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TITLE: PCR (POLYMERASE CHAIN REACTION) TESTING FOR LEISHMANIASIS

SUBTITLE: Services to Develop, Standardize, and Validate Polymerase Chain Reaction (PCR) Protocols for the Detection of Leishmaniasis in Clinical Samples

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The disease Leishmaniasis, endemic in Africa, South West Asia, and South America, is caused by transmission of a parasite of the Leishmania species via the bite of an infected sandfly. The severity of the disease ranges from cutaneous lesions to a frequently fatal visceralization of the internal organs unless treated at an early stage. Detection of the parasite is made difficult due to very low numbers of infected cells in peripheral blood. Existing tests for Leishmania parasites are time consuming and have high (50%) false negative rates. We report here the development of a rapid polymerase chain reaction (PCR) based diagnostic capable of detecting 1 infected cell in more than 5 mls of peripheral blood. The test has an accuracy greater than 92%, and a false negative rate of less than 8%, when validated against known clinical samples.

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Frank G. Autin III — Oct 18th 1993

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1. Objectives

As set forth in the Statement of Work for contract DAMD17-92-C-2097 entitled "Services to develop, standardize, and validate polymerase chain reaction (PCR) protocols for the detection of Leishmaniasis in clinical samples", the objective of this study is comprised of two phases. In the first phase, the contractor shall "Develop and standardize the technology known as the polymerase chain reaction (PCR) for the detection of leishmaniasis in clinical samples". Phase II defines the task of the contractor to "validate PCR testing protocols developed as a result of the first objective in a large select military population of approximately 2,500 to 3,000 individuals". At this point in time, Phase I of the study is nearly complete, but Phase II has not yet started. Taking this into consideration, this Midterm report will be confined to the objectives stated for Phase I of the study and the progress made toward those goals.

2. Introduction

Leishmaniasis is a zoonotic disease which afflicts over 12 million persons throughout the tropical and subtropical regions of the world. Over 400,000 new infections of this clinically ill-defined group of diseases are diagnosed yearly, and the actual infection rate in developing nations may be significantly higher. This disease has been a particular concern following Operation Desert Storm (ODS)(1).

The etiologic agent of leishmaniasis is a protozoan of the genus *Leishmania* . *Leishmania* are grouped broadly into the New (*L. mexicana* and *L. braziliensis* complexes) and Old (*L. donovani* and *L. tropica*) World species. Four species and at least 15 subspecies are generally recognized. All are similar in morphology and life history. The parasites which infect humans are transmitted by the Old and New World sandflies, *Phlebotomus* and *Lutzomyia*. In the insect vector, the protozoan is found as the flagellated or promastigote form. Upon introduction into the human host, and if phagocytized by a macrophage, the parasite transforms into the non-flagellated, ovoid form, the amastigote.

Three major types of pathologies result from active infection with *Leishmania* . Cutaneous leishmaniasis is characterized by fairly localized cutaneous lesions. If the organism spreads from the site of infection to the nasal cavity and naso-pharyngeal region, the severely disfiguring mucocutaneous leishmaniasis can result. The most severe form of the disease occurs if the parasite metastasizes through the reticuloendothelial system, to infect the spleen, bone marrow, and other tissues. Visceral leishmaniasis, or kala-azar, is frequently fatal, if untreated.

Increased travel between the industrialized countries of the West and the developing nations, by businessmen and tourists, mandates the development of more appropriate tools for laboratory diagnosis

of this potentially very serious disease. Effective drug treatments to control the infection are available, but a proper diagnosis is required before starting treatment. To apply laboratory diagnostic methods in developing nations, simplified assay formats are desirable. A further reason to pursue development of nucleic acid based tests which are simplified and amenable to large scale screening is the potential for dissemination of these and similar parasitic infections through the blood donor pool.

For the purposes of this study, the primary interest is in Old World species as these are the organisms native to the geographic region where ODS took place. The strains *L. Tropica*, *L. Major*, and *L. Donovani* predominate in south west Asia, and are the 3 species against which most of the PCR primer sets described in this report were tested. When patient samples were used as positive controls, the majority of these were from geographic regions where *L. Donovani* predominates. During the course of this contract however, several New World species have also been examined, as well as patient samples from South and Central America.

2.1 Background on the Polymerase Chain Reaction (PCR) Assay

In order to better appreciate the methodologies utilized in this contract, some general information on the Polymerase Chain Reaction (PCR) is provided here, along with the rational used in doing PCR in a clinical setting and the algorithm used to test the samples under this contract.

The polymerase chain reaction (PCR) is *in vitro* DNA replication. Rather than the DNA double helix being unwound by replication-associated enzyme complexes, DNA is heat-denatured in the presence of thermostable DNA dependent DNA polymerase, oligonucleotide primers complementary to target sequences to be amplified, and deoxynucleoside building blocks for DNA synthesis. Alternating

cycles of denaturation, primer annealing (hybridization), and extension, result in the accumulation of double-stranded DNA fragments of discrete length, termed amplicons. This process operates under defined conditions and for a limited number of cycles as a quantitative exponential amplification of the target sequences. PCR thus results in a vast increase in copies of the target sequence and probably constitutes the most sensitive analytical technique currently available for molecular diagnostics, being capable of detection of a single copy of nucleic acid per reaction.

2.1.1 Quality Control For PCR

The exquisite sensitivity of PCR coupled with its ability to amplify DNA sequences many million fold can lead to false positive reactions due to contamination with amplifiable DNA not derived from the specimens to be tested. Such contaminating DNA generally is derived from two possible sources: native DNA template introduced through specimen cross-contamination or previously amplified amplicons. SRA has more than 5 years experience using PCR in both a diagnostic and a developmental testing atmosphere, and is well versed in techniques required to minimize or eliminate the chances of contamination from both sources. As a consequence of meticulous implementation of the techniques described in this section, for example, during three years of testing, our PCR laboratory has never reported a confirmed false positive HIV-1 test result to the WRAIR, Department of Retrovirology. To date under the provisions of this contract, the same performance record exists for *Leishmania* PCR testing when using known samples.

2.1.2 Mechanical/Physical Barriers to Contamination

Procedures have been given in the literature for avoiding false positives in PCR (2). In SRA's PCR laboratory, all PCR reagents are tested prior to use for contamination with amplifiable DNA.

Single-use aliquots are then frozen for future use with specimen diagnostics. Sample preparation, PCR reaction setup, and PCR product analysis are all performed in separate rooms with dedicated equipment, including certified Biosafety cabinets equipped with HEPA filters for all steps involving potentially infectious materials. For all pipetting steps prior to specimen amplification, either positive displacement pipettors with single-use tips and pistons or special commercially-available pipet tips with an aerosol-barrier are used.

Positive controls to be included in each PCR run are samples which are reproducibly detectable, but not of excessively high copy number, to avoid generating extremely high amounts of PCR product.

A negative control for sample preparation is included with each panel of specimens tested. Negative controls for contamination during reaction set-up consist of DNA from specimens known from previous testing to be negative for the sequences being amplified. Such negative controls are inserted between each set of specimen duplicate reactions. Spacial proximity of negative controls to specimen reactions is essential to control for contamination arising through handling and/or aerosol generation during reaction set-up.

2.1.3 Biochemical Methods to Prevent False Positives

DNA polymerases are unable to distinguish native DNA templates from previously-amplified PCR products. Recently, several techniques have been suggested for either inactivating amplicons or modifying them to render them unamplifiable, though still available for hybridization to specific probes. Such techniques include gamma irradiation of reaction mixes prior to PCR (3), UV crosslinking of amplified DNA (4), digestion of PCR reaction mixes with nucleolytic enzymes prior to specimen addition (5), treatment of amplified products with isopsoralens which form monoadducts and thus prevent

re-amplification (6), or a method analogous to the excision repair system of living cells, utilizing the enzyme uracil-N-glycosylase (uracil DNA glycosylase, UNG) (7).

In the course of our work for WRAIR, Department of Retrovirology, we have evaluated UV irradiation, isopsoralen inactivation of PCR products, and the use of UNG; in our experience, UNG is more efficient and presents a more generally applicable method for PCR product carryover prevention. In this method, dUTP substitutes for dTTP in all PCR reactions, and UNG is included in all PCR reactions. Prior to temperature cycling, this moderately heat stable enzyme selectively excises uracil residues which have been incorporated into DNA during previous amplifications. During the initial heating step in PCR, the DNA backbone of any contaminating previously amplified material is broken at these apyrimidinic sites, thus preventing the U-containing DNA from serving as a template for polymerization. Since native DNA templates do not contain U residues and since Taq DNA polymerase efficiently incorporates dUTP as well as dTTP during PCR, this technique can be made to operate without decreasing sensitivity or specificity of PCR reactions. Pretreatment of all PCR reactions eliminates the most common cause of a false positive result - carryover of amplified DNA from a previous amplification. SRA has incorporated this technique into PCR protocols for the amplification of HIV-1 and HTLV-I/II, as well as all PCR methods which have been used by us to amplify *Leishmania* sequences.

2.1.4 Design of an Appropriate Testing Algorithm

In the course of conducting PCR testing prior to the advent of UNG-mediated carryover prevention, we found it necessary to design PCR testing algorithms to minimize the chance of false positive test results arising from PCR product contamination (8). Specifically, we have tested (and continue to test) all specimens as duplicate reactions plus a negative control spatially unique to the duplicate

reaction set, with a primary primer set. Reaction products are subjected to hybridization analysis using an oligonucleotide probe to sequences bracketed by but not overlapping the primers. A result of "reactive" is then defined as the detection of specific hybridization signal in both duplicates, with no specific signal in the corresponding negative control reaction. The detection of specific hybrids in only one duplicate is defined as a result of "non-diagnostic," necessitating repeat testing. "Non-reactive" refers to the absence of specific signal in both duplicates, with low copy number positive controls being detected. In spite of the introduction of sophisticated biochemical methods for detection of product cross-contamination, the ever-present possibilities for operator error support the continued use of carefully-designed PCR testing algorithms.

2.1.5 Optimization of Reaction Parameters

In addition to the techniques designed to eliminate contamination and resulting false positives, all PCR reactions are optimized for both specificity and product yield. These procedures include empirical determination of optimal oligonucleotide ratios and concentrations, magnesium ion concentration, Taq polymerase concentration, and annealing temperature. Despite these precautions, some non-specific annealing of PCR primers does occur, even with single copy gene detection, and more so with the detection of retrovirus or parasite DNA in the presence of a high background of human genomic DNA. Since annealing of primers to template is not 100% specific under all conditions encountered during the course of a PCR reaction, it is necessary to adjust reaction conditions to maximize synthesis of specific product.

While post-PCR hybridization detection ensures that non-specific products will not be detected, the synthesis of these spurious amplicons affects the amplification process. Non-specific reaction products do incorporate PCR primers such that subsequent

amplification cycles result in their specific amplification as "quasi-specific" templates. This detracts from the overall efficiency of the reaction, as both non-specific and specific products compete for primer and Taq binding. Careful adjustment of reactant concentrations to strike a balance between maximization of primer hybridization and minimization of non-specific annealing can significantly increase PCR product yield and also extend sensitivity into the < 10 copy range. In our experience, with some primer sets, rigorous optimization can extend the detection limit 2 to 3 orders of magnitude. SRA has pioneered the development of HPLC protocols for quantitation of PCR products (9). The analytical precision of HPLC analysis allows more precise determination of PCR product yield, with very fast turnaround, often allowing complete optimization of reaction conditions for a new primer set within two days.

