - LOT #: PDK-10, FRI2-2/17. Myc#: 202

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>LOT #</th>
<th>Aerobic</th>
<th>Anaerobic</th>
<th>On Test</th>
<th>Off Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPLO Agar</td>
<td>2</td>
<td>2</td>
<td>7/9/90</td>
<td>7/24/90</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>PPLO Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/16/90</td>
<td>7/31/90</td>
<td></td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D5 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/16/90</td>
<td>7/31/90</td>
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</tr>
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</tr>
<tr>
<td>D10 Subpass to Broth</td>
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<td></td>
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</tr>
<tr>
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<td>8/8/90</td>
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</tr>
<tr>
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<td>8/8/90</td>
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<tr>
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</table>

Positive Control (+): **M. arginini**  
Negative Control (-): **FB 2910/070**

Date: **8/18/90**  
Signed: **Jim Goodley**
### Mycoplasma Test Record Sheet

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>LOT #</th>
<th>No. ml Tested</th>
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<th>Results</th>
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<tbody>
<tr>
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<td>900523</td>
<td>2</td>
<td>7/19/90</td>
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</tr>
<tr>
<td>PPLO Broth</td>
<td>900523</td>
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<td>7/19/90</td>
<td>NEGATIVE</td>
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<td>D5 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/11/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/18/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D10 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/19/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
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<td>2</td>
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<td>NEGATIVE</td>
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<tr>
<td>D15 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
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<td>2</td>
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</table>

**DENGUE-4 (#341750) Control Fluid - LOT # Myc# 204**

<table>
<thead>
<tr>
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<th>No. ml Tested</th>
<th>Date</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPLO Agar</td>
<td>900523</td>
<td>2</td>
<td>7/19/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>PPLO Broth</td>
<td>900523</td>
<td>25.0</td>
<td>7/19/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D5 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/11/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>7/18/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>D10 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/19/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>8/3/90</td>
<td>NEGATIVE</td>
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<tr>
<td>D15 Subpass to Broth</td>
<td>25.0</td>
<td>25.0</td>
<td>7/24/90</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>to Agar</td>
<td>2</td>
<td>2</td>
<td>8/8/90</td>
<td>NEGATIVE</td>
</tr>
</tbody>
</table>

Positive Control (+): **M. arginini**

Negative Control (-): **Fb 29101 C070**

Date: 8/8/90

Signed: Jim Carley
APPENDIX

B

ANIMAL SAFETY TEST IN ADULT RABBITS

Study NO.: ZA359.005101
Dengue-4 Prod Seed: PDK-6, FRhL-2/d7 ........ pages 21 - 31

Study NO.: ZA360.005101
Dengue-4 Prod Seed: PDK-10, FRhL-2/d7 ........ pages 32 - 42

Study NO.: ZA361.005101
Dengue-4 Prod Seed: PDK-15, FRhL-2/d7 ........ pages 43 - 53
ANIMAL SAFETY TEST IN ADULT RABBITS

Study No.: ZA359.005101

Test Article: Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7

Final Report
For

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9000 Blackwell Road
Rockville, Maryland 20850
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III. Procedures .......................................... 25
IV. Results ............................................... 26
V. Conclusions ........................................... 26
VI. Approvals ............................................ 27
VII. Quality Assurance Statement ....................... 31
SUMMARY

The purpose of this assay is to detect the presence of adventitious agent(s) in the test article pre-clarified bulk live virus vaccine and/or fluids, other than the specific virus in the product. The test article was inoculated into adult rabbits.

No evidence of contamination with adventitious agent(s) was observed due to the test article Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilized inoculation of adult rabbits. The test is performed as described in CFR Title 21, Section 630.16.

Adult rabbits are utilized in this assay to detect possible contamination of the test article with B-virus or other adventitious agent(s) including other Simian agents, adenovirus(es), etc. which might be present in the test article. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Rabbits

B. Study Number: ZA359.005101

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7 was received at Microbiological Associates, Inc. 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:

1. Positive Control: none
2. Negative Control: none
3. Vehicle Control: none

F. Test System: Rabbits, four females, SPF NZW, 1.5 - 2.5 kg.
Source: Buckshire Corp.
P.O. Box 155
Perkasie, PA 18944

G. Sponsor: Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

Authorized Representative: Dr. Louis Potash
II. H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:
1. Study Director: Mary D. Whiteman
2. Associate Study Director: Janet Luczak, M.T. (ASCP), M.G.A.

J. Schedule:
1. Study Initiation Date: 07/16/90
2. Lab Initiation Date: 08/03/90
3. Lab Completion Date: 08/31/90
4. Study Completion Date: See Study Director’s Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:
All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:
Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

A. Objective:
The study objective is to detect inapparent adventitious agent(s) which might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.
B. Methods:

1. Test System Identification and Randomization

Each rabbit was housed individually. Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Each rabbit was identified by a unique number tattooed on its ear. The rabbit's number was recorded on the cage card.

The rabbits were randomized according to SOP #OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. The rabbits were observed for 28 days for clinical signs of illness or distress.

3. Animal Husbandry

a. Rabbits were fed certified rabbit chow ad libitum and water was supplied via water bottles, ad libitum.

b. Rabbit's cages were changed weekly.

c. Animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All rabbits inoculated with the test article and the uninoculated control rabbit remained normal and healthy for the 28 day observation period.

See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article, Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7, was observed.
VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration’s Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director
<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
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</thead>
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<td>1</td>
<td>1</td>
<td>I.D.</td>
<td>1.0 ml</td>
<td>Test Article</td>
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<tr>
<td></td>
<td></td>
<td>S.Q.</td>
<td>9.0 ml</td>
<td>Observe for Illness</td>
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<td>1</td>
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<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above  
I.D. = Intradermal Inoculation  
S.Q. = Subcutaneous Inoculation
# TABLE 2

**Survival Summary for Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7**

<table>
<thead>
<tr>
<th>RABBITS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Article</td>
<td>3/3(^a)</td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>1/1(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Number of surviving, healthy animals after 28 days/Number of animals inoculated.

\(^b\) Number of surviving, healthy animals after 28 days/Number of animals on lab initiation date.
### TABLE 3
Summary of Daily Observations for Dengue-4 (341750) Prod Seed, PDK-6, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
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<tr>
<td>Rabbit</td>
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<td></td>
<td>Uninoculated Control</td>
<td>4</td>
<td>Normal</td>
<td></td>
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</tr>
</tbody>
</table>
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT RABBITS

Study Number: ZA359.005101

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/16/90 - 08/16/90, TO STUDY DIR 08/16/90, TO MGMT 08/21/90
PHASES: ANIMAL OBSERVATIONS

INSPECT ON 09/21/90 - 09/24/90, TO STUDY DIR 09/24/90, TO MGMT 09/28/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Ed Warburton
Quality Assurance Unit

Date 9/24/90
ANIMAL SAFETY TEST IN ADULT RABBITS

Study No.: ZA360.005101

Test Article: Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7

Final Report

For

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By

Microbiological Associates, Inc.
Life Sciences Center
9000 Blackwell Road
Rockville, Maryland 20850
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<td>I. Introduction</td>
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<td>II. Study Information</td>
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<td>III. Procedures</td>
<td>36</td>
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<tr>
<td>IV. Results</td>
<td>37</td>
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<td>V. Conclusions</td>
<td>37</td>
</tr>
<tr>
<td>VI. Approvals</td>
<td>38</td>
</tr>
<tr>
<td>VII. Quality Assurance Statement</td>
<td>42</td>
</tr>
</tbody>
</table>
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   2. Negative Control: none
   3. Vehicle Control: none

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   Authorized Representative: Dr. Louis Potash

- 35 -
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Mary D. Whiteman
Study Director

9/28/90  Date
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<td>1</td>
<td>1</td>
<td>I.D.</td>
<td>1.0 ml</td>
<td>Test for Illness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.Q.</td>
<td>9.0 ml</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
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<td>SAA</td>
<td>SAA</td>
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</tr>
<tr>
<td>4</td>
<td>1</td>
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<th>Animal Number</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Test</td>
<td>14</td>
<td>Normal</td>
<td></td>
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<tr>
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<td>Article</td>
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<td>Uninoculated</td>
<td>Control</td>
<td>4</td>
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</tr>
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QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT RABBITS

Study Number: ZA360.005101

Study Director: Mary D. Whitema:

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PHASES: ANIMAL OBSERVATIONS

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PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Ed Warburton
Quality Assurance Unit

- 42 - Microbiological Associates Inc.
ANIMAL SAFETY TEST IN ADULT RABBITS

Study No.: ZA361.005101

Test Article: Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7

Final Report
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<tr>
<td>VII. Quality Assurance Statement</td>
<td>53</td>
</tr>
</tbody>
</table>
SUMMARY

The purpose of this assay is to attempt to detect the presence of adventitious agent(s) in the test article pre-clarified bulk live virus vaccine and/or fluids, other than the specific virus in the product. The test article was inoculated into adult rabbits.

No evidence of contamination with adventitious agent(s) was observed due to the test article Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilized inoculation of adult rabbits. The test is performed as described in CFR Title 21, Section 630.16.

