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Defining PCR reaction conditions for the amplification of a 3000-4000 ntp sequence of Drosophila melanogaster mtDNA

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Senior Research Project

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ABSTRACT

Mitochondrial DNA (mtDNA) is extremely vulnerable to large deletions. These mutations seem to contribute to the physiological effects of aging as the mtDNA is increasingly exposed to free radicals generated by degenerate respiratory enzymes. The Polymerase Chain Reaction (PCR) and agarose gel electrophoresis are used to optimize reaction conditions necessary to amplify a 3000-4000 nucleotide pair (ntp) segment of *Drosophila melanogaster* mtDNA. Ten primers are designed to amplify different portions of the mitochondrial genome. The reaction conditions necessary to successfully amplify a 216 ntp DNA sequence are confirmed, and the ten primers are examined for PCR amplification compatibility.

INTRODUCTION

Mitochondria are the principal energy transducers for eukaryotic cells. These double-membraned organelles contain their own DNA, which differs significantly from nuclear DNA. Although double-stranded, the mitochondrial genome is circular and much smaller than its nuclear counterpart. The 19,517 ntp genome of *D. melanogaster*, which codes for 13 polypeptides, 22 tRNAs and 2 rRNAs, exists in the matrix of the mitochondrion as a singular chromosome with multiple copies. Furthermore, mitochondrial DNA (mtDNA) does not contain histones or other DNA-packaging proteins (8). Since only 22 of the usual 30 tRNAs are present in the mitochondrial genetic system, relaxed pairing at the third (wobble) position permits fewer tRNA molecules to be involved in translation. In fact, four of the 64 codons actually have a different meaning than predicted from the universal genetic code (1).

In addition to generating an H^+ gradient, the electron transport system (ETS) is a major source of reactive oxygen species (ROS). These ROS, or free radicals, are characterized by an unpaired electron and are highly unstable. Approximately 90% of a cell's oxygen consumption

occurs within the mitochondria, and an estimated 2-3% of the oxygen consumed by the mitochondria is converted into ROS such as superoxide and hydrogen peroxide (3). In order to neutralize these highly reactive free radicals, the body produces natural antioxidants that remove the unpaired electron from an ROS. Antioxidant defenses include naturally-produced superoxide dismutase and glutathione peroxidase as well as vitamins C and E, and ubiquinone (7).

Due to its proximity to the ETS and its lack of protective packaging, the vulnerable mitochondrial genome is prone to fragmentation and deletions caused by escaped ROS (8). Unfortunately, the ETS is not only the generator of free radicals, but also the target. In 1956, Harman proposed the mitochondrial theory of aging. He postulated a cycle where ROS produced by the ETS attack the exposed mtDNA causing mutations. This action, along with the direct modification of inner membrane proteins, compromises the efficiency of respiratory enzymes, producing a rapidly increasing number of free radicals (6). Because the mitochondrial genetic system possesses limited DNA repair mechanisms and the genome is exposed to ROS, mtDNA has a 10 fold higher mutation rate when compared with nuclear DNA (2). Since no introns or noncoding regions are found in mtDNA except for the area surrounding the origin of replication, mutations would be more likely to modify a gene or other critical regions.

ROS interaction with the mtDNA results in accumulated mutations, specifically large deletions. The polymerase chain reaction (PCR) is used to amplify certain sequences of mtDNA and detect the presence of deletions in fruitfly mtDNA. The purpose of this project is to optimize PCR parameters to allow the stable amplification of a relatively large region (3000-4000 ntp) of the larval *Drosophila melanogaster* mitochondrial genome.

MATERIALS AND METHODS

***Drosophila* Cultures.** *Drosophila melanogaster* larvae were incubated using the procedure outlined by Flagg (4). All fruitflies were obtained from the Carolina Biological Supply Company, Burlington, NC.

DNA Extraction. DNA was extracted from twenty wild-type *D. melanogaster* larvae at the third instar stage as described by Sammer (12).

Primer