At the start of this contract, HPLC was used to evaluate PCR reaction products. While it has the aforementioned advantages of precise quantitation, it does not have the sensitivity of our microplate based capture assays which we now use. A basic protocol for using the HPLC for PCR product detection is provided, however, in the Methods and Results section because of its use initially for this protocol.

2.1.6 Design of PCR Primers and Probes

Design of synthetic oligonucleotide primers and probes is facilitated by the use of computer software dedicated to that purpose (e.g., Oligo™, National Biosciences; Primer Detective™, Clontech). After candidate sequences are designed, these sequences are compared to DNA sequences of both related and unrelated organisms by computer homology searchs from the Genbank database using the Lasergene DNAStar program running on a Macintosh IIci. These preliminary steps reduce the chance of PCR artifacts (primer-dimers) due to primers that share significant sequence homology, or

secondary structure that would reduce the overall efficiency of the PCR amplification. In addition, with highly variable sequences or sequences which are only partially known (e.g., *Leishmania* minicircle kDNA), it has been suggested that PCR primers preferentially end in 3'-T, to minimize the effects of possible 3'-mismatch (10). Other strategies to lessen the effect of random non-homologies with the target sequence include the synthesis of primers with degenerate positions and/or inosine substitutions (11). However, excessive degeneracy should be avoided, in order to maintain specificity. We have exploited this later technique in the design of some "second generation" primers that show improved detection of New World *Leishmania* strains in our testing.

In the case of *Leishmania* , almost 20 different PCR primer combinations have been evaluated to date. These include multiple sets that amplify sequences found in the kinetoplast (kDNA) minicircles, one set directed against sequences found in the kDNA maxicircles (equivalent to mitochondrial DNA), one set directed against ribosomal RNA sequences (rRNA), and one set specific for conserved sequences from one nuclear gene (DHFR). Finally, it should be noted that, even though primer and probe sequences have been carefully chosen based on predicted homology to the desired sequences and the lack of homology to other (especially human) sequences in GenBank, it is still necessary to test these primers sets against actual specimens of related and unrelated organisms. This has been done for all primer sets that show acceptable sensitivity against the *Leishmania* strains of interest.

3. Methods and Results

While there has been significant improvement in the sensitivity of the PCR detection protocol over the course of the first year of this contract, the basic elements of the procedure are used commonly for many of the PCR protocols used by SRA. The sample preparation steps and basic PCR protocol remain essentially unchanged from the beginning of the contract, as they have already been validated for other protocols. The capture plate procedure for PCR product detection was developed for other applications and was adapted to this use solely by the design and implementation of the Leishmania-specific probes. The PCR reaction and detection protocols are given in the following sections, with a detailed discussion of the results of the various PCR primer sets tested to date following the protocols.

3.1 Sample Preparation

The sensitivity of PCR permits the detection of the low level of parasites in the peripheral blood, at least during active infection. In many cases, however, peripheral blood samples from a given individual were negative, while splenic or bone marrow aspirates were positive by PCR. Although the major specimen type is whole blood, the protocol given below works equally well for bone marrow and splenic aspirates, as well as cutaneous lesion lavage specimens, thus simplifying the overall test.

We have successfully employed differential lysis for the selective removal of RBC's from blood and bone marrow samples prior to DNA extraction for PCR. This method is based on the specific RBC-lytic activity of saponin, and is quite simple, requiring only the use of a tabletop centrifuge. The blood is gently mixed with 0.3% saponin (Mallinckrodt) in slightly hypotonic saline and allowed to remain at ambient temperature for 5 minutes, during which time RBC's are lysed. Centrifugation recovers leukocytes, which are again washed

with saponin to remove residual RBC's. The final cell pellet contains total leukocytes, and appears to be free of inhibition to the PCR, by either heme or the saponin itself. The cell pellet is then lysed by the addition of proteinase K, and, following heat inactivation of the proteinase K, the crude lysate can be used directly in diagnostic PCR reactions.

3.1.1 Sample Preparation by Total Leukocyte Separation

1. For specimens received in Leukoprep tubes
    a. Centrifuge at 3000 rpm for 20 min. Pipet off the supernatant into a 50mL polypropylene centrifuge tube
    b. Count cells using Zapaglobin. (40μL specimen + 20mL Isoton II + 5-6 drops Zapaglobin)
    c. Add 20mL of 0.1% saponin in 0.6% NaCl. Mix well by inversion. Maintain at room temperature 5 min.
    d. Centrifuge at 1500 rpm for 15 min. Decant the supernatant.
2. Whole Blood specimens
    a. Count cells using Zapaglobin. (40μL specimen + 20mL Isoton II + 5-6 drops Zapaglobin)
    b. Add 10 volumes of 0.1% saponin in 0.6% NaCl. Mix well by inversion. Maintain at room temperature 5 min.
    c. Centrifuge 1500 rpm for 15 minutes. Decant the supernatant.
    d. Resuspend the pellet with 15mL 0.1% saponin in 0.6% NaCl
    e. Centrifuge 1500 rpm for 15 minutes. Decant the supernatant.

3.1.2 Cell Lysis

1. Use the cell count taken at the beginning of this procedure and determine lysis buffer volume for 30 X 10^6 cells/ ml. Add the determined volume of lysis buffer containing 2X proteinase K. Vortex briefly.

3. Incubate in water bath at 55°C - 60°C. for 1 h. Vortex briefly. If a large number of cells are being lysed, it may be necessary to vortex several times during this hour or extend the incubation time.

4. Transfer lysate to 1.5 mL screw-cap microcentrifuge tube. Label tube with specimen number, date lysed, tech initials.

   NOTE: Lysates prepared by this protocol should be labelled with an "S."

5. Heat-inactivate the proteinase K by keeping the tubes at 95°C for 15 min. in dry-bath.

6. Quench on ice. Store at -20°C in freezer boxes in pre-PCR lab.

3.1.3 PCR Cycling Conditions

1. Prepare lower layer PCR mix as follows:

H_2O	10.9 µl	
10X buffer (Promega)	4.0 µl	
MgCl(25mM)	4.0 µl	
dNTP (AUCG)	16.0 µl	
UNG	0.1 µl	
JW11/b-JW12 (10µM each)	5.0 µl	
	40.0 µl	TOTAL

2. Prepare an upper layer PCR mix as follows:

H_2O	7.9 µl	
10X buffer (Promega)	1.0 µl	
UNG	0.1 µl	
Taq polymerase	1.0 µl	
	10.0 µl	TOTAL

3. Aliquot 40 µL of the lower mix to each tube.

4. Add one bead of ampliwax. Number tubes. Place in the heating block at 65°C for up to 5 minutes to melt the wax. Allow to cool.

5. Pipet 10 µL of the upper mix in the tube.

6. Add 50 µL of the appropriate specimen lysate to each tube. Do not add positive control template in the pre-PCR lab.

7. In the positive control lab add 50µL *Leishmania* copy 1, 10, 100 to the appropriate PCR tubes.

8. Immediately carry the reactions to the cycler. Proofread the program before starting. Fill out cycler log book.

CYCLER CONDITIONS

PRIMERS	JW 11/12b
Time Delay file	
94°C	5'0"
Step Cycles	
97°C	0'15"
55°C	1'0"
72°C	1'0'
CYCLES	10
Step Cycles	
92°C	0'15"
55°C	1'0"
72°C	1'0'
CYCLES	30
SOAK 72°C	

NOTE: products be frozen immediately on removal from the cycler, unless they can be assayed within 1 h due to the presence of un-denatured UNG and it's ability to degrade PCR products at room temperature and 4°C over time.

3.2 PCR Product Detection

PCR products may be analyzed using the affinity-based hybrid capture assay. We have used two slightly different detection systems with equivalent results. These utilize either an Alkaline Phosphatase (AP) labeled specific oligonucleotide and a chemiluminescent substrate (Lumiphos), or the same oligo labeled with Horseradish Peroxidase (HRP) and a colorimetric substrate (OPD, TMB). The sensitivity obtained with either probe system is approximately equivalent. Both can be read in automatic plate readers or luminometers that are readily available. One distinct advantage with using and HRP probe is that the color produced from even 3 to 10 initial copies, after 40 cycles of amplification, is readily discernible by eye. Thus, it becomes possible to interpret results visually, by comparison with standards. Such an approach

is acceptable for qualitative, though not quantitative, assays, and may be advantageous for application in developing nations. An example of the sensitivity of this system is given in Figure 1. showing the detection of *L. Tropica* by PCR using the AP-labeled probe and capture plate system.

3.2.1 Capture Plate Procedure

For simplicity, only the Alkaline Phosphatase-coupled protocol is described here. The primary differences include, obviously, use of a horseradish peroxidase (HRP) labeled oligonucleotide probe, OPD or TMB for colorimetric detection, and the use of clear rather than opaque plastic microwells in an ELISA-type plate reader rather than a luminometer. A diagram of the principles of operation of the capture plate is given in Figure 3 at the end of this report.

1. Prepare an avidin-coated and blocked microwell plate according to the following procedure.

   a. Pipet 120 µl of 100 g/ml avidin D (Vector Labs) into each well of a high-binding plate (e.g., MaxiSorp, Nunc; Immulon 4, Dynatech). Incubate overnight at ambient temperature.

   b. Remove the solution, and wash 4 times with Wash Buffer (1% Tween 20 in PBS).

   c. Pipet 200 µl of 1% casein (Hammarsten Grade, BDH) in PBS into each well. Incubate for 1 h to overnight at ambient temperature.

   d. Remove the solution. Store the plate frozen, under which conditions it remains stable for at least several weeks.

2. Heat denature PCR products by incubation at 95°C for 5 min., followed by quick-cooling to approximately 4°C.

3. Pipet 90 µl of Hybridization Buffer (1% casein in PBS) containing 1 pmol of AP- or HRP- conjugated probe JW14 (Synthetic Genetics) into each well.

4. Pipet 10 µl of PCR product into the appropriate well.

5. Incubate at 42°C for 20 min. to allow both hybridization and capture.

6. Remove the hybridization solution and discard. Wash the plate 4 times with Wash Buffer.

7. Pipet 100 µl LumiPhos into each well. For HRP-labeled probes, 100 µl of either OPD or TMB are used as the substrate.

8a. AP-labeled Probes: Incubate at 37°C for 30 min. Read immediately in the ML1000 microplate luminometer (Dynatech).

8b. HRP-labeled Probes: Incubate at room temperature for 5-15 min (depending upon the substrate used) and OD is read in a Molecular Devices ELISA plate reader.

3.2.2 PCR Product Detection by HPLC

1. Inject 30 µl of each PCR product onto a TSK-DEAE NPR column. For greater precision, an automatic sample injector should be used, such as the ISS-200 (Perkin Elmer).

2. The following gradient (requiring approximately 9 minutes per run) is used to separate specific and non-specific PCR products:

a. Equilibrate column 5 minutes at 46% A.

b. Ramp linearly to 54% A over 0.1 minute following injection.

c. Ramp linearly to 60% A over 3.9 minutes.

d. Ramp linearly to 75% A over 1 minute.

e. Return to 46% A over 0.1 minute.