Adult rabbits are utilized in this assay to detect possible contamination of the test article with B-virus or other adventitious agent(s) including other Simian agents, adenovirus(es), etc. which might be present in the test article. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Rabbits

B. Study Number: ZA361.005101

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7 was received at Microbiological Associates, Inc. 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:

1. Positive Control: none
2. Negative Control: none
3. Vehicle Control: none

F. Test System: Rabbits, four females, SPF NZW, 1.5 - 2.5 kg.
Source: Buckshire Corp.
P.O. Box 155
Perkasie, PA 18944

G. Sponsor: Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department
   Microbiological Associates, Inc.
   Life Sciences Center
   9900 Blackwell Road
   Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
   5221 River Road
   Bethesda, Maryland 20816

I. Personnel:

1. Study Director: Mary D. Whiteman

2. Associate Study Director: Janet Luczak, M.T. (ASCP), M.G.A.

J. Schedule:

1. Study Initiation Date: 07/16/90

2. Lab Initiation Date: 08/03/90

3. Lab Completion Date: 08/31/90

4. Study Completion Date: See Study Director’s Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

A. -Objective:

The study objective is to detect inapparent adventitious agent(s) which might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.
B. Methods:

1. Test System Identification and Randomization

   Each rabbit was housed individually. Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Each rabbit was identified by a unique number tattooed on it's ear. The rabbit's number was recorded on the cage card.

   The rabbits were randomized according to SOP #OPBT0213.

2. Animal Inoculation with Test Article

   Animals were inoculated according to Table 1. The rabbits were observed for 28 days for clinical signs of illness or distress.

3. Animal Husbandry

   a. Rabbits were fed certified rabbit chow ad libitum and water was supplied via water bottles, ad libitum.
   b. Rabbit's cages were changed weekly.
   c. Animal facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

   All rabbits inoculated with the test article and the uninoculated control rabbit remained normal and healthy for the 28 day observation period.

   See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

   No evidence of contamination with adventitious agent(s) due to the test article, Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7, was observed.
VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice Regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director

Date
**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I.D. I.D.</td>
<td>1.0 ml 9.0 ml</td>
<td>Test</td>
<td>Observe Article for Illness</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>SAA</td>
<td>SAA SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>SAA</td>
<td>SAA SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>None</td>
<td>None None</td>
<td>None</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above  
I.D. = Intradermal Inoculation  
S.Q. = Subcutaneous Inoculation
TABLE 2

Survival Summary for Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7

<table>
<thead>
<tr>
<th>RABBITS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Article</td>
<td>3/3(^a)</td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>1/1(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Number of surviving, healthy animals after 28 days/Number of animals inoculated.

\(^b\) Number of surviving, healthy animals after 28 days/Number of animals on lab initiation date.
TABLE 3

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
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<tr>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>19</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Uninoculated Control 4</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
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</table>
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT RABBITS

Study Number: ZA361.005101

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/16/90 - 08/16/90, TO STUDY DIR 08/16/90, TO MGMT 08/21/90
PHASES: ANIMAL OBSERVATIONS

INSPECT ON 09/21/90 - 09/24/90, TO STUDY DIR 09/24/90, TO MGMT 09/28/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Ed Warburton
Quality Assurance Unit

Date 9-25-90

Microbiological Associates Inc.
APPENDIX C

ANIMAL SAFETY TEST IN
ADULT MICE AND SUCKLING MICE

Study NO.: ZA359.005100
Dengue-4 Prod Seed: PDK- 6, FRhL-2/d7 .......... pages 55 - 67

Study NO.: ZA360.005100
Dengue-4 Prod Seed: PDK-10, FRhL-2/d7 .......... pages 68 - 81

Study NO.: ZA361.005100
Dengue-4 Prod Seed: PDK-15, FRhL-2/d7 .......... pages 82 - 99
ANIMAL SAFETY TEST IN
ADULT MICE AND SUCKLING MICE

Study No.: ZA359.005100

Test Article: Dengue-4 (341750) Prod Seed, PDK-6, FRhL-2/d7

Final Report
For
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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<tr>
<td>VII. Quality Assurance Statement</td>
<td>67</td>
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</tbody>
</table>
SUMMARY

The purpose of this assay is to detect the presence of adventitious agent(s) in the test article, pre-clarified bulk live virus vaccines and/or fluids, other than the specific virus in the product. The test article was inoculated into adult mice and suckling mice. The suckling mouse portion of the assay included a subpassage of homogenized tissue after 14 days into a new group of suckling mice, followed by an additional 14 day observation period.

No evidence of contamination with adventitious agents due to the test article Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7 was observed.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent virus(es). The experimental design utilizes inoculations of adult and suckling mice. The test is performed as described in CFR Title 21, Section 630.35 (a)(1)(2).

Adult mice are included in the assay to detect possible contamination of the test article with neurotropic or other viruses such as lymphocytic choriomeningitis virus. Suckling mice are used to detect Coxsackie or other viruses. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Mice and Suckling Mice

B. Study Number: ZA359.005100

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Mice

Suckling litters (Primary Inoculation): Tac(SW)fBR, three adult females each with ten <24 hour old suckling pups,
   Source: Taconic Farms, Germantown, New York

Suckling litters (Blind Passage): Tac:(SW)fBR, four adult females each with ten <24 hour old suckling pups,
   Source: Taconic Farms, Germantown, New York
Adult - HSD:ICR, Fifteen males and fifteen females, Body Weight range: 15-20 grams.
Source: Harlan Sprague Dawley, Frederick, Maryland

G. Sponsor: Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

Authorized Representative: Dr. Louis Potash

H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:
1. Study Director: Mary D. Whiteman
2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:
1. Study Initiation Date: 07/16/90
2. Lab Initiation Date: 07/24/90
3. Lab Completion Date: 08/23/90
4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:
All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.
L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

With approval of the sponsor, a previously thawed test article sample which was frozen back and stored at \(-70^\circ C \pm 10^\circ C\) was utilized in the inoculation of the suckling mouse portion of the assay. In addition, at the request of the sponsor, in the suckling mouse portion of the assay, 1.3 ml of the test article was combined with 1.3 ml of sponsor supplied antisera Den-4 #814669 Carec, SM5 of 11-21-82 and heated at 37°C for 90 minutes, prior to inoculation of the suckling mice. The remaining untreated sample was again frozen back and was utilized along with a previously unthawed aliquot of the test article to inoculate the adult mouse portion of the assay.

A. Objective:

The study objective is to detect inapparent virus(es) that might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.

B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Adult mice were ear-tagged but housed in groups according to inoculum type and sex. Suckling mice were not individually identified.

Suckling and adult mice were randomized according to SOP #OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. The adult mice were observed every working day for 28 days for clinical signs. In the suckling mouse portion of the assay, the animals were inoculated according to Table 1 and were then observed every working day for 14 days for clinical signs. Fourteen days post-inoculation, all surviving
suckling mice from each group were euthanized using cervical dislocation. Following euthanasia their skin and gastrointestinal were removed, the carcasses cut into pieces and placed in a sterile pre-weighed bowl. After determining the weight of the entire group of mice from a cage, enough Hank's Balanced Salt Solution (HBSS) with gentamicin was added to make a 20% w/v suspension. The entire content of the bowl was then homogenized in a sterile blender, clarified by centrifugation, diluted 1:2 in HBSS and subsequently inoculated into a new group of suckling mice by the same routes and in the same volumes as the original group. These newly inoculated mice were observed for a period of fourteen days. Ten suckling mice were held as uninoculated controls and observed for fourteen days.

3. Animal Husbandry

a. All animals were fed the following diet ad libitum:

   Mice - autoclavable chow.

b. Water was supplied ad libitum via fresh apples.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All adult mice inoculated with the test article and all uninoculated control adult mice remained normal and healthy for the twenty-eight day observation period.

All uninoculated control suckling mice and test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. The surviving mice of each group were homogenized and the homogenate of each group was passaged into a new group of suckling mice. The remainder of the homogenates was frozen at -70°C ± 10°C.
In the blind passage all of the uninoculated control suckling mice, all of the suckling mice inoculated with the homogenate of the uninoculated control suckling mice, and nineteen of the twenty suckling mice inoculated with the homogenate of the test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. One of the test article homogenate inoculated suckling mice was missing and presumed cannibalized day four post-inoculation.

See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

No evidence of contamination with adventitious agents was observed due to the test article, Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice Regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director

Date 10/23/90
<table>
<thead>
<tr>
<th>Group #</th>
<th>Number of Animals</th>
<th>Sex</th>
<th>Species</th>
<th>Route(s) of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM 1</td>
<td>1</td>
<td>female</td>
<td>mouse</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(lactating)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10</td>
<td>various</td>
<td>i.p.</td>
<td>0.1 ml</td>
<td>test article</td>
<td>Observed for illness after 14 days passage a single pool of emulsified tissue (minus skin and gastrointestinal) of all surviving mice onto at least 10 additional suckling mice. Use same routes and volumes as original.</td>
</tr>
<tr>
<td>SM 2</td>
<td>SAA*</td>
<td>SA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
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<tr>
<td>SM 3</td>
<td>SAA</td>
<td>SA</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AM 1</td>
<td>5</td>
<td>male</td>
<td>mouse</td>
<td>i.p.</td>
<td>0.5 ml</td>
<td>test article</td>
<td>Observe for illness</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>i.c.</td>
<td>0.03 ml</td>
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<td></td>
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<td>5</td>
<td>male</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>uninoc control</td>
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<tr>
<td>AM 6</td>
<td>5</td>
<td>female</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same as above
i.c. = Intracranial
i.p. = Intraperitoneal
### TABLE 2

Survival Summary for Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7

<table>
<thead>
<tr>
<th>Suckling Mice&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Adult Mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primary Inoculation</th>
<th>Blind Passage</th>
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</thead>
<tbody>
<tr>
<td>Test Article</td>
<td>20/20</td>
<td>20/20</td>
<td>19/20</td>
</tr>
<tr>
<td>Uninoculated Control</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Uninoculated Control&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of surviving, healthy animals after 28 days/Number of animals inoculated.