Buffer A: 25 mM Tris-Cl, pH 9.0, 1.0 M NaCl
1% acetonitrile
Buffer B: 25 mM Tris-Cl, pH 9.0
1% acetonitrile

3. Products are detected by UV absorbance monitoring at 260 nm.

4. Integration of chromatographic peaks is by an automatic integrator (Perkin Elmer Nelson Model 1020). Specific peaks are identified by characteristic retention times as compared with strong positives and molecular weight standards (2.5 µg of 250 µg/ml HaeIII digest of pBR322).

5. If quantitation is desired, data should be plotted as "peak area vs. log initial copy number." A linear plot should be obtained over the range of 30 to 30,000 initial DNA template copies. Linear regression permits the estimation of copy number in unknown samples.

3.3 PCR Primer Selection and Development

The principle area of development for this contract has been in the design and testing of various Leishmania-specific PCR primer and probe combinations. For all designs, the following rational was used. Since the *Leishmania* parasites are suspected to be present in very low numbers in peripheral blood of infected individuals, it was deemed that maximal sensitivity was the key requirement for the assay.

Toward this end, it was reasoned that directing the PCR primers against a "pre-amplified" target was, if possible, the best way to increase signal strength, and hence assay sensitivity, going into the PCR reactions themselves. Then the reaction conditions would be optimized as described previously in the Introduction, to produce the maximum specific yield from each primer set. For these reasons, several PCR primer sets directed against *Leishmania* target sequences that exist in more than one copy per parasite were designed. These included sequences in the ribosomal RNA (rRNA) genes, present in 5-20 copies per organism, nuclear Dihydrofolate Reductase (DHFR) genes, that exist in 2-10 copies per parasite, certain maxicircle sequences, present in 10-100 copies per organism, and several different minicircle sequences.

The minicircle sequences offer the highest possible target number as they are present in 100-10000 copies per organism. One significant problem targeting minicircle sequences, however, is the extreme sequence heterogeneity and the observation that multiple distinct "families" of minicircles exist not only within a given parasite, but also between differing species of Leishmania, with

the largest differences found between the New World and Old World strains. A diagram showing a comparison of the "conserved" sequence regions of a number of *Leishmania* strains is given in Figure 2 in the Appendix of this report. A summary of the sequences tested is given in Table 1 on the following page.

Table 1. Sequences of *Leishmania* Specific PCR Primers.

Primer Name	Primer Sequences	Primer Location
JW11	CCTATTTTACACCAACCCC(C/T)AGTTT	minicircle
JW12	CGGGTAGGGGCGTTCTGCGAAA(A/T)T	minicircle
TW01	GCGTCTCCGACCCTCATCTTCAAGG	DHFR (nuclear)
TW02	GACACCCTCCTCTCTCTATACGGC	DHFR (nuclear)
TW03	ATTGAAATAATAAAAGGTTCGAGC	maxicircle
TW04	AATTACAAATAATAGATCCTTGCG	maxicircle
JW16	GAATTCGATTTTCGCAGAACGCCCCT	minicircle
JW17	GAATTCAAACTGGGGGTTGGTGTGTAAAAT	minicircle
R222	TATTGGAGATTATGGAGCTG	rRNA gene
R332	GGCCGGTAAAGGCCGAATAG	rRNA gene
LK1S	CCTATTTTACACCAACCCC	minicircle
LK2R	GGGTAGGGGCGTTCTGCGA	minicircle
LS1	GGGGTTGGTGTAAAATAG	minicircle
LS2	CCAGTTTCCCGCCCCG	minicircle
B1	GGGGTTGGTGTAATATAGTGG	minicircle NW
B2	CTAATTGTGCACGGGGAGG	minicircle NW
B3	CCCGACATGCCTCTGGGTAG	minicircle NW
PROBE P1	CAGAAACCCCGTTCAAAAAT	minicircle NW
JW-11-i	CCTATTTTACACCAACCCCIAGTTT	minicircle
JW-12-i	CGGGTAGGGGCGTTCTGCGAAAIT	minicircle
C-JW11-1	CCTATITTACACCAACCCCIAITTI	minicircle
C-JW11-2	CCTATITTACACCAACCCCIAITT	minicircle
C-JW11-3	CCTATITTACACCAACCCCIAI	minicircle
C-JW12-1	CGGGIAGGGGCGTTCTGCGAAAI	minicircle
C-JW12-2	CGGGTAGGGGCGTTCTGCGAAAA	minicircle
C-JW14-1	ATTGAACGGGITTTCTGTATICITTTTTCGAA	minicircle
C-JW14-2	ATTIGAACGGGITTTCTGIAIICIATTTTTIGAA	minicircle
C-JW14-3	GAACGGGITTTCTGIAIICIATTTTCGITTTT	minicircle
JW-21	TGAACGGGITTTCTGIAIICATTT	minicircle
JW-22	GGGGTTGGTGTAAAATAGGICIG	minicircle
JW-24	CATTTTTCIIITTTCGCAGAACGCCCCTACC	minicircle

The sequences of the individual primers are given in the first section of the table, along with the target of amplification. In the JW11&12 primer set, the sequences indicated in parenthesis are mixed base positions in the synthetic oligonucleotides. The I in

several other sequences indicate an inosine (I) base at that position, that allows hybridization with any other base. An NW in the amplification target site indicates those sequences are specific for New World *Leishmania* strains. The JW and TW series of primers were designed in-house at SRA Technologies utilizing computer software for PCR primer design (Primer Detective, Oligo) and evaluated against potential cross-reactive sequences in Genbank using the Lasergene DNAstar molecular biology software package running on a Mac IIci. The sources of the other primer sequences are as follows: *R222/332;* ref (12) . *LK1S/2R;* Personal communication from G. van Eys to Maj. E. Nuzum. *LS1/LS2;* ref (13). Summaries the sensitivity of all other primers evaluated to date are given in Tables 2 and 3 that follow.

To date, the most consistent and sensitive primer set against the strains of Old World *Leishmania* examined is the JW 11&12 PCR primer pair. The detection limits of that PCR primer set are given in the following table when compared against control strains of *Leishmania* parasites. A plus (+) indicates that signal at least 5 fold above assay background was consistently obtained against that species, with lower numbers indicating greater sensitivity.

Table 2. Detection of Various *Leishmania* Species using the minicircle-specific PCR primers JW11/12 .

Species	# *Leishmania* Detected (JW 11/12)			
	1000	*100*	*10*	*1*
746	+	+		
842	+			
1031	+	+	+	+
1041	+			
1063	+	+	+	+
1077	+	+	+	
2053	+	+		
669	+			
7Y6	+	+	+	
1003	+	+		
2086a	+	+	+	
2086b	+	+	+	

The next table indicates the sensitivity of various primer combinations against the control strain of *L. Tropica* used for these evaluations (1063). The sensitivities of the various combinations of inosine-substituted primer sets are too complex for this table and are given separately in 3 figures at the end of this report.

Table 3. Sensitivity of various primer sets against *L. Tropica*

Primer Combinations	Detection Limits on *L. Tropica*
JW11/12	Less than 1 parasite
TW03/04	Approximately 100 parasites
TW01/02	Greater than 1000 parasites
R222/332	Greater than 1000 parasites
JW16/17	Greater than 1000 parasites
LK1S/2R	Greater than 1000 parasites
LS1/2	Greater than 1000 parasites
B1/B2	Greater than 1000 parasites
JW11i/JW12i	Less than 1 parasite
JW21/JW22	Approximately 100 parasites *

As can be readily seen from the previous tables, the primer set JW 11&12 is very sensitive when tested against *L. Tropica*, and by extension due to the accuracy of detection when used against control samples from Kala-Azar patients, *L. Donovani* (presumably the causative agent of Kala-Azar). Its sensitivity is, however, fairly limited when tested against other strains, particularly New World strains, of Leishmania, as seen in Table 2 (eg. 842, 669). Other primer sets tested to date are significantly less sensitive when tested against *L. Tropica* parasites than the JW 11&12 set, as seen in the preceding table (Table 3). The asterisk (*) given for primer set JW21/JW22 indicates a preliminary result as testing is not yet completed on that primer set.

3.3.1 PCR Primers with Multiple Inosine Substitutions

During the course of Phase I of this study, a number of samples from South and Central America, both as controls and as patients with suspicious disease, were provided for testing. It became readily obvious when testing against control strains provided by MAJ M. Grogl that the sensitivity of the basic JW11&JW12 PCR primer set was much better against Old World than the New World strains.

It was the opinion of the Leishmania Working Group that in addition to the original requirement for a PCR based detection test that was sensitive against the Old World strains prevalent in South West Asia, it would be useful to have a test usable against at least some of the New World strains of *Leishmania* for use with patient samples obtained from service personnel stationed in South and Central America. Toward these ends, it was decided to try designing PCR primers with multiple inosine substitutions at those DNA base positions that differ between characterized New and Old World *Leishmania* strains (see Figure 2). A collection of inosine-substituted primers based on the most sensitive PCR primer set tested to date (JW11&12) was developed and tested in various pairwise combinations.

The results of those tests are given in 3 charts comparing the pairwise combinations of these primers (Figures 4, 5 and 6) given at the end of this report. These combinations were tested using our optimized PCR protocol and the HRP-coupled capture plate assay against 10 and 100 copy equivalents of parasite DNA for each of 3 different control species (*L. Tropica*, *L. Donovani*, *L. Braziliensis*). Higher signal indicated greater PCR yield, and therefore greater sensitivity of that particular PCR primer combination against the species of *Leishmania* in question.

Briefly summarized, the more conservatively inosine-substituted primers give at least equivalent sensitivity (detection of a single parasite equivalent) to the JW 11&12 primer set for the species *L. Tropica* and in most cases *L. Donovani*. Some of the more extensively inosine-substituted primers give increased sensitivity against the New World strain *L. Braziliensis*, but show reduced signal when tested against the Old World strains of *L. Tropica* and *L. Donovani*. Based on these results, it was decided that since sensitivity against the Old World strains was of primary importance, the use of the more extensively inosine-substituted primers (eg. C-JW11-3/C-JW12-1) would be restricted to samples with

origins in geographic regions where New World stains of *Leishmania* predominate.

We have completed testing of all possible combinations of these inosine-substituted primers and obtained a pair that is slightly better in terms of product yield (strength of the positive reaction) and increased cross-reactivity against the largest number of both New and Old World *Leishmania* species than the original JW 11&12 primer set. The original primer set (JW 11&12) and the new "optimized" set (JW 11-2 & 12-i) are compared below. The sequence differences are indicated in ***bold italics***. The A/T indicates a mixed base composition at this position.

JW-11 CCTATTTTACACCAACCCCA/TAGTTT
JW-12 CGGGTAGGGGCGTTCTGCGAAAA/TT

JW-11-2 CCTAT***I***TTACACCAACCCC***IA***TT
JW-12-i CGGGTAGGGGCGTTCTGCGAA***AI***T

This second primer set, while not sufficiently different to be a truly independent second set, is apparently optimized for better cross-species amplification, particularly on some of the New World species.