<sup>b</sup> In the suckling mice portion of the assay, animals are inoculated and observed for 14 days. On day 14 post-inoculation a homogenate is prepared from the surviving sucklings from each group. This homogenate was used to inoculate another group of suckling mice which were observed for an additional 14 days. The number of surviving sucklings/number of animals inoculated is presented.

<sup>c</sup> In the blind passage of the suckling mouse portion of the assay an uninoculated control group was held with the blind passage animals inoculated with the homogenate of the test article and the homogenate of the uninoculated control group from the primary inoculation.
### TABLE 3

Summary of Daily Observations for Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Sacrifice (Post-inoc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Mice</td>
<td>Test</td>
<td>10601</td>
<td>Normal</td>
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<tr>
<td></td>
<td>Article</td>
<td>10602</td>
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### TABLE 3 (Cont.)

**Summary of Daily Observations**  
for Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7

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<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Number/Cage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-Inoc.)</th>
<th>Day of Sacrifice (Post-Inoc.)</th>
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<td>SM1 (10)</td>
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<td>Uninoculated Control</td>
<td>SM3 (10)</td>
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<sup>a</sup> Ten suckling mice inoculated per cage.

<sup>b</sup> Surviving suckling mice from primary inoculation were sacrificed on day 14 for preparation of blind passage tissue homogenate.

<sup>c</sup> One suckling mouse was missing and presumed cannibalized.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study Number: ZA359.005100

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/23/90
PHASES: ANIMAL OBSERVATIONS

INSPECT ON 10/17/90 - 10/17/90, TO STUDY DIR 10/17/90, TO MGMT 10/25/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Dana H. Hamlen
Quality Assurance Unit

Date 10-25-90

Microbiological Associates Inc.
ANIMAL SAFETY TEST IN 
ADULT MICE AND SUCKLING MICE

Study No.: ZA360.005100
Test Article: Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7

Final Report
For
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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<td>VII. Quality Assurance Statement</td>
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SUMMARY

The purpose of this assay is to attempt to detect the presence of adventitious agent(s) in the test article, pre-clarified bulk live virus vaccines and/or fluids, other than the specific virus in the product. The test article was inoculated into adult mice and suckling mice. The suckling mouse portion of the assay included a subpassage of homogenized tissue after 14 days into a new group of suckling mice, followed by an additional 14 day observation period.

No evidence of contamination with adventitious agents due to the test article Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7 was observed in the adult mouse portion of the assay. The results of the suckling mouse portion of the assay were inconclusive due to the lethal effect of the specific virus in the product, on the suckling mice.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent virus(es). The experimental design utilizes inoculations of adult and suckling mice. The test is performed as described in CFR Title 21, Section 630.35 (a)(1)(2).

Adult mice are included in the assay to detect possible contamination of the test article with neurotropic or other viruses such as lymphocytic choriomeningitis virus. Suckling mice are used to detect Coxsackie or other viruses. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Mice and Suckling Mice

B. Study Number: ZA360.005100

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Mice

   Suckling litters (Primary Inoculation): Tac:(SW)fBR, three adult females each with ten <24 hour old suckling pups,
   Source: Taconic Farms, Germantown, New York

   Suckling litters (Blind Passage): Tac:(SW)fBR, four adult females each with ten <24 hour old suckling pups,
   Source: Taconic Farms, Germantown, New York
Adult - HSD:ICR, Fifteen males and fifteen females, Body Weight range: 15-20 grams. 
Source: Harlan Sprague Dawley, Frederick, Maryland

G. Sponsor: Program Resources, Inc. 
Biomedical Services Division 
7655 Old Springhouse Road 
McLean, VA 22102

Authorized Representative: Dr. Louis Potash

H. Testing Facility: Biotechnology Services Department 
Microbiological Associates, Inc. 
Life Sciences Center 
9900 Blackwell Road 
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc. 
5221 River Road 
Bethesda, Maryland 20816

I. Personnel:

1. Study Director: Mary D. Whiteman

2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:

1. Study Initiation Date: 07/16/90

2. Lab Initiation Date: 07/24/90

3. Lab Completion Date: 08/23/90

4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.
L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

With approval of the sponsor, a previously thawed test article sample which was frozen back and stored at -70°C ± 10°C was utilized in the inoculation of the suckling mouse portion of the assay. In addition, at the request of the sponsor, in the suckling mouse portion of the assay, 1.3 ml of the test article was combined with 1.3 ml of sponsor supplied antisera Den-4 #814669 Carec, SM5 of 11-21-82 and heated at 37°C for 90 minutes, prior to inoculation of the suckling mice. The remaining untreated sample was again frozen back and was utilized along with a previously unthawed aliquot of the test article to inoculate the adult mouse portion of the assay.

A. Objective:

The study objective is to detect inapparent virus(es) that might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.

B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Adult mice were ear-tagged but housed in groups according to inoculum type and sex. Suckling mice were not individually identified.

Suckling and adult mice were randomized according to SOP #OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. The adult mice were observed every working day for 28 days for clinical signs. In the suckling mouse portion of the assay, the animals were inoculated according to Table 1 and were then observed every working day for 14 days for clinical signs.
Fourteen days post-inoculation, all surviving suckling mice from each group were euthanized using cervical dislocation. Following euthanasia their skin and gastrointestinal were removed, the carcasses cut into pieces and placed in a sterile pre-weighed bowl. After determining the weight of the entire group of mice from a cage, enough Hank's Balanced Salt Solution (HBSS) with gentamicin was added to make a 20% w/v suspension. The entire content of the bowl was then homogenized in a sterile blender, clarified by centrifugation, diluted 1:2 in HBSS and subsequently inoculated into a new group of suckling mice by the same routes and in the same volumes as the original group. These newly inoculated mice were observed for a period of fourteen days. Ten suckling mice were held as uninoculated controls and observed for fourteen days.

3. Animal Husbandry

a. All animals were fed the following diet ad libitum:

   Mice - autoclavable chow.

b. Water was supplied ad libitum via fresh apples.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All adult mice inoculated with the test article and all uninoculated control adult mice remained normal and healthy for the twenty-eight day observation period.

All of the uninoculated control suckling mice and nineteen of the twenty test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. One of the test article inoculated suckling mice appeared lethargic and had a swollen head day 14 post-inoculation, prior to sacrifice of all surviving mice of
each group. The surviving mice were homogenized and the homogenate of each group was passaged into a new group of suckling mice. The remainder of the homogenates was frozen at \(-70^\circ\text{C} \pm 10^\circ\text{C}\).

In the blind passage, all of the uninoculated control suckling mice, all of the suckling mice inoculated with the homogenate of the uninoculated control suckling mice and seven of the twenty suckling mice inoculated with the homogenate of the test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. Two of the suckling mice inoculated with the homogenate of the test article inoculated suckling mice were found partially cannibalized; one on day 9 post-inoculation and one on day 10 post-inoculation. Four additional suckling mice in the test article homogenate group were moribund day 10 post-inoculation and were sacrificed. Necropsy was performed. Four of the test article homogenate inoculated suckling mice were found dead day 12 post-inoculation. One of the test article homogenate inoculated suckling mice appeared lethargic day 13 post-inoculation and was found partially cannibalized day 14 post-inoculation. Of the remaining suckling mice, two appeared lethargic on day 14 post-inoculation. It was requested by the sponsor that no histopathologic examination be performed on the surviving, dead, or moribund suckling mice. The death of the suckling mice was most likely caused by the specific virus in the product, and was not an unexpected response to the intracranial inoculation of suckling mice with this product.

See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article, Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7, was observed in the adult mouse portion of the assay. The results of the suckling mouse portion of the assay were inconclusive due to the lethal effect of the specific virus in the product, on the sucking mice.
VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice Regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman    10/23/90  
Study Director
<table>
<thead>
<tr>
<th>Group #</th>
<th>Number of Animals</th>
<th>Sex</th>
<th>Species</th>
<th>Route(s) of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
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<tbody>
<tr>
<td>SM 1</td>
<td>1</td>
<td>female mouse (lactating)</td>
<td>None</td>
<td>None</td>
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<td>None</td>
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<tr>
<td></td>
<td>+ 10</td>
<td>various mouse (suckling)</td>
<td>i.p.</td>
<td>0.1 ml</td>
<td>test article</td>
<td>Observed for illness after 14 days passage a single pool of emulsified tissue (minus skin and gastrointestinal) of all surviving mice onto at least 10 additional suckling mice. Use same routes and volumes as original.</td>
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<tr>
<td>SM 2</td>
<td>SAA*</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
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<tr>
<td>AM 1</td>
<td>5 male mouse</td>
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<td>0.5 ml</td>
<td>test article</td>
<td>Observe for illness</td>
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<td>AM 2</td>
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SAA = Same as above
i.c. = Intracranial
i.p. = Intraperitoneal
**TABLE 2**

**Survival Summary**
for Dengue-4 (§341750) Prod Seed, PDK-10, FRhL-2/d7

<table>
<thead>
<tr>
<th>Test Article</th>
<th>Adult Mice(^a)</th>
<th>Primary Inoculation</th>
<th>Blind Passage</th>
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<tr>
<td>Test Article</td>
<td>20/20</td>
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<td>9/20</td>
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<td>Uninoculated</td>
<td>10/10</td>
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<tr>
<td>Uninoculated</td>
<td>10/10</td>
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</tbody>
</table>

\(^{a}\) Number of surviving, animals after 28 days/Number of animals inoculated.