An additional idea for a primer set has been explored recently. While it was requested in the contract Statement of Work that at least 2 PCR primer sets should be developed, it has not been possible to find 2 truly independent primer sets of approximately equivalent sensitivity. Working within the constraints placed on PCR primer selection due to limited DNA sequence homology across strains, and alternate idea is being pursued. Specifically, the idea of developing a second, partially overlapping but distinct minicircle primer set for use against Old World strains. This set is composed of PCR primers JW21, JW22, and probe JW24, with the sequences indicated in Table 1 seen previously. The basic idea for

this primer set takes advantage of a small conserved region downstream (3') of the site of the JW12 primer, on the same strand (see Table 2). By biotinylating and shortening the JW14 probe slightly, in order to bring its T_m closer to that calculated for the new primer, termed tentatively JW 22, a primer pair yielding an approximately 140 bp product is produced. A probe can be constructed by lengthening the original JW 12 sequence, making its reverse complement (so it binds to the other, biotinylated strand) and coupling it to HRP. Other arrangements might also be possible by switching which primer is biotinylated, and reversing the probe sequence.

One advantage of this approach is that a second, independent PCR product can be made, still using the conserved sequences in the kDNA minicircles. The partial overlap would prevent false reactions due to amplification of carry-over products from the other primer set; only 1 of the 2 primers needed to amplify the sequence is capable of binding to the PCR product of the other set. Utilizing variations of the same sequences should permit more rapid PCR optimization as well. Most importantly, it should provide us with another primer set with which to test patients exposed to Old World Leishmania.

One potentially significant disadvantage is that the downstream "conserved" sequence is only conserved in Old World strains. It is significantly different in the kDNA sequences from New World strains that we have been able to examine. The would almost assuredly render this new primer set ineffective against New World *Leishmania* strains. This primer set is currently under evaluation and optimization in our laboratories. Initial results have been disappointing in terms of sensitivity when compared to the basic JW 11&12 primer set, but it is hoped that by varying reaction conditions and/or slightly modifying the PCR primer sequences, increased sensitivity will be seen.

3.4 Decatenation of *Leishmania* kDNA minicircles

Additional experiments were done to further increase the sensitivity of the PCR detection test. One set of experiments proposed in SRA's response to the contract proposal was the use of procedures to "decatenate" the *Leishmania* kinetoplast DNA, theoretically releasing most of the minicircle and maxicircle DNA fragments into solution. This could potentially increase the chance of detection of *Leishmania* parasites by making more target DNA templates accessible for PCR. After evaluating a number of potential treatments our results are reported below.

3.4.1 Decatenation Protocol

Equal quantities of *Leishmania* parasite lysates were used for each treatment. Controls were lysates stored at -20°C or 4°C overnight (no difference was seen at either temperature). Treatments included;

1. Incubation at 37°C overnight.
2. Digestion with the restriction enzymes Dra I, Eco RI, or Bam HI at 37°C overnight using 20-50 units of enzyme. These enzymes have each been reported to introduce a single specific cut in some minicircles, linearizing the DNA.
3. Treatment with Topoisomerase II (20 units) overnight at 37°C. Topoisomerase II introduces double stranded transient breaks in DNA allowing decatenation of concatenated circles and reduction of supercoiling induced torsional strain in circular molecules, facilitating denaturation of the circular DNA.
4. Limited digestion with an inorganic Iron nuclease to introduce random double stranded breaks in all DNA present in the reaction.
5. Following treatments, each lysate was serially diluted 10 fold to give a range of 10 to 0.001 parasite equivalents, and subjected to PCR analysis as described.

3.4.1.1 Results of the Decatenation Experiments

Unfortunately, multiple experiments failed to show any significant difference in PCR product yield, and hence sensitivity of the reaction, when compared to untreated controls with the equivalent amounts of input DNA. All differences were within +/- 20% of the signal produced by the untreated controls at each dilution point, and were not constantly repeatable from experiment to experiment when using different batches of parasite lysates and/or enzymes (data not shown). Based on these results, we think further experimentation along these lines in not warranted. Our efforts will focus on finding additional PCR primer sets with equivalent sensitivity to the minicircle set JW 11&12.

3.5 Heterologous DNA Testing

It was suggested in the Statement of Work and by the Leishmania Working Group that we test a variety of heterologous DNAs using our PCR detection technique to ensure specificity of the primers and probes being used. A list of possible organisms was provided by the Leishmania Working Group and included the following;

Trypanosoma cruzi, T. gambiense, T. rhodesiense T rangeli. Leptomone, Crithidia, Herpetomonas, Toxoplasma, Plasmodium falciparum, Babesia, Pneumocystis carinii, Herpes, Salmonella, Histoplasma capsulatum, Mycobacterium tuberculosis, HIV, and Hepatitis B and C.

After receiving information from MAJ Grogl regarding the availability of certain heterologous DNAs from the ATCC, we obtained the necessary permit applications to purchase samples of these organisms as many of them are classified as Class II, III, and IV pathogens. Testing the DNA obtained by protocols appropriate for DNA isolation from each type of organism at 1μg concentrations in our standard PCR reactions did not produce any detectable signal from any of the organisms listed. Additional tests against blind negative control patients with Malaria and

several other tropical diseases also produced no false positive reactions, indicating that the current PCR primers and conditions are specific for *Leishmania* kDNA and do not cross-react to any significant degree with the heterologous DNAs tested. Any additional primer sets that produce promising results when tested against the appropriate *Leishmania* controls will also be tested against samples of these heterologous DNAs to ensure specificity.

3.6 PCR Results on Patient Samples

During the course of Phase I of the contract, a number of patient samples have been received and tested using the PCR protocols described in this report. A complete listing of all samples tested to date is included in the Appendix of this report.

These samples fall into several categories including positive and negative control samples obtained from patients with known diseases including Kala-Azar, Malaria, HIV infection, etc, canine samples used for potential animal model work, spiked controls, and a large number of "rule out" patient samples. This latter category is comprised of samples (blood, bone marrow, and on occasion, spleen, liver and other tissue specimens) from patients who present with clinical symptoms that may indicate *Leishmania* infection.

While the unknown patient samples comprise the most important aspect of the assay development, they are also the hardest to evaluate in terms of test accuracy. A large number of the unknown patient samples have tested negative, while a few have tested positive. In all cases of a positive result, the diagnosis has been confirmed by clinical data such as IFA, culture, etc (LTC E. Nuzum, personal communication). Some of the negative PCR results can be considered "biological false negatives". That is, the negative PCR result is false negative caused by the "biology" of the infection; in some cases, very few or no circulating parasite-

infected cells are present in the patient samples, as is the case following successful treatment. This is particularly true also for samples from patients having cutaneous lesions but no visceralization of the disease. From such patients, aspirates of the lesion itself test positive for PCR while peripheral blood samples test negative.

This result is to be distinguished from a "technical false negative" which is defined as a sample that does have some level of *Leishmania* DNA in it, but presents a false negative result when tested by PCR. These results are extremely rare (less than 4% to date) and may be explained in several ways. As indicated previously (Figures 4, 5 and 6) different species of *Leishmania* exhibit differing limits of detection using our current PCR protocol. It is possible that in some cases, the particular species of *Leishmania* present in a given sample is at a level undetectable using our current primer sets. Another possible explanation is the presence of inhibition in the patient sample that prevents PCR amplification of the *Leishmania* DNA present in the sample. This possibility has been evaluated in all "false negative" results seen to date by re-assaying the sample with a fixed level of *Leishmania* control DNA spiked into the sample. In all cases, the spiked DNA was detected with approximately the same signal strength as seen from a parallel reaction containing the same level of *Leishmania* control DNA in reaction buffer, effectively ruling out the presence of significant inhibition in those samples.

During the course of Phase I of this contract, a number of blind negative samples have been assayed. To date, the false positive rate is 0% (0/15 samples tested). Several samples from patients with Malaria or other tropical diseases prevalent in the region where the *Leishmania* parasites are found were also tested. In all cases, no reaction was seen (0% false positive). These results are indicative of the care with which PCR is conducted in our

laboratory and the effectiveness of the containment procedures, described in the Introduction, in place to prevent contamination from various sources in our laboratories, and the specificity of the PCR reactions.

When assaying blind control samples (total of 64 tested to date) obtained from several locations (Kenya, India, Brazil) of patients with Kala-Azar disease at various stages, the following statistics were obtained (provided by COL J. Berman); When samples come from patients prior to therapy, between 92-96% of samples are positive by our PCR assay, with a false negative rate of 4-8% percent, depending upon the source of the patient sample. If the patient has completed therapy by 2-6 months, 0-4 patients (0%) are positive, indicating the effectiveness of the therapy at removing detectable levels of *Leishmania* DNA from peripheral blood. The detection rate for purely cutaneous disease is approximately 11% (1 out of 9), indicating the absence of detectable *Leishmania* DNA in peripheral blood if the disease is confined to skin lesions. If aspirates are taken of cutaneous lesions, these samples do test positive using our PCR protocol, indicating presence of parasite DNA in an actively infected lesion.

4. Conclusions

The need for sensitivity in detecting the very low levels of *Leishmania* present in peripheral blood suggests that the optimal target sequence would be "pre-amplified", such as DNA present in multiple copies in each parasite. The kinetoplast DNA (kDNA) found in *Leishmania* and related organisms presents a good example of this kind of target. One inherent limitation this target sequence presents, however, is the genetic diversity of the sequences present in kDNA. Differences exist over the largest amount of this sequence, not only between different species of Leishmania, but also potentially within a given organism. The concatenated nature of the target, where some minicircles may not be available to the PCR primers, presents yet another potential problem reducing sensitivity for the PCR reaction. Within these limitations, some regions of conserved DNA sequence have been observed across multiple *Leishmania* species, although there is more similarity between New World species and Old World species than there is across these divisions.

Utilizing DNA sequence information and computer homology searches, multiple PCR primers have been designed within these kDNA sequences, and tested to produce specific PCR products with no significant cross-reaction with non-Leishmania DNA. The reaction conditions have been optimized to detect single copy parasite equivalents in peripheral blood and tested with blinded control patient samples from various geographical locations. The current test is capable of detecting *Leishmania* in these patient samples at very low levels with very good confidence in the result.

Based on the results obtained to date during the first year of this contract, SRA Technologies has developed and is in the process of validating a sensitive and specific PCR based diagnostic test against Leishmaniasis. While we have not yet received large numbers of patient samples to screen for *Leishmania* infection, and

there are certain biological factors regarding the low level of Leishmanial DNA present in peripheral blood in some cases, we are confident that the test can be used on the patient samples as proposed in the original Request for Proposal.

Among the information gained so far as a result of our development and validation of the assay, are novel observations regarding the level of *Leishmania* parasites (as indicated by the presence of *Leishmania* DNA detectable by the PCR assay). It is evident even from our limited number of samples, that *Leishmania* are either absent, or present in extremely low levels (less than 1 parasite or infected cell in 8 mls of blood) in cases of cutaneous disease, or in patients during and after suitable anti-Leishmanial therapies. These biological effects may limit the use of any test that detects *Leishmania* in peripheral blood to cases of visceral disease prior to treatment. The test could be used, however, to monitor the success of therapy due to the observation that the parasites are largely cleared from circulation following successful therapy. Despite these limitations, the PCR based test described in this report exhibits sensitivity to a single parasite equivalent or a single infected cell in 8 mls of blood, as demonstrated by spiking control experiments.