\(^{b}\) In the suckling mice portion of the assay, animals are inoculated and observed for 14 days. On day 14 post-inoculation a homogenate is prepared from the surviving sucklings from each group. This homogenate was used to inoculate another group of suckling mice which were observed for an additional 14 days. The number of surviving sucklings/number of animals inoculated is presented.

\(^{c}\) In the blind passage of the suckling mouse portion of the assay an uninoculated control group was held with the blind passage animals inoculated with the homogenate of the test article and the homogenate of the uninoculated control group from the primary inoculation.
<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Sacrifice (Post-inoc.)</th>
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TABLE 3 (Cont.)
Summary of Daily Observations
for Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number/ Cagea</th>
<th>Clinical Signs</th>
<th>Day of Signs Onset</th>
<th>Day of Sacrifice/ (Post-Inoc.)</th>
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<tbody>
<tr>
<td>Sucklingb Mice (Primary Inoculation)</td>
<td>Test Article</td>
<td>SM1 (10)</td>
<td>Normal</td>
<td>14</td>
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<tr>
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<td>Uninoculated Control</td>
<td>SM3 (10)</td>
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<td>(Blind Passage)</td>
<td>Test Article</td>
<td>SM1 (10)</td>
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<td></td>
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<td>(9)c</td>
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<td>(0)e</td>
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<td>(9)h</td>
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<td>Homo- genate</td>
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</table>

- a Ten suckling mice inoculated per cage.
- b Surviving suckling mice from primary inoculation were sacrificed on day 14 for preparation of blind passage tissue homogenate.
- c One suckling mouse was found partially cannibalized.
- d Four suckling mice appeared moribund and were sacrificed. Necropsy was performed.
- e Four suckling mice were found dead.
- f One suckling mouse appeared runted and hunched day 13 post-inoculation and was found partially cannibalized day 14.
- g One suckling mouse was lethargic and appeared to have a swollen head.
- h Two suckling mice were lethargic and had hunched postures.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study Number: ZA360.005100

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/23/90, TO MGMT 08/23/90
PHASES: ANIMAL OBSERVATIONS

INSPECT ON 10/17/90 - 10/17/90, TO STUDY DIR 10/17/90, TO MGMT 10/25/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Dana H. Hamblen
Quality Assurance Unit

Date

10-25-90

Microbiological Associates Inc.
ANIMAL SAFETY TEST IN
ADULT MICE AND SUCKLING MICE

Study No.: ZA361.005100

Test Article: Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7

Final Report
For

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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SUMMARY

The purpose of this assay is to attempt to detect the presence of adventitious agent(s) in the test article, pre-clarified bulk live virus vaccines and/or fluids, other than the specific virus in the product. The test article was inoculated into adult mice and suckling mice. The suckling mouse portion of the assay included a subpassage of homogenized tissue after 14 days into a new group of suckling mice, followed by an additional 14 day observation period.

No evidence of contamination with adventitious agents due to the test article Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7 was observed in the adult mouse portion of the assay. The results of the suckling mouse portion of the assay were inconclusive, due to the lethal effect of the specific virus in the product on the suckling mice.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilizes inoculations of adult and suckling mice. The test is performed as described in CFR Title 21, Section 630.35 (a)(1)(2).

Adult mice are included in the assay to detect possible contamination of the test article with neurotropic or other viruses such as lymphocytic choriomeningitis virus. Suckling mice are used to detect Coxsackie or other viruses. All animals are observed for signs of illness and any that become sick or show any abnormalities are examined in an attempt to establish cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Mice and Suckling Mice

B. Study Number: ZA361.005100

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-15, FRhl-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Mice

Suckling litters (Primary Inoculation): Tac: (SW)fBR, three adult females each with at least eight <24 hour old suckling pups,
/Source: Taconic Farms, Germantown, New York
Suckling litters (Blind Passage): Tac(SW)fBR, six adult females each with ten <24 hour old suckling pups,
Source: Taconic Farms, Germantown, New York

Adult - HSD:ICR, Fifteen males and fifteen females,
Body Weight range: 15-20 grams.
Source: Harlan Sprague Dawley, Frederick, Maryland

G. Sponsor: Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

Authorized Representative: Dr. Louis Potash

H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:
1. Study Director: Mary D. Whiteman

2. Associate
Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:
1. Study Initiation Date: 07/16/90
2. Lab Initiation Date: 07/24/90
3. Lab Completion Date: 08/28/90
4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:
All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of
samples of the test article is the responsibility of the sponsor.

L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

With approval of the sponsor, a previously thawed test article sample which was frozen back and stored at \(-70^\circ\text{C} \pm 10^\circ\text{C}\) was utilized in the inoculation of the suckling mouse portion of the assay. In addition, the sponsor requested that in the suckling mouse portion of the assay 1.3 ml of the test article be combined with 1.3 ml of the sponsor supplied antisera Den-4 #814669 Carec, SM5 of 11-21-82 and heated at \(37^\circ\text{C}\) for 90 minutes prior to inoculation of the suckling mice. This procedure was followed except that due to an insufficient volume of antisera, 1.2 ml of antisera was combined with 1.2 ml of the test article. This procedural change was approved by the sponsor. The remaining untreated sample was frozen back and was utilized along with a previously unthawed aliquot of the test article to inoculate the adult mouse portion of the assay.

A. Objective:

The study objective is to detect inapparent virus(es) that might be present in the test article, pre-clarified bulk live virus and/or fluids, other than the specific virus in the product.

B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Adult mice were ear-tagged but housed in groups according to inoculum type and sex. Suckling mice were not individually identified.

Suckling and adult mice were randomized according to SOP #OPBT0213.
2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. The adult mice were observed every working day for 28 days for clinical signs. In the suckling mouse portion of the assay, the animals were inoculated according to Table 1 and were then observed every working day for 14 days for clinical signs.

Note: After preparation of a 1:1 dilution of the remaining antisera Den-4 #814669 Carec, SM5 of 11-21-82 and the test article, the resulting volume was not sufficient to inoculate 10 suckling mice per group. After consultation with the sponsor, and with the sponsors approval, eight suckling mice were inoculated per group.

Fourteen days post-inoculation, all surviving suckling mice from each group were euthanized using cervical dislocation. Following euthanasia their skin and gastrointestinal were removed, the carcasses cut into pieces and placed in a sterile pre-weighed bowl. After determining the weight of the entire group of mice from a cage, enough Hank's Balanced Salt Solution (HBSS) with gentamycin was added to make a 20% w/v suspension. The entire content of the bowl was then homogenized in a sterile blender, clarified by centrifugation, diluted 1:2 in HBSS and subsequently inoculated into a new group of suckling mice by the same routes and in the same volumes as the original group. These newly inoculated mice were observed for a period of fourteen days. Ten suckling mice were held as uninoculated controls and observed for fourteen days.

3. Animal Husbandry

a. All animals were fed the following diet ad libitum:

Mice - autoclavable chow.

b. Water was supplied ad libitum via fresh apples.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.
d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

IV. RESULTS

All adult mice inoculated with the test article and all uninoculated control adult mice remained normal and healthy for the twenty-eight day observation period.

All of the uninoculated control suckling mice and six of the sixteen test article inoculated suckling mice appeared normal and healthy for the 14 day observation period. One of the test article inoculated suckling mice was missing and presumed cannibalized day one post-inoculation. On day 13 post-inoculation, five of the test article inoculated suckling mice appeared moribund and were sacrificed and homogenized for passage into new groups of suckling mice. Four of the test article inoculated suckling mice were found dead; three on day 13 and one on day 14 post-inoculation. Necropsy and histopathology were performed on the dead suckling mice. The cause of death of these animal could not be determined due to the degree of autolysis in the tissues examined. (See Pathology Report in Appendix.)

In the blind passage, all of the uninoculated control suckling mice and all of the suckling mice inoculated with the homogenate of the uninoculated control suckling mice appeared normal and healthy for the 14 day observation period. One of the test article homogenate inoculated suckling mice was missing and presumed cannibalized day 9 post-inoculation. By day 10 post-inoculation, all but one of the remaining suckling mice appeared lethargic and hunched and were staggering. On day 11 post-inoculation, one test article homogenate inoculated suckling mouse was found dead. Eight test article homogenate inoculated suckling mice were found dead day 12 post-inoculation. Two of the dead suckling mice were partially cannibalized. On day 13 post-inoculation, of the ten remaining test article homogenate inoculated suckling mice, five were found dead, one was found partially cannibalized and four were moribund. Two of the moribund suckling mice inoculated with the test article homogenate, were sacrificed. Necropsy was performed. On day 14 post-inoculation, the remaining two suckling mice were found dead.
Of the twenty suckling mice inoculated with the homogenates of the test article inoculated mice which were moribund day 13 post-inoculation, eight appeared normal and healthy for the 14 day observation period. Two of the suckling mice inoculated with the homogenate of the test article moribund sucklings were missing and presumed cannibalized day 1 post-inoculation and one was missing and presumed cannibalized day 4 post-inoculation. Of the seventeen remaining suckling mice inoculated with the homogenate of the moribund test article suckling mice, on day 13 post-inoculation, two were found partially cannibalized, four were moribund and one was runted and lethargic. One of the four moribund suckling mice and the runted suckling mouse were sacrificed. Necropsy was performed. On day 14 post-inoculation, three suckling mice were found dead and two were moribund.

It was requested by the sponsor that no histopathologic examination be performed on the dead, sacrificed or moribund suckling mice. The death of the suckling mice was most likely caused by the specific virus in the product and was not an unexpected response to the intracranial inoculation of suckling mice with this product.

See Tables 2 and 3 for a summary of the data.

V. CONCLUSIONS

No evidence of contamination with adventitious agents due to the test article, Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7, was observed in the adult mouse portion of the assay. The results of the suckling mouse portion of the assay were inconclusive due to the lethal effect of the specific virus in the product on the suckling mice.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice Regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director

10/34/50
<table>
<thead>
<tr>
<th>Group #</th>
<th>Number of Animals</th>
<th>Sex</th>
<th>Species</th>
<th>Route(s) of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
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<tbody>
<tr>
<td>SM 1</td>
<td>1</td>
<td>female</td>
<td>mouse (lactating)</td>
<td>None</td>
<td>None</td>
<td>None</td>
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<tr>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td>i.p.</td>
<td>0.1 ml test article</td>
<td>Observed for illness after 14 days passage a single pool of emulsified tissue (minus skin and gastrointestinal) of all surviving mice onto at least 10 additional suckling mice. Use same routes and volumes as original.</td>
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<tr>
<td></td>
<td>10</td>
<td>various</td>
<td>Mouse (suckling)</td>
<td>i.c.</td>
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<td>SM 3</td>
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<td>SAA</td>
<td>SAA</td>
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<td>None</td>
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<td>5</td>
<td>female</td>
<td>SAA</td>
<td>None</td>
<td>None</td>
<td>SAA</td>
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SAA = Same as above  
i.c. = Intracranial  
i.p. = Intraperitoneal

MICROBIOLOGICAL ASSOCIATES, INC.
**TABLE 2**

Survival Summary for Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7

<table>
<thead>
<tr>
<th>Suckling Mice&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Adult Mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primary Inoculation</th>
<th>Blind Passage</th>
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<td>20/20</td>
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<td>Uninoculated Control</td>
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<td>10/10</td>
<td>10/10</td>
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<tr>
<td>Test Article Homogenate of Moribund Sucklings</td>
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<sup>a</sup> Number of surviving animals after 28 days/Number of animals inoculated.

<sup>b</sup> In the suckling mice portion of the assay, animals are inoculated and observed for 14 days. On day 14 post-inoculation a homogenate is prepared from the surviving sucklings from each group. This homogenate was used to inoculate another group of suckling mice which were observed for an additional 14 days. The number of surviving sucklings/number of animals inoculated is presented.

<sup>c</sup> In the blind passage of the suckling mouse portion of the assay an uninoculated control group was held with the blind passage animals inoculated with the homogenate of the test article and the homogenate of the uninoculated control group from the primary inoculation.
## TABLE 3


<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Sacrifice (Post-inoc.)</th>
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### TABLE 3 (Cont.)

**Summary of Daily Observations for Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7**

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Number/ Cage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-Inoc.)</th>
<th>Day of Sacrifice (Post-Inoc.)</th>
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</thead>
<tbody>
<tr>
<td>Suckling Mice</td>
<td>Test</td>
<td>SM1 (8)</td>
<td>Normal</td>
<td>13</td>
<td>13</td>
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<tr>
<td>(Primary Article)</td>
<td>(6)&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>13</td>
<td>13</td>
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<tr>
<td>Inoculation)</td>
<td></td>
<td>(3)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>SM2 (8)</td>
<td>Normal</td>
<td>(7)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>1</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>(6)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13</td>
<td>13</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(4)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

Uninoculated Control SM3 (10) Normal

---

<sup>a</sup> Eight to ten suckling mice inoculated per cage.

<sup>b</sup> Surviving suckling mice from primary inoculation were sacrificed on day 14 for preparation of blind passage tissue homogenate.

<sup>c</sup> One suckling mouse missing and presumed cannibalized.

<sup>d</sup> One suckling mouse found dead and sent for necropsy and histopathology.

<sup>e</sup> Two moribund suckling mice were homogenized for passage.

<sup>f</sup> Two suckling mice found dead and sent for necropsy and histopathology.

<sup>g</sup> Three moribund suckling mice were homogenized for passage.
TABLE 3 (Cont.)
Summary of Daily Observations
for Denque-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Number/ Cage</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-Inoc.)</th>
<th>Day of Death/ Sacrifice (Post-Inoc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Blind Passage)</td>
<td>Test</td>
<td>SM1 (10)</td>
<td>Normal</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Article</td>
<td>(10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homo- genate</td>
<td>(9)</td>
<td>(10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0)</td>
<td>(9)</td>
<td>(10)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SM2</td>
<td>(10)</td>
<td>Normal</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>(9)</td>
<td>(10)</td>
<td>Normal</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>(6)</td>
<td>(2)</td>
<td>(10)</td>
<td>13</td>
<td>13</td>
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<tr>
<td>(0)</td>
<td>(0)</td>
<td>(10)</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>Control</td>
<td>Homo- genate</td>
<td>SM3 (10)</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>Control</td>
<td>SM4 (10)</td>
<td>Normal</td>
<td></td>
<td></td>
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<tr>
<td>Test</td>
<td>SM5</td>
<td>(10)</td>
<td>Normal</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Article</td>
<td>(8)</td>
<td>(10)</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>(5)</td>
<td>(10)</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Moribund SM6</td>
<td>(10)</td>
<td>Normal</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mice</td>
<td>(9)</td>
<td>(10)</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

h All ten sucklings were lethargic, hunched and were staggering.

i One suckling mouse was found dead.

j Five suckling mice were found dead.

k Three suckling mice were found dead; remaining suckling was moribund and was sacrificed and sent for necropsy.

l Eight suckling mice were lethargic, hunched, and were staggering.

m Three suckling mice were found dead; of the three, two were partially cannibalized.

n Two suckling mice were found dead and one was found partially cannibalized; three were moribund and one of the three moribund mice was sacrificed and sent for necropsy.

o Two suckling mice were found dead.

p Two suckling mice were missing and presumed cannibalized.

q Two suckling mice found partially cannibalized; four appeared moribund; one appeared lethargic and runted; one of the four moribund sucklings was sacrificed and sent for necropsy.

r Three suckling mice were found dead; remaining two sucklings appeared moribund.

s One suckling mouse appeared lethargic and slightly runted, and was sacrificed and sent for necropsy.
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT MICE AND SUCKLING MICE

Study Number: ZA361.005100

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/19/90 - 07/19/90, TO STUDY DIR 07/19/90, TO MGMT 07/19/90
PHASES: PROTOCOL REVIEW

INSPECT ON 07/24/90 - 07/24/90, TO STUDY DIR 07/24/90, TO MGMT 07/24/90
PHASES: ADMINISTRATION OF THE TEST ARTICLE TO THE TEST SYSTEM

INSPECT ON 10/17/90 - 10/17/90, TO STUDY DIR 10/17/90, TO MGMT 10/25/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

[Signature]
Dana H. Hamblén
Quality Assurance Unit

[Signature]
Date

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Microbiological Associates Inc.
IX. APPENDIX
PATHOLOGY REPORT

CAHS 2187  DATE RECEIVED:  8/6/90

SOURCE: Biotech Services  DATE NECROPSIED:  8/7/90
ZA361.005100

SPECIES: Mouse  DATE REPORTED:  8/28/90

RESULTS:

Organs examined microscopically: Nose, eyes, ears, trachea, brain, heart, kidney, lung, liver, spleen, ileum, cecum, colon, gross lesions.

2187-1  Suckling  Test Article  Cage 6  5g.

Gross: Animal received dead.
Abdominal organs partially autolyzed.
Brain tissue soft.
Red color over top of nasal cavity.

Microscopic: All tissues examined -- Autolysis

2187-2  Suckling  Test Article  Cage 6  4g.

Gross: Animal received dead.
Abdominal organs partially autolyzed.
Brain tissue soft and dark red in color.

Microscopic: All tissues examined -- Autolysis

2187-3  Suckling  Test Article  Cage 7  5g.

Gross: Animal received dead.
Organs partially autolyzed.
Brain tissue soft and darkly colored red.

Microscopic: All tissues examined -- Autolysis
## PATHOLOGY REPORT

<table>
<thead>
<tr>
<th>CAHS 2187</th>
<th>DATE RECEIVED:</th>
<th>8/7/90</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOURCE: Biotech Services ZA361.005100</td>
<td>DATE NECROPSIED:</td>
<td>8/8/90</td>
</tr>
<tr>
<td>SPECIES: Mouse</td>
<td>DATE REPORTED:</td>
<td>8/28/90</td>
</tr>
</tbody>
</table>

### RESULTS:

<table>
<thead>
<tr>
<th>2187-4</th>
<th>Suckling Test Article Cage 6 5g.</th>
</tr>
</thead>
</table>

**Gross:**
- Animal received dead.
- Abdominal tissues darkly colored.
- All tissues partially autolyzed.
- Brain tissue soft.