Additional work on developing a second diagnostic primer set is continuing despite disappointing results with almost all of the possible primer sets designed to date. In order for a second primer set to be truly useful, it must possess several characteristics. It must be of approximately equivalent sensitivity as the primary set, in order to function in a confirmatory role. If the sensitivity is significantly different between the two sets, discordant results become impossible to resolve due to the chance that one primer set is able to pick up a low level of infection while the other is not. Accordingly, all primer sets should exhibit similar cross-reactive sensitivity, both in terms of detecting multiple *Leishmania* species while not

producing false positive results with heterologous DNA samples. Discordance of results between multiple primer sets is impossible to resolve if either of these two possibilities exists as well. Finally, the two primer sets should not produce cross-reactive PCR products to reduce the chance of cross-contaminating the PCR reactions, leading to false positive results.

For the second year of this contract, SRA Technologies proposes to continue assay development and control sample testing as directed by the Leishmania Working Group. It is anticipated that at some time in the near future, the Group will provide large numbers of potentially infected patient samples for screening using our PCR test. Several manuscripts detail the application of this technology for the detection and diagnosis of Leishmanial infections are currently in preparation.

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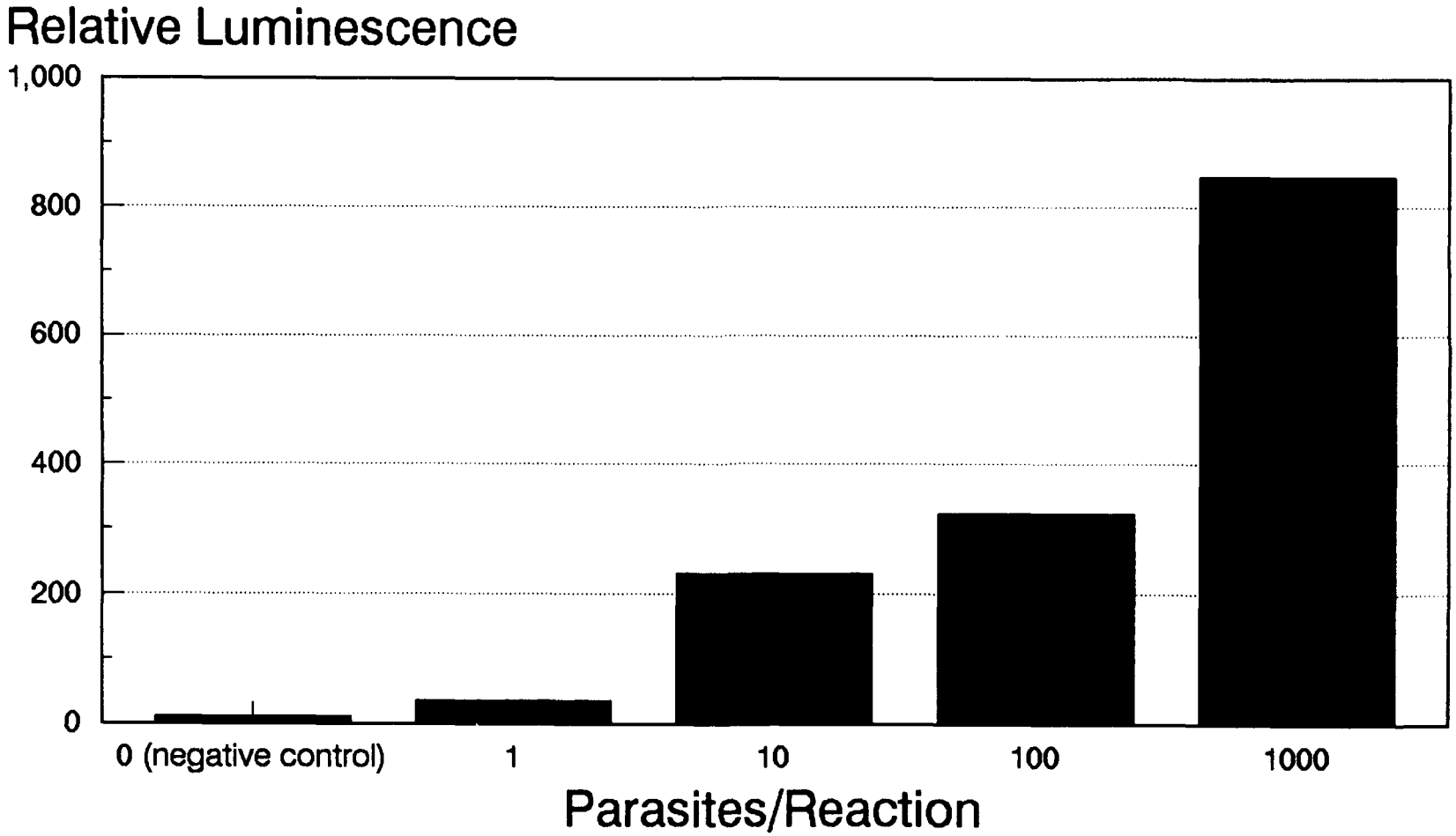
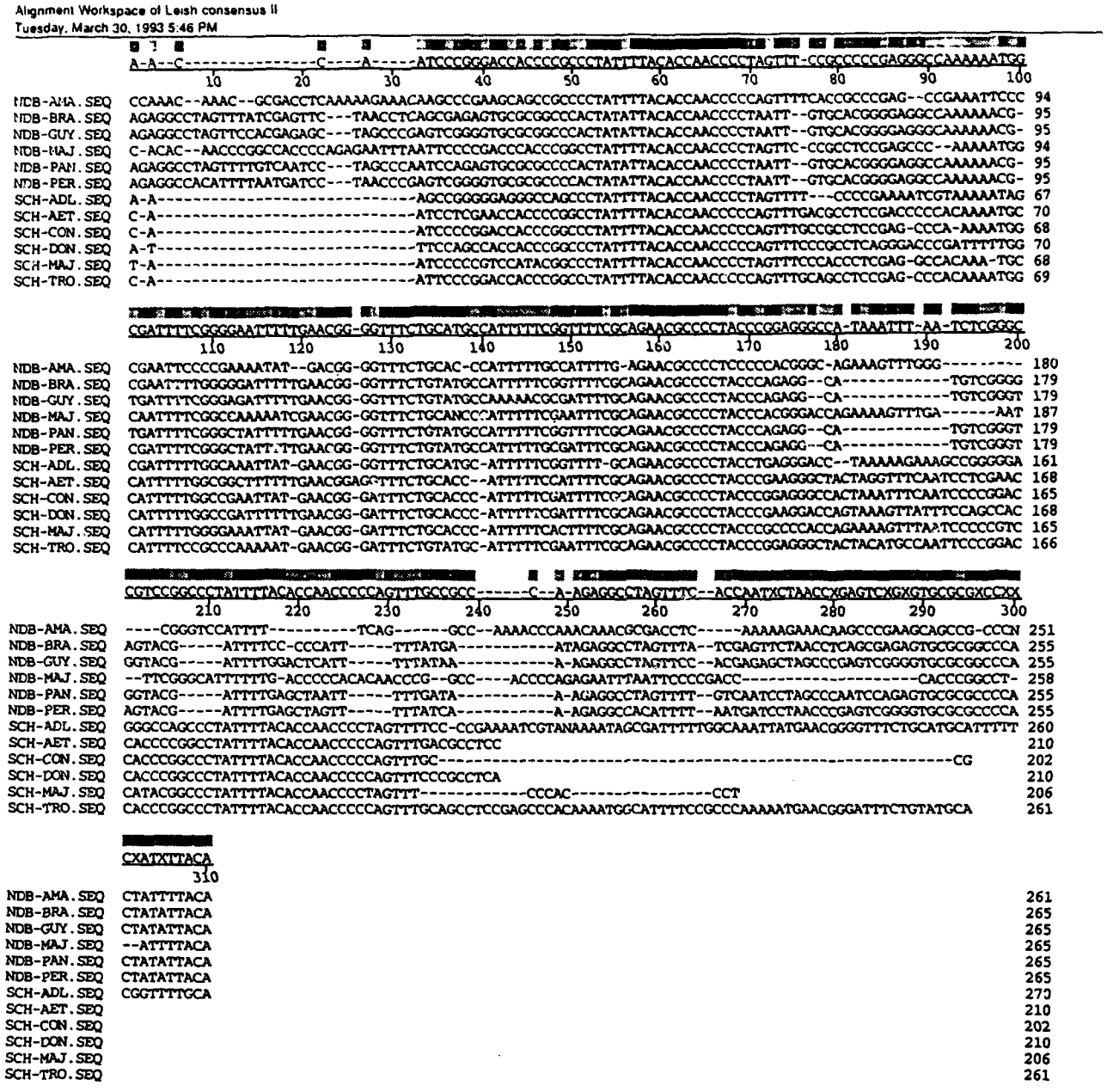
Figure 1. Detection of Leishmania tropica minicircle DNA by PCR



Dilutions of total DNA extracted from L. tropica promastigotes were prepared to correspond to the indicated numbers of parasites per reaction. Amplification was performed for 40 cycles using primers designed to amplify Old World Leishmania, and designated as sequences JW11 (5'-CCTATTTTACACCAACCCCMAGTTT, where M denotes a mixed base position: C,T) and JW12 (5'-CGGGTAGGGGCGTTCTGCGAAAMT, where M denotes A,T). Specific detection of the amplified sequences by alkaline phosphatase-labelled JW14 (5'-ATTGAACGGGGTTTCTGTATGCATTTTTCGAA) was performed in a 96 well plate, with chemiluminescent detection.

Figure 2. Sequence Alignment of Conserved Region of Leishmania Minicircle kDNA.

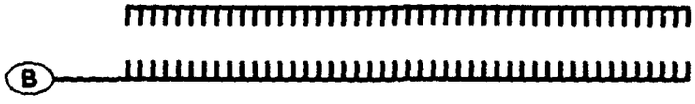


Published sequences of Leishmania minicircle conserved regions were compared using LaserGene (DNAStar, LTD), generating the underlined consensus sequence. The sequences designated "NDB" are the complementary strands to those reported by De Bruijn, et al (11); those designated "SCH" have been reported by Schoone, et al (9). The following abbreviations were used to refer to species and subspecies: AMA: amazonensis; BRA: braziliensis; GUY: guyanensis; MAJ: major; PAN: panamensis; PER: peruviana; ADL: adleri; AET: aethopica; CON: Schoone consensus sequence; DON: donovani.

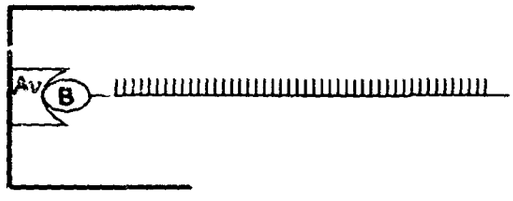
Figure 3. Capture Plate PCR Product Analysis

① PCR reaction is run using 1 biotinylated primer
Specific product is produced

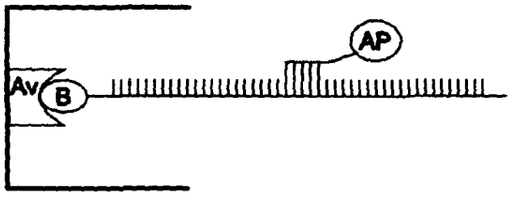
② PCR products are denatured by heating



③ The biotinylated strand is captured in an avidin-coated microplate well



④ A specific synthetic oligonucleotide coupled to Alkaline Phosphatase is allowed to hybridize to the bound PCR product



⑤ A chemiluminescent substrate is added. The breakdown of this substrate produces light, which is detected by a microplate luminometer

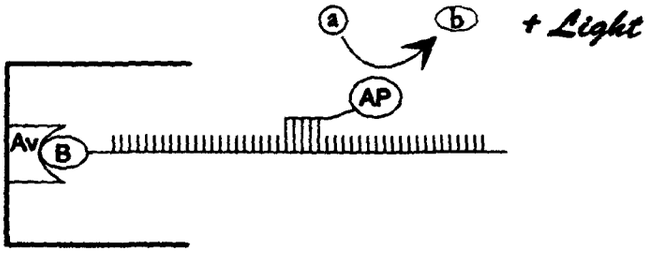


Figure 4. PCR Primers Tested Against L. Tropica

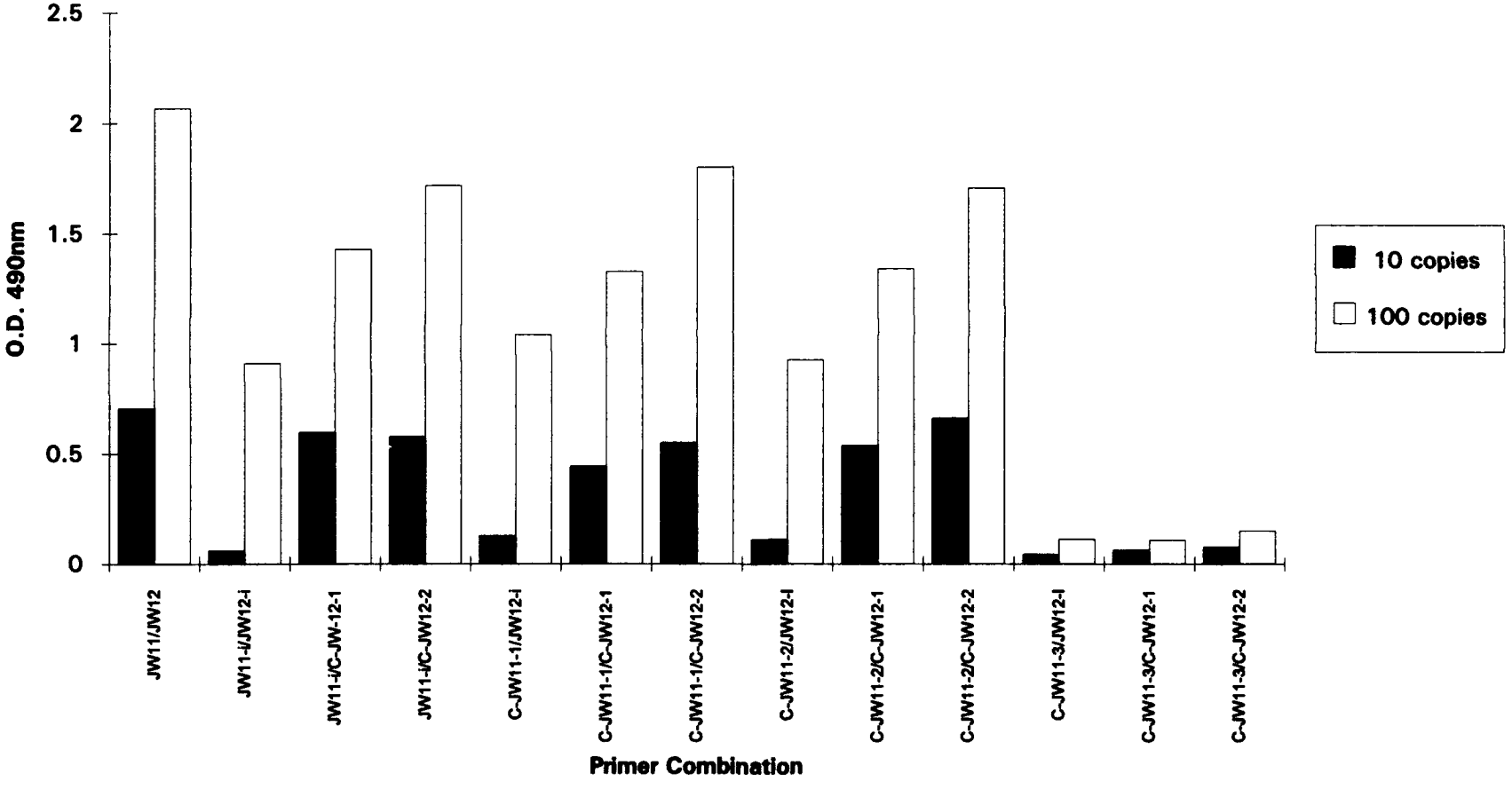


Figure 5. PCR Primers Against L. Donovani

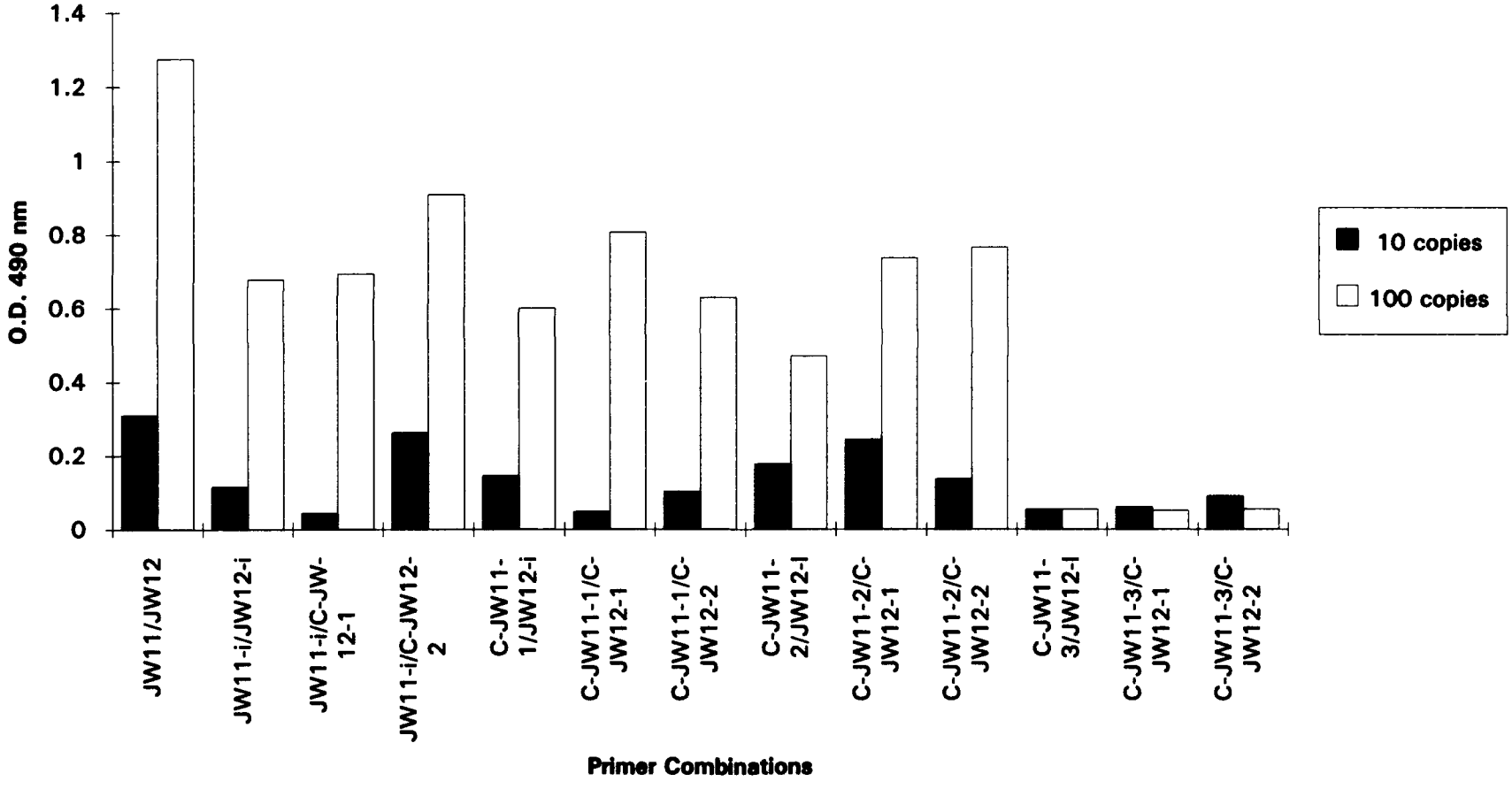
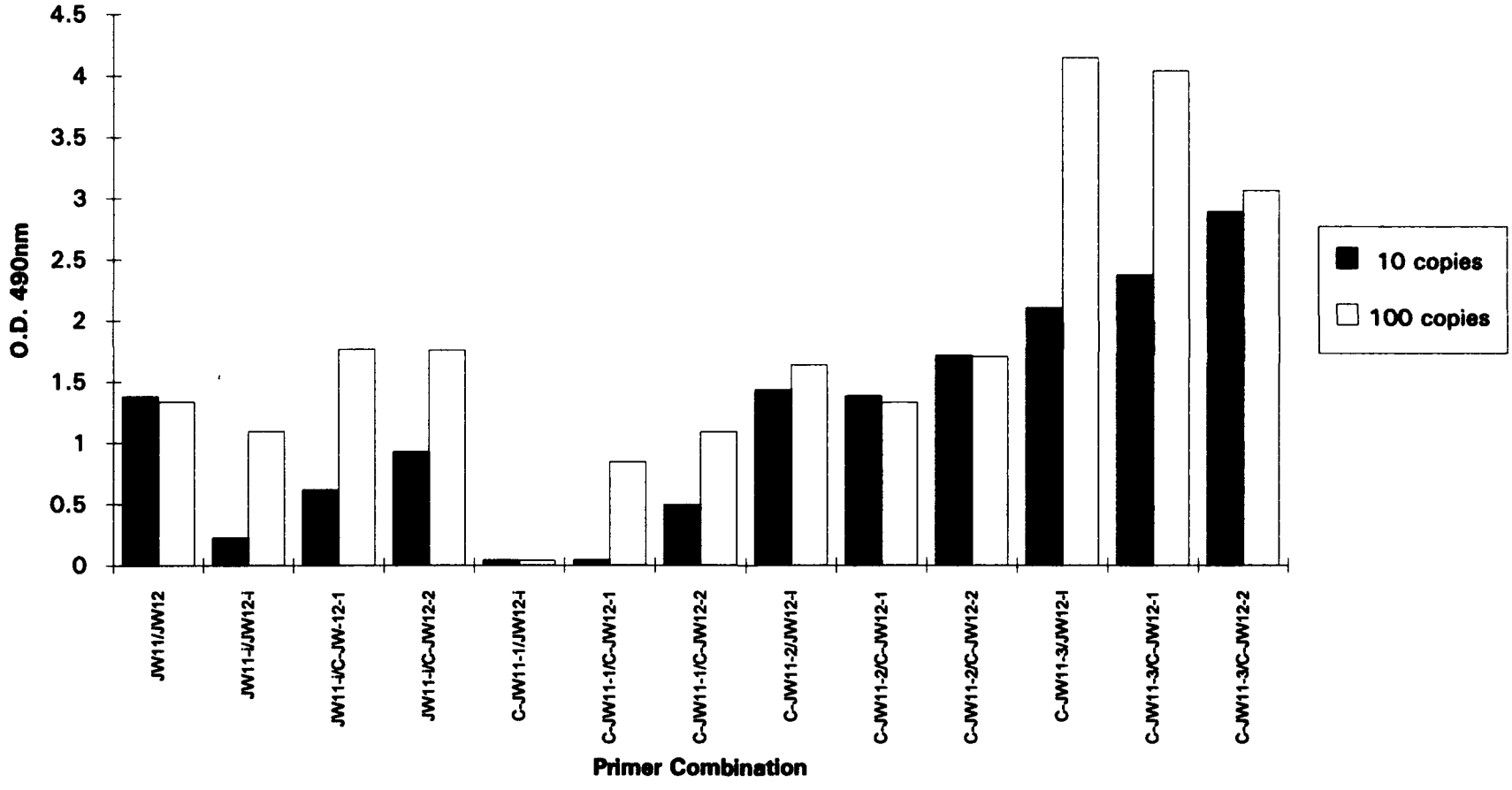