**Microscopic:** All tissues examined -- Autolysis

**COMMENT:** The cause of death cannot be determined due to the marked autolysis in the examined tissues.

Lynda L. Pippin, DVM

Lynda L. Pippin, DVM
APPENDIX D

ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study NO.: ZA359.005102
Dengue-4 Prod Seed: PDK- 6, FRhL-2/d7 ........ pages 101 - 114

Study NO.: ZA360.005102
Dengue-4 Prod Seed: PDK-10, FRhL-2/d7 ........ pages 115 - 128

Study NO.: ZA361.005102
Dengue-4 Prod Seed: PDK-15, FRhL-2/d7 ........ pages 129 - 142
ANIMAL SAFETY TEST IN
ADULT GUINEA PIGS

Study No.: ZA359.005102
Test Article: Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7

Final Report

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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<td>VIII. Appendix</td>
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</table>
SUMMARY

The purpose of this test is to attempt to detect the presence of adventitious agent(s) in the test article, preclarified bulk live virus and/or fluids, other than the specific virus in the product, by the inoculation and observation of guinea pigs.

No evidence of contamination with adventitious agents was observed due to the test article Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agent(s). The experimental design utilizes the inoculation of adult guinea pigs. The test is performed as described in CFR Title 21, Section 630.16(a)(4).

Adult guinea pigs are used in this assay to detect possible contamination of the test article with M. tuberculosis or other adventitious agent(s). Animals are examined for signs of illness, and any that become sick or show any abnormalities are examined in an attempt to determine the cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Guinea Pigs

B. Study Number: ZA359.005102

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-6, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Guinea Pigs
   Hartley Albino
   6 adult females,
   Body weight range: 350-400 g
   Source: Hazleton Research Animals
           Denver, Pennsylvania

G. Sponsor: Program Resources, Inc.
           Biomedical Services Division
           7655 Old Springhouse Road
           McLean, VA 22102

Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

I. Personnel:

1. Study Director: Mary D. Whiteman

2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:

1. Study Initiation Date: 07/11/90

2. Lab Initiation Date: 07/12/90

3. Lab Completion Date: 09/06/90

4. Study Completion Date: See Study Director’s Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

A. Objective:

The study objective is to detect adventitious agents that might be present in the test article.
B. Methods:

1. Test System Identification and Randomization

   Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Animals were housed separately and were identified by ear tags.

   The guinea pigs were randomized according to SOP OPBT0213.

2. Animal Inoculation with Test Article

   Animals were inoculated according to Table 1. Three were held as uninoculated controls. All animals were observed every working day of the 42 day test period for death or clinical signs of illness or distress. Beginning day 21 post inoculation, rectal temperatures were recorded through day 42 post-inoculation. All remaining guinea pigs were sacrificed. Gross pathological examinations were performed on all guinea pigs.

3. Animal Husbandry

   a. Animals were fed Ralston Purina Certified Guinea Pig Chow.

   b. Water was supplied ad libitum via water bottles. Water was disinfected with 7 ppm chlorine.

   c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

   d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.
<table>
<thead>
<tr>
<th>Group #</th>
<th>Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>I.C. 0.1 ml</td>
<td>Test Article</td>
<td></td>
<td>Observe for Illness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I.P. 5.0 ml</td>
<td></td>
<td></td>
<td>Record Rectal Temp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>On Days 21-42 Post-Inoculation</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
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<td>SAA</td>
<td>SAA</td>
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<td>4</td>
<td>1</td>
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<td>None</td>
<td>Uninoc</td>
<td>SAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control</td>
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<td>1</td>
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<td>SAA</td>
<td>SAA</td>
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<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above
I.C. = Intracranial
I.P. = Intraperitoneal
IV. RESULTS

All of the uninoculated control guinea pigs and all of the test article inoculated guinea pigs appeared normal and healthy throughout the 42 day observation period. See Table 2 for a summary of the data.

None of the uninoculated control guinea pigs and none of the test article inoculated guinea pigs had significant temperature rises indicative of either viral or bacterial infections during the 21 day recording period from day 21 through day 42 post-inoculations. See Table 2 for a summary of the data.

At examination on day 42 for gross pathology, no lesions were found in the uninoculated control or test article guinea pigs. (See Pathology Report in Appendix.)

V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article Dengue-4 (#341750) Prod Seed, PdK-6, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. White 9/13/90
Study Director
TABLE 2

Summary of Daily Observations for Dengue-4 (#341750) Prod Seed, PDK-6, PRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs (Post-inoc.)</th>
<th>Day of Onset</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
<th>Range of Body Temp °C D-21 to D-42</th>
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<tr>
<td>Guinea Test</td>
<td>10421</td>
<td>Normal</td>
<td></td>
<td></td>
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<td>37.2 - 38.7</td>
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<tr>
<td>Pig Article</td>
<td>10422</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td>38.1 - 38.6</td>
</tr>
<tr>
<td></td>
<td>10423</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td>38.1 - 38.6</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>10424</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td>38.1 - 38.6</td>
</tr>
<tr>
<td>Control</td>
<td>10425</td>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td>37.5 - 38.7</td>
</tr>
<tr>
<td></td>
<td>10426</td>
<td>Normal</td>
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<td>38.1 - 38.5</td>
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QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study Number: ZA359.005102

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/16/90 - 07/16/90, TO STUDY DIR 07/16/90, TO MGMT 07/16/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/14/90 - 08/14/90, TO STUDY DIR 08/14/90, TO MGMT 08/21/90
PHASES: RECTAL TEMPERATURE DETERMINATION

INSPECT ON 09/12/90 - 09/12/90, TO STUDY DIR 09/12/90, TO MGMT 09/14/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan
Quality Assurance Unit

Date 9/4/90
VIII. APPENDIX
PATHOLOGY REPORT

CAHS-2190

DATE RECEIVED: 08/23/90

SOURCE: Biotecn Services

DATE NECROPSIED: 08/23/90

ZA356.005102 ZA357.005102

ZA358.005102 ZA359.005102

DATE REPORTED: 09/06/90

ZA360.005102 ZA361.005102

SPECIES: Guinea Pig

Results of gross examination according to SOP #865.201.

ZA356.005102

2190-1 (10401) Test Article 563.9 g
Gross: No lesions found

2190-2 (10402) Test Article 684.2 g
Gross: No lesions found

2190-3 (10403) Test Article 614.8 g
Gross: No lesions found

2190-4 (10404) Control 614.4 g
Gross: No lesions found

2190-5 (10405) Control 673.3 g
Gross: No lesions found

2190-6 (10406) Control 646.2 g
Gross: No lesions found

ZA357.005102

2190-7 (10407) Test Article 629.0 g
Gross: No lesions found

2190-8 (10408) Test Article 583.6 g
Gross: No lesions found

2190-9 (10409) Test Article 528.1 g
Gross: No lesions found
ZA358.005102

2190-10 (10410) Test Article 636.3 g
Gross: No lesions found

2190-11 (10411) Test Article 490.5 g
Gross: Retroperitoneal abscess (4x6 cm) and peritonitis

2190-12 (10412) Test Article 595.5 g
Gross: No lesions found

ZA359.005102

2190-13 (10421) Test Article 605.3 g
Gross: No lesions found

2190-14 (10422) Test Article 631.8 g
Gross: No lesions found

2190-15 (10423) Test Article 547.4 g
Gross: No lesions found

2190-16 (10424) Control 651.5 g
Gross: No lesions found

2190-17 (10425) Control 623.6 g
Gross: No lesions found

2190-18 (10426) Control 615.9 g
Gross: No lesions found

ZA360.005102

2190-19 (10427) Test Article 561.8 g
Gross: No lesions found

2190-20 (10428) Test Article 594.6 g
Gross: No lesions found

2190-21 (10429) Test Article 598.4 g
Gross: No lesions found
Gross: No lesions found

2190-22 (10430)  Test Article  559.8 g
Gross: No lesions found

2190-23 (10431)  Test Article  547.9 g
Gross: No lesions found

2190-24 (10432)  Test Article  624.8 g
Gross: No lesions found

COMMENT: The retroperitoneal abscess in guinea pig #2190-11 is believed to be due to a rectal perforation (no longer visible) caused by insertion of a temperature measuring probe. This is a common lesion in guinea pigs in which the probe is inserted on multiple occasions.

Anton M. Allen, DVM, Ph.D.
Director of Veterinary Services
ANIMAL SAFETY TEST IN
ADULT GUINEA PIGS

Study No.: ZA360.005102

Test Article: Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7

Final Report

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
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Rockville, Maryland 20850
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<td>VII. Quality Assurance Statement</td>
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<td>VIII. Appendix</td>
<td>125</td>
</tr>
</tbody>
</table>
SUMMARY

The purpose of this test is to attempt to detect the presence of adventitious agent(s) in the test article, preclarified bulk live virus and/or fluids, other than the specific virus in the product, by the inoculation and observation of guinea pigs.

No evidence of contamination with adventitious agents was observed due to the test article Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agents. The experimental design utilizes the inoculation of adult guinea pigs. The test is performed as described in CFR Title 21, Section 630.16(a)(4).

Adult guinea pigs are used in this assay to detect possible contamination of the test article with \textit{M. tuberculosis} or other adventitious agent(s). Animals are examined for signs of illness, and any that become sick or show any abnormalities are examined in an attempt to determine the cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Guinea Pigs

B. Study Number: ZA360.005102

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. Medium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Guinea Pigs
   Hartley Albino
   6 adult females, Body weight range: 350-400 g
   Source: Hazleton Research Animals
   Denver, Pennsylvania

G. Sponsor: Program Resources, Inc.
   Biomedical Services Division
   7655 Old Springhouse Road
   McLean, VA 22102

   Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department  
Microbiological Associates, Inc.  
Life Sciences Center  
9900 Blackwell Road  
Rockville, Maryland 20850

Animal Facility: Microbiological Associates, Inc.  
5221 River Road  
Bethesda, Maryland 20816

I. Personnel:

1. Study Director: Mary D. Whiteman

2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.

J. Schedule:

1. Study Initiation Date: 07/11/90
2. Lab Initiation Date: 07/12/90
3. Lab Completion Date: 09/06/90
4. Study Completion Date: See Study Director’s Signature Date, in the "Approvals" Section.

K. Raw Data, Records and Test Article Samples:

All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.

L. Archive:

Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.

III. PROCEDURES

A. Objective:

The study objective is to detect adventitious agents that might be present in the test article.
B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Animals were housed separately and were identified by ear tags.

The guinea pigs were randomized according to SOP OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. Three were held as uninoculated controls. All animals were observed every working day of the 42 day test period for death or clinical signs of illness or distress. Beginning day 21 post inoculation, rectal temperatures were recorded through day 42 post-inoculation. All remaining guinea pigs were sacrificed. Gross pathological examinations were performed on all guinea pigs.

3. Animal Husbandry

a. Animals were fed Ralston Purina Certified Guinea Pig Chow.

b. Water was supplied ad libitum via water bottles. Water was disinfected with 7 ppm chlorine.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.
### TABLE 1

<table>
<thead>
<tr>
<th>Group #</th>
<th>No. of Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
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<tr>
<td>1</td>
<td>1</td>
<td>I.C.</td>
<td>0.1 ml</td>
<td>Observe for Illness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I.P.</td>
<td>5.0 ml</td>
<td>Record Rectal Temp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>On Days 21-42 Post-Inoculation</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
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<tr>
<td>4</td>
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<td>None</td>
<td>None</td>
<td>Uninoc Control</td>
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</tr>
<tr>
<td>6</td>
<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above  
I.C. = Intracranial  
I.P. = Intraperitoneal
IV. RESULTS

All of the uninoculated control guinea pigs and all of the test article inoculated guinea pigs appeared normal and healthy throughout the 42 day observation period. See Table 2 for a summary of the data.

None of the uninoculated control guinea pigs and none of the test article inoculated guinea pigs had significant temperature rises indicative of either viral or bacterial infections during the 21 day recording period from day 21 through day 42 post-inoculation. See Table 2 for a summary of the data.

At examination on day 42 for gross pathology, no lesions were found in the inoculated control or test article guinea pigs. (See Pathology Report in Appendix.)

V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article, Dengue-4 (#341750) Prod Seed, PDK-10, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration’s Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary b. Whiteman
Study Director

Date: 9/13/90
### TABLE 2

Summary of Daily Observations for Dengue-4 (#341750) Prod Seed, FDK-10, FRhL-2/d7

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset (Post-inoc.)</th>
<th>Day of Death/Sacrifice (Post-inoc.)</th>
<th>Range of Body Temp in °C</th>
<th>D-21 to D-42</th>
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<td>Guinea Test</td>
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<td>Normal</td>
<td>38.0 - 38.6</td>
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<tr>
<td>Pig Article</td>
<td>10428</td>
<td>Normal</td>
<td>38.0 - 38.5</td>
<td></td>
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<tr>
<td></td>
<td>10429</td>
<td>Normal</td>
<td>38.2 - 38.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>10424</td>
<td>Normal</td>
<td>38.1 - 38.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10425</td>
<td>Normal</td>
<td>37.5 - 38.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>10426</td>
<td>Normal</td>
<td>38.1 - 38.5</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study Number: ZA360.005102

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/16/90 - 07/16/90, TO STUDY DIR 07/16/90, TO MGMT 07/16/9C
PHASES: PROTOCOL REVIEW

INSPECT ON 07/30/90 - 07/30/90, TO STUDY DIR 07/30/90, TO MGMT 08/06/9C
PHASES: ANIMAL OBSERVATIONS

INSPECT ON 09/12/90 - 09/12/90, TO STUDY DIR 09/12/90, TO MGMT 09/14/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan  
Quality Assurance Unit  

9/14/90  
Date
VIII. APPENDIX
### PATHOLOGY REPORT

**CAHS-2190**

**SOURCE:** Biotech Services  
ZA356.005102  ZA357.005102  ZA358.005102  ZA359.005102  ZA360.005102  ZA361.005102

**SPECIES:** Guinea Pig  
Results of gross examination according to SOP #865.201.

**ZA356.005102**

- **2190-1 (10401)** Test Article  
  Gross: No lesions found  
  Weight: 563.9 g

- **2190-2 (10402)** Test Article  
  Gross: No lesions found  
  Weight: 684.2 g

- **2190-3 (10403)** Test Article  
  Gross: No lesions found  
  Weight: 614.3 g

- **2190-4 (10404)** Control  
  Gross: No lesions found  
  Weight: 614.4 g

- **2190-5 (10405)** Control  
  Gross: No lesions found  
  Weight: 673.3 g

- **2190-6 (10406)** Control  
  Gross: No lesions found  
  Weight: 646.2 g

**ZA357.005102**

- **2190-7 (10407)** Test Article  
  Gross: No lesions found  
  Weight: 629.0 g

- **2190-8 (10408)** Test Article  
  Gross: No lesions found  
  Weight: 583.6 g

- **2190-9 (10409)** Test Article  
  Gross: No lesions found  
  Weight: 528.1 g
ZA358.005102

2190-10 (10410) Test Article 636.3 g
Gross: No lesions found

2190-11 (10411) Test Article 490.5 g
Gross: Retroperitoneal abscess (4x6 cm) and peritonitis

2190-12 (10412) Test Article 595.5 g
Gross: No lesions found

ZA359.005102

2190-13 (10421) Test Article 605.3 g
Gross: No lesions found

2190-14 (10422) Test Article 631.8 g
Gross: No lesions found

2190-15 (10423) Test Article 547.4 g
Gross: No lesions found

2190-16 (10424) Control 651.5 g
Gross: No lesions found

2190-17 (10425) Control 623.6 g
Gross: No lesions found

2190-18 (10426) Control 615.9 g
Gross: No lesions found

ZA360.005102

2190-19 (10427) Test Article 561.8 g
Gross: No lesions found

2190-20 (10428) Test Article 594.6 g
Gross: No lesions found

2190-21 (10429) Test Article 598.4 g
Gross: No lesions found
<table>
<thead>
<tr>
<th>ZA361.005102</th>
</tr>
</thead>
<tbody>
<tr>
<td>2190-22 (10430) Test Article</td>
</tr>
<tr>
<td>Gross: No lesions found</td>
</tr>
<tr>
<td>2190-23 (10431) Test Article</td>
</tr>
<tr>
<td>Gross: No lesions found</td>
</tr>
<tr>
<td>2190-24 (10432) Test Article</td>
</tr>
<tr>
<td>Gross: No lesions found</td>
</tr>
</tbody>
</table>

**COMMENT:** The retroperitoneal abscess in guinea pig #2190-11 is believed to be due to a rectal perforation (no longer visible) caused by insertion of a temperature measuring probe. This is a common lesion in guinea pigs in which the probe is inserted on multiple occasions.

Anton M. Allen, DVM, Ph.D.
Director of Veterinary Services
ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study No.: ZA361.005102

Test Article: Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7

Final Report

Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22102

By
Microbiological Associates, Inc.
Life Sciences Center
9900 Blackwell Road
Rockville, Maryland 20850
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<tr>
<td>VIII. Appendix</td>
<td>139</td>
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</tbody>
</table>
SUMMARY

The purpose of this test is to attempt to detect the presence of adventitious agent(s) in the test article, preclarified bulk live virus and/or fluids, other than the specific virus in the product, by the inoculation and observation of guinea pigs.

No evidence of contamination with adventitious agents was observed due to the test article Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7.
I. INTRODUCTION

It is the purpose of this study to detect the presence of latent or inapparent adventitious agents. The experimental design utilizes the inoculation of adult guinea pigs. The test is performed as described in CFR Title 21, Section 630.16(a)(4).

Adult guinea pigs are used in this assay to detect possible contamination of the test article with M. tuberculosis or other adventitious agent(s). Animals are examined for signs of illness, and any that become sick or show any abnormalities are examined in an attempt to determine the cause of illness or death.

II. STUDY INFORMATION

A. Title: Animal Safety Test in Adult Guinea Pigs

B. Study Number: ZA361.005102

C. Test Article: Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7 was received at Microbiological Associates, Inc. on 07/06/90. Determination of the stability, purity and concentration of the test article is the responsibility of the sponsor.