Figure 6. PCR Primers Against L. Brazilliensis



SRA #	Type	Received Date	Stdy	Assay Date	Probe	Tube	Tube	Dx
17309	SM	15-OCT-92	LM	10-DEC-92	JW11/12	-	-	NEG
17310	SM	15-OCT-92	LM	10-DEC-92	JW11/12	-	-	NEG
17311	SM	15-OCT-92	LM	10-DEC-92	JW11/12	+	+	POS
17311	SM	15-OCT-92	LM	16-DEC-92	JW11/12	+	+	POS
17312	SM	15-OCT-92	LM	10-DEC-92	JW11/12	+	+	POS
17313	SM	15-OCT-92	LM	10-DEC-92	JW11/12	+	+	POS
18117	SM	06-NOV-92	LM	10-DEC-92	JW11/12	-	-	NEG
18118	SM	06-NOV-92	LM	10-DEC-92	JW11/12	-	-	NEG
18544A	BL	23-NOV-92	LM	02-DEC-92	JW11/12	-	-	NEG
18544B	BL	23-NOV-92	LM	02-DEC-92	JW11/12	-	-	NEG
18661A	BM	24-NOV-92	LM	16-DEC-92	JW11/12	-	-	NEG
18661B	BM	24-NOV-92	LM	02-DEC-92	JW11/12	-	-	NEG
18662	PL	24-NOV-92	LM	02-DEC-92	JW11/12	-	-	NEG
20027	BL	13-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20028	BM	13-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20278	BL	26-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20279	BM	26-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20280	BL	26-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20281	BM	26-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20344	BL	27-JAN-93	LM	03-FEB-93	JW11/12	-	+	IND
20345	BM	27-JAN-93	LM	03-FEB-93	JW11/12	+	-	IND
20366	BL	28-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20367	BM	28-JAN-93	LM	03-FEB-93	JW11/12	-	-	NEG
20401	BL	01-FEB-93	LM	19-FEB-93	JW11/12	-	-	NEG
20402	BM	01-FEB-93	LM	19-FEB-93	JW11/12	-	-	NEG
21243	BL	03-MAR-93	LM	09-MAR-93	JW11/12	-	-	NEG
21244	BM	03-MAR-93	LM	09-MAR-93	JW11/12	-	-	NEG
21459	BL	10-MAR-93	LM	15-MAR-93	JW11/12	-	-	NEG
21460	BM	10-MAR-93	LM	15-MAR-93	JW11/12	-	-	NEG
21551	BL	15-MAR-93	LM	25-MAR-93	JW11/12	-	-	NEG
21552	BM	15-MAR-93	LM	25-MAR-93	JW11/12	-	-	NEG
22745	BL	04-MAY-93	LM	05-MAY-93	JW11/12	-	-	NEG
22955	BL	12-MAY-93	LM	03-AUG-93	JW11/12	+	+	POS
22956	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22957	BL	12-MAY-93	LM	13-MAY-93	JW11/12	-	-	NEG
22968	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22969	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22970	BL	12-MAY-93	LM	20-MAY-93	JW11/12	+	+	POS
22972	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22973	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22974	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22975	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22976	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22977	BL	12-MAY-93	LM	20-MAY-93	JW11/12	+	+	POS
22978	BL	12-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
22979	BL	12-MAY-93	LM	13-MAY-93	JW11/12	-	-	NEG
23000	BL	13-MAY-93	LM	14-MAY-93	JW11/12	-	-	NEG
23011	BL	14-MAY-93	LM	15-MAY-93	JW11/12	-	-	NEG
23012	BL	14-MAY-93	LM	20-MAY-93	JW11/12	-	-	NEG
23019	BL	14-MAY-93	LM	15-MAY-93	JW11/12	-	-	NEG
23046	BL	17-MAY-93	LM	20-MAY-93	JW11/12	+	+	POS
23056	BM	18-MAY-93	LM	20-MAY-93	JW11/12	+	+	POS
23084	BL	18-MAY-93	LM	20-MAY-93	JW11/12	-	-	NEG
23085	BM	18-MAY-93	LM	20-MAY-93	JW11/12	-	-	NEG
23250	BL	21-MAY-93	LM	25-MAY-93	JW11/12	+	+	POS
23251	BL	21-MAY-93	LM	01-JUL-93	JW11/12	-	-	NEG
23324	BL	26-MAY-93	LM	27-MAY-93	JW11/12	-	-	NEG
23328A	BL	26-MAY-93	LM	30-MAY-93	JW11/12	+	+	POS
23328B	BL	26-MAY-93	LM	30-MAY-93	JW11/12	+	+	POS

SRA #	Type	Received Date	Stdy	Assay Date	Probe	Tube	Tube	Dx
23344	BL	27-MAY-93	LM	01-JUL-93	JW11/12	-	-	NEG
23345	BM	27-MAY-93	LM	03-AUG-93	JW11/12	-	-	NEG
23384A	SM	01-JUN-93	LM	02-JUL-93	JW11/12	-	-	NEG
23384B	SM	01-JUN-93	LM	02-JUL-93	JW11/12	-	-	NEG
23385	BL	01-JUN-93	LM	02-JUL-93	JW11/12	-	-	NEG
23573	BL	07-JUN-93	LM	08-JUN-93	JW11/12	-	-	NEG
23574	BL	07-JUN-93	LM	08-JUN-93	JW11/12	-	-	NEG
23575	BL	07-JUN-93	LM	08-JUN-93	JW11/12	-	-	NEG
23576	BM	07-JUN-93	LM	08-JUN-93	JW11/12	-	-	NEG
23677	BL	11-JUN-93	LM	15-JUN-93	JW11/12	-	-	NEG
23678	BL	11-JUN-93	LM	15-JUN-93	JW11/12	-	-	NEG
23757	BM	15-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23758	BL	15-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23827	BL	17-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23828	BM	17-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23877	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23878	BM	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23879A	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23879B	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23880	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23881	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23882	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23883	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23884A	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23884B	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23885	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23886	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23887	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23888	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23889	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
23890	BL	21-JUN-93	LM	24-JUN-93	JW11/12	-	-	NEG
24068	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24069	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24070	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24071	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24072	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24073	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24074	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24075	BL	25-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24102	BL	28-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24125	BM	29-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24126	BL	29-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24127	BM	29-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
24175	BL	02-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
24176	BL	02-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
24177	BL	02-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
24178	BL	02-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
24223	BL	06-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
24224	BM	06-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
24454A	BL	14-JUL-93	LM	29-JUL-93	JW11/12	-	-	NEG
24454B	BL	14-JUL-93	LM	29-JUL-93	JW11/12	-	-	NEG
24566	BL	21-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
24567	BM	21-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
24930	BL	09-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
24931	BL	09-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
24932	BL	09-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
24932	BL	09-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
25286	BL	24-AUG-93	LM	01-SEP-93	JW11/12	-	-	NEG
25391	BL	30-AUG-93	LM	08-SEP-93	JW11/12	-	-	NEG

SRA #	Type	Received Date	Stdy	Assay Date	Probe	Tube	Tube	Dx
25474	BL	03-SEP-93	LM	08-SEP-93	JW11/12	-	+/-	IND
25475	BM	03-SEP-93	LM	08-SEP-93	JW11/12	-	-	NEG
Z000101	BL	09-DEC-92	LM	10-DEC-92	JW11/12	+	+	POS
Z000102	BL	09-DEC-92	LM	10-DEC-92	JW11/12	-	-	NEG
Z000103	BL	09-DEC-92	LM	10-DEC-92	JW11/12	-	-	NEG
Z000104	BL	09-DEC 92	LM	10-DEC-92	JW11/12	+	+	POS
Z000105	BL	09-DEC-92	LM	10-DEC-92	JW11/12	+	+	POS
Z000106	BL	09-DEC-92	LM	10-DEC-92	JW11/12	+	+	POS
Z000107	BL	09-DEC-92	LM	10-DEC-92	JW11/12	+	+	POS
Z000108	BL	09-DEC-92	LM	10-DEC-92	JW11/12	-	-	NEG
Z000109	BL	09-DEC-92	LM	10-DEC-92	JW11/12	-	-	NEG
Z000110	BL	09-DEC-92	LM	10-DEC-92	JW11/12	+	+	POS
Z000111	BL	09-DEC-92	LM	10-DEC-92	JW11/12	+	+	POS
Z000112	BL	09-DEC-92	LM	10-DEC-92	JW11/12	+	+	POS
Z000117	BL	16-DEC-92	LM	17-DEC-92	JW11/12	+	+	POS
Z000118	BL	16-DEC-92	LM	17-DEC-93	JW11/12	+	+	POS
Z000119	BL	16-DEC-92	LM	17-DEC-93	JW11/12	-	-	NEG
Z000120	BL	16-DEC-92	LM	17-DEC-93	JW11/12	+	+	POS
Z000139	BL	16-DEC-92	LM	17-DEC-92	JW11/12	+	+	POS
Z000140	BL	16-DEC-92	LM	17-DEC-92	JW11/12	-	-	NEG
Z000141	BL	16-DEC-92	LM	17-DEC-92	JW11/12	+	+	POS
Z000142	BL	16-DEC-92	LM	17-DEC-92	JW11/12	-	-	NEG
Z000143	BL	16-DEC-92	LM	17-DEC-92	JW11/12	-	-	NEG
Z000144	BL	16-DEC-92	LM	17-DEC-92	JW11/12	+	+	POS
Z000145	BL	16-DEC-92	LM	17-DEC-92	JW11/12	+	+	POS
Z000146	BL	16-DEC-92	LM	17-DEC-92	JW11/12	-	-	NEG
Z000161	BL	30-DEC-92	LM	06-JAN-93	JW11/12	-	-	NEG
Z000162	BL	30-DEC-92	LM	06-JAN-93	JW11/12	+	+	POS
Z000163	BL	30-DEC-92	LM	06-JAN-93	JW11/12	+	+	POS
Z000164	BL	30-DEC-92	LM	06-JAN-93	JW11/12	-	-	NEG
Z000165	BL	30-DEC-92	LM	06-JAN-93	JW11/12	+	+	POS
Z000166	BL	30-DEC-92	LM	06-JAN-93	JW11/12	+	+	POS
Z000167	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000168	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000169	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000170	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000171	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000172	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000173	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000174	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000175	BL	11-JAN-93	LM	11-JAN-93	JW11/12	-	-	NEG
Z000176	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000177	BL	11-JAN-93	LM	11-JAN-93	JW11/12	-	-	NEG
Z000178	BL	11-JAN-93	LM	11-JAN-93	JW11/12	+	+	POS
Z000257	BL	18-FEB-93	LM	19-FEB-93	JW11/12	+	+	POS
Z000258	BL	18-FEB-93	LM	19-FEB-93	JW11/12	+	+	POS
Z000259	BL	19-FEB-93	LM	19-FEB-93	JW11/12	+	+	POS
Z000260	BL	18-FEB-93	LM	19-FEB-93	JW11/12	+	+	POS
Z000261	BL	18-FEB-93	LM	19-FEB-93	JW11/12	-	-	NEG
Z000262	BL	18-FEB-93	LM	19-FEB-93	JW11/12	-	-	NEG
Z000263	BL	18-FEB-93	LM	19-FEB-93	JW11/12	-	-	NEG
Z000264	BL	18-FEB-93	LM	19-FEB-93	JW11/12	-	-	NEG
Z000308	BL	11-MAR-93	LM	15-MAR-93	JW11/12	+	+	POS
Z000309	BL	11-MAR-93	LM	15-MAR-93	JW11/12	+	+	POS
Z000310	BL	11-MAR-93	LM	15-MAR-93	JW11/12	+	+	POS
Z000311	BL	11-MAR-93	LM	15-MAR-93	JW11/12	-	-	NEG
Z000312	BL	11-MAR-93	LM	15-MAR-93	JW11/12	+	+	POS
Z000313	BL	12-MAR-93	LM	15-MAR-93	JW11/12	-	-	NEG
Z000320	BL	23-MAR-93	LM	25-MAR-93	JW11/12	+	+	POS

SRA #	Type	Received Date	Stdy	Assay Date	Probe	Tube	Tube	Dx
Z000321	BL	23-MAR-93	LM	25-MAR-93	JW11/12	-	-	NEG
Z000336	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000337	BL	26-MAR-93	LM	06-APR-93	JW11/12	-	-	NEG
Z000338	BL	26-MAR-93	LM	08-APR-93	JW11/12	-	-	NEG
Z000339	BL	26-MAR-93	LM	08-APR-93	JW11/12	+	+	POS
Z000340	BL	26-MAR-93	LM	08-APR-93	JW11/12	+	+	POS
Z000341	BL	26-MAR-93	LM	08-APR-93	JW11/12	-	-	NEG
Z000342	BL	26-MAR-93	LM	08-APR-93	JW11/12	+	+	POS
Z000343	BL	26-MAR-93	LM	08-APR-93	JW11/12	+	+	POS
Z000344	BL	26-MAR-93	LM	08-APR-93	JW11/12	+	+	POS
Z000345	BL	26-MAR-93	LM	08-APR-93	JW11/12	+	+	POS
Z000346	BL	26-MAR-93	LM	08-APR-93	JW11/12	+	+	POS
Z000347	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000348	BL	26-MAR-93	LM	08-APR-93	JW11/12	-	-	NEG
Z000349	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000350	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000351	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000352	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000353	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000354	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000355	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000356	BL	26-MAR-93	LM	06-APR-93	JW11/12	+	+	POS
Z000357	BL	26-MAR-93	LM	06-APR-93	JW11/12	-	-	NEG
Z00035	BL	26-MAR-93	LM	06-APR-93	JW11/12	-	-	NEG
Z00035	BL	26-MAR-93	LM	06-APR-93	JW11/12	-	-	NEG
Z00036	BL	26-MAR-93	LM	06-APR-93	JW11/12	-	-	NEG
Z000361	BL	26-MAR-93	LM	06-APR-93	JW11/12	-	-	NEG
Z000362	BL	29-MAR-93	LM		JW11/12	-	-	NEG
Z000363	BL	29-MAR-93	LM		JW11/12	-	-	NEG
Z000364	BL	29-MAR-93	LM		JW11/12	-	-	NEG
Z000365	BL	29-MAR-93	LM		JW11/12	-	-	NEG
Z000367	BL	01-APR-93	LM	05-APR-93	JW11/12	-	-	NEG
Z000368	BL	01-APR-93	LM	05-APR-93	JW11/12	-	-	NEG
Z000369	BL	01-APR-93	LM	05-APR-93	JW11/12	-	-	NEG
Z000370	BL	01-APR-93	LM	05-APR-93	JW11/12	-	-	NEG
Z000375	BL	05-APR-93	LM	08-APR-93	JW11/12	-	-	NEG
Z000376	BL	05-APR-93	LM	08-APR-93	JW11/12	-	-	NEG
Z000377	BL	08-APR-93	LM	09-APR-93	JW11/12	-	-	NEG
Z000378	BL	12-APR-93	LM	09-APR-93	JW11/12	-	-	NEG
Z000379	BL	12-APR-93	LM	15-APR-93	JW11/12	+	+	POS
Z000380	BL	12-APR-93	LM	15-APR-93	JW11/12	-	-	NEG
Z000381	BL	12-APR-93	LM	15-APR-93	JW11/12	-	-	NEG
Z000413	BL	23-APR-93	LM	28-APR-93	JW11/12	-	-	NEG
Z000414	BL	23-APR-93	LM	28-APR-93	JW11/12	-	-	NEG
Z000439	BL	30-APR-93	LM	05-MAY-93	JW11/12	-	-	NEG
Z000440	BL	30-APR-93	LM	05-MAY-93	JW11/12	-	-	NEG
Z000460	BM	21-MAY-93	LM	25-MAY-93	JW11/12	-	-	NEG
Z000461	BL	21-MAY-93	LM	25-MAY-93	JW11/12	-	-	NEG
Z000466	LI	25-MAY-93	LM	26-MAY-93	JW11/12	+	+	POS
Z000473	BL	26-MAY-93	LM	27-MAY-93	JW11/12	+	+	POS
Z000474	BL	26-MAY-93	LM	27-MAY-93	JW11/12	+	+	POS
Z000475	BL	26-MAY-93	LM	27-MAY-93	JW11/12	+	+	POS
Z000476	BL	26-MAY-93	LM	27-MAY-93	JW11/12	+	+	POS
Z000501	BL	09-JUN-93	LM	15-JUN-93	JW11/12	-	-	NEG
Z000502	BM	09-JUN-93	LM	15-JUN-93	JW11/12	-	-	NEG
Z000503	BL	11-JUN-93	LM	15-JUN-93	JW11/12	+	+	POS
Z000504	BL	14-JUN-93	LM	15-JUN-93	JW11/12	+	+	POS
Z000505	BL	11-JUN-93	LM	15-JUN-93	JW11/12	+	+	POS
Z000506	BL	11-JUN-93	LM	15-JUN-93	JW11/12	+	+	POS

SRA #	Type	Received Date	Stdy	Assay Date	Probe	Tube	Tube	Dx
Z000507	TE	11-JUN-93	LM	15-JUN-93	JW11/12	-	-	NEG
Z000508	TE	11-JUN-93	LM	15-JUN-93	JW11/12	-	-	NEG
Z000511	PL	24-JUN-93	LM	07-JUL-93	JW11/12	-	-	NEG
Z000517	BL	07-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
Z000518	BL	07-JUL-93	LM	09-JUL-93	JW11/12	-	-	NEG
Z000519	BL	07-JUL-93	LM	09-JUL-93	JW11/12	+	+	POS
Z000520	BL	07-JUL-93	LM	09-JUL-93	JW11/12	+	+	POS
Z000541	BL	12-JUL-93	LM	16-JUL-93	JW11/12	+	+	POS
Z000542	BL	12-JUL-93	LM	16-JUL-93	JW11/12	+	+	POS
Z000543	BL	12-JUL-93	LM	16-JUL-93	JW11/12	+	+	POS
Z000550	BL	16-JUL-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000550	BL	16-JUL-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000551	BL	16-JUL-93	LM	29-JUL-93	JW11/12	-	-	NEG
Z000551	BL	16-JUL-93	LM	29-JUL-93	JW11/12	-	-	NEG
Z000558	BL	22-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000559	BL	22-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000569	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000570	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000572	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000573	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000574	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000575	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000576	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000577	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000578	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000579	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000580	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000581	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000582	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000583	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000584	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000585	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000586	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000587	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000588	BL	30-JUL-93	LM	10-AUG-93	JW11/12	-	-	NEG
Z000589	BL	30-JUL-93	LM	10-AUG-93	JW11/12	+	+	POS
Z000590	BL	30-JUL-93	LM	10-AUG-93	JW11/12	+	+	POS
Z000591	BL	05-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000592	BL	05-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000593	BL	05-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000594	BL	05-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000595	BL	05-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000596	BL	05-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000597	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000598	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000599	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	NEG
Z000600	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000601	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000602	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000603	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000604	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000605	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000606	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000607	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000608	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000609	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000610	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000611	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000612	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG

SRA #	Type	Received Date	Stdy	Assay Date	Probe	Tube	Tube	Dx
Z000613	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000614	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000615	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000616	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000617	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000618	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000619	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000620	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000625	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000626	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000627	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000628	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000629	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000630	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000632	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000633	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000634	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000635	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000636	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000637	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000638	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000639	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000640	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000641	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000642	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000643	BL	10-AUG-93	LM	16-AUG-93	JW11/12	-	-	NEG
Z000644	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000645	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000646	BL	10-AUG-93	LM	16-AUG-93	JW11/12	+	+	POS
Z000647	BL	10-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000648	BL	10-AUG-93	LM	23-AUG-93	JW11/12	+	+	POS
Z000649	BL	10-AUG-93	LM	23-AUG-93	JW11/12	+	+	POS
Z000650	BL	10-AUG-93	LM	23-AUG-93	JW11/12	+/-	-	POS
Z000651	BL	10-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000652	BL	10-AUG-93	LM	23-AUG-93	JW11/12	+	+	IND
Z000653	BL	10-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000654	BL	10-AUG-93	LM	23-AUG-93	JW11/12	+	+	POS
Z000655	BL	10-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000656	BL	10-AUG-93	LM	23-AUG-93	JW11/12	+	+	POS
Z000657	BL	10-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000658	BL	10-AUG-93	LM	23-AUG-93	JW11/12	+	+	POS
Z000659	BL	13-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000660	BM	13-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000668	BL	13-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000669	BL	13-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000673	BL	17-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000674	BM	17-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG
Z000675	LI	18-AUG-93	LM	23-AUG-93	JW11/12	-	-	NEG