D. M.dium Test Article: none

E. Control Articles:
   1. Positive Control: none
   2. Negative Control: none
   3. Vehicle Control: none

F. Test System: Guinea Pigs
   Hartley Albino
   6 adult females,
   Body weight range: 350-400 g
   Source: Hazleton Research Animals
          Denver, Pennsylvania

G. Sponsor: Program Resources, Inc.
            Biomedical Services Division
            7655 Old Springhouse Road
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Authorized Representative: Dr. Louis Potash
H. Testing Facility: Biotechnology Services Department  
Microbiological Associates, Inc.  
Life Sciences Center  
9900 Blackwell Road  
Rockville, Maryland 20850  

Animal Facility: Microbiological Associates, Inc.  
5221 River Road  
Bethesda, Maryland 20816  

I. Personnel:  
1. Study Director: Mary D. Whiteman  
2. Associate Study Director: Janet Luczak, M.T.(ASCP), M.G.A.  

J. Schedule:  
1. Study Initiation Date: 07/11/90  
2. Lab initiation Date: 07/12/90  
3. Lab Completion Date: 09/06/90  
4. Study Completion Date: See Study Director's Signature Date, in the "Approvals" Section.  

K. Raw Data, Records and Test Article Samples:  
All raw data, records, protocol and all report copies will be maintained by the testing facility, Regulatory Affairs/Quality Assurance Department. Retention of samples of the test article is the responsibility of the sponsor.  

L. Archive:  
Study records will be archived by the Regulatory Affairs/Quality Assurance Department, Microbiological Associates, Inc., 9900 Blackwell Road, Rockville, Maryland 20850.  

III. PROCEDURES  
A. Objective:  
The study objective is to detect adventitious agent(s) that might be present in the test article.
B. Methods:

1. Test System Identification and Randomization

Each animal cage was assigned a number and labelled as "test article" or "uninoculated control". Animals were housed separately and were identified by ear tags.

The guinea pigs were randomized according to SOP OPBT0213.

2. Animal Inoculation with Test Article

Animals were inoculated according to Table 1. Three were held as uninoculated controls. All animals were observed every working day of the 42 day test period for death or clinical signs of illness or distress. Beginning day 21 post inoculation, rectal temperatures were recorded through day 42 post-inoculation. All remaining guinea pigs were sacrificed. Gross pathological examinations were performed on all guinea pigs.

3. Animal Husbandry

a. Animals were fed Ralston Purina Certified Guinea Pig Chow.

b. Water was supplied ad libitum via water bottles. Water was disinfected with 7 ppm chlorine.

c. Bedding - Bed-o-Cobs, Anderson Cob Mills. Cages were changed as necessary, usually twice per week.

d. Animal facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.
### TABLE 1

<table>
<thead>
<tr>
<th>Group #</th>
<th>No. of Animals</th>
<th>Routes of Inoculation</th>
<th>Volume of Inoculum</th>
<th>Treatment after Inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I.C.</td>
<td>0.1 ml</td>
<td>Observe for Illness</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>I.P.</td>
<td>5.0 ml</td>
<td>Record Rectal Temp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>On Days 21-42 Post-Inoculation</td>
</tr>
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<td>SAA</td>
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<td>SAA</td>
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<td>Uninoc Control</td>
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<td>1</td>
<td>SAA</td>
<td>SAA</td>
<td>SAA</td>
</tr>
</tbody>
</table>

SAA = Same As Above  
I.C. = Intracranial  
I.P. = Intraperitoneal
IV. RESULTS

All of the uninoculated control guinea pigs and all of the test article inoculated guinea pigs appeared normal and healthy throughout the 42 day observation period. See Table 2 for a summary of the data.

None of the uninoculated control guinea pigs and none of the test article inoculated guinea pigs had significant temperature rises indicative of either viral or bacterial infections, during the 21 day recording period from day 21 through day 42 post-inoculation. See Table 2 for a summary of the data.

At examination on day 42 for gross pathology, no lesions were found in the uninoculated control or test article guinea pigs. (See Pathology Report in Appendix.)

V. CONCLUSIONS

No evidence of contamination with adventitious agent(s) due to the test article, Dengue-4 (#341750) Prod Seed, PDK-15, FRhL-2/d7, was observed.

VI. APPROVALS

This study met the criteria for a valid test and was performed in compliance with the requirements of the Food and Drug Administration's Good Laboratory Practice regulations as found in Title 21 CFR Part 58.

Mary D. Whiteman
Study Director

9/13/90
### TABLE 2


<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Inoculum</th>
<th>Animal Number</th>
<th>Clinical Signs</th>
<th>Day of Onset</th>
<th>Day of Death/ Sacrifice</th>
<th>Range of Body Temp in °C</th>
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<td>Guinea</td>
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<td>38.1 - 38.8</td>
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<td>Pig</td>
<td>Article</td>
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<td>38.1 - 38.7</td>
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<td>10432</td>
<td>Normal</td>
<td></td>
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<td>38.1 - 38.6</td>
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<td>Uninoculated</td>
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<td>Normal</td>
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<td>38.1 - 38.6</td>
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<td>Control</td>
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<td>38.1 - 38.5</td>
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QUALITY ASSURANCE STATEMENT

Study Title: ANIMAL SAFETY TEST IN ADULT GUINEA PIGS

Study Number: ZA361.005102

Study Director: Mary D. Whiteman

This study has been divided into a series of phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLPs (40 CFR 792 and 40 CFR 160), and the OECD Guidelines and to assure that the study is conducted according to the protocol.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

INSPECT ON 07/16/90 - 07/16/90, TO STUDY DIR 07/16/90, TO MGMT 07/16/90
PHASES: PROTOCOL REVIEW

INSPECT ON 08/23/90 - 08/23/90, TO STUDY DIR 08/24/90, TO MGMT 08/27/90
PHASES: EXAM. OF ABDOMINAL AND THORACIC VISCERA AT DAY 42 POST-INOCULATION FOR OBVIOUS OR SUGGESTIVE ABNORMALITIES

INSPECT ON 09/12/90 - 09/12/90, TO STUDY DIR 09/12/90, TO MGMT 09/14/90
PHASES: FINAL REPORT

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Joan M. McGowan
Quality Assurance Unit

Date: 9/14/90
VIII. APPENDIX
PATHOLOGY REPORT

CAHS-2190

SOURCE: Biotech Services

ZA356.005102 ZA357.005102
ZA358.005102 ZA359.005102
ZA360.005102 ZA361.005102

DATE RECEIVED: 08/23/90
DATE NECROPSIED: 08/23/90
DATE REPORTED: 09/06/90

SPECIES: Guinea Pig

Results of gross examination according to SOP #865.201.

ZA356.005102

2190-1 (10401) Test Article 563.9 g
Gross: No lesions found

2190-2 (10402) Test Article 684.2 g
Gross: No lesions found

2190-3 (10403) Test Article 614.8 g
Gross: No lesions found

2190-4 (10404) Control 614.4 g
Gross: No lesions found

2190-5 (10405) Control 673.3 g
Gross: No lesions found

2190-6 (10406) Control 646.2 g
Gross: No lesions found

ZA357.005102

2190-7 (10407) Test Article 629.0 g
Gross: No lesions found

2190-8 (10408) Test Article 583.6 g
Gross: No lesions found

2190-9 (10409) Test Article 528.1 g
Gross: No lesions found
PATHOLOGY REPORT
CAHS-2190
SOURCE: Biotech Services
PAGE 2

ZA358.005102

2190-10 (10410) Test Article 636.3 g
Gross: No lesions found

2190-11 (10411) Test Article 490.5 g
Gross: Retroperitoneal abscess (4x6 cm) and peritonitis

2190-12 (10412) Test Article 595.5 g
Gross: No lesions found

ZA359.005102

2190-13 (10421) Test Article 605.3 g
Gross: No lesions found

2190-14 (10422) Test Article 631.8 g
Gross: No lesions found

2190-15 (10423) Test Article 547.4 g
Gross: No lesions found

2190-16 (10424) Control 651.5 g
Gross: No lesions found

2190-17 (10425) Control 623.6 g
Gross: No lesions found

2190-18 (10426) Control 615.9 g
Gross: No lesions found

ZA360.005102

2190-19 (10427) Test Article 561.8 g
Gross: No lesions found

2190-20 (10428) Test Article 594.6 g
Gross: No lesions found

2190-21 (10429) Test Article 598.4 g
Gross: No lesions found
COMMENT: The retroperitoneal abscess in guinea pig #2190-11 is believed to be due to a rectal perforation (no longer visible) caused by insertion of a temperature measuring probe. This is a common lesion in guinea pigs in which the probe is inserted on multiple occasions.

Anton M. Allen, DVM, Ph.D.
Director of Veterinary Services
December 17, 1990

Dr. Louis Potash
Program Resources, Inc.
Biomedical Services Division
7655 Old Springhouse Road
McLean, VA 22101

Dear Dr. Potash,

Microbiological Associates, Inc. is an AAALAC accredited animal facility, and all studies are performed in accordance with the "Guide for the Care and Use of Laboratory Animals", U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH Publication No. 86-23.

Sincerely,

Mary D. Whitman
Study Director, In Vivo Assays
Biotechnology Division
May 14, 1991

TO: Mr. Donald Holzworth, Vice President
    Dr. Louis Potash, Study Director

FROM: James R. Plautz
    Sr. QA Advisor

RE: GLP Compliance Audit of Final Reports for Safety Testing of Dengue Virus Type 1 and Type 4

On April 14, 1991 a complete audit for GLP compliance (21 CFR, Part 58) was conducted for the subject final reports and their respective raw data.

Our complete findings indicate that the studies were conducted under the guidance of the referenced Standard Operating Procedures (SOPs), the variations from the SOPs had no apparent effect on study outcome, and that the final report for each study is substantiated by the raw data.

Animal safety testing was conducted and reported separately from these final reports.

[Signature]
May 14, 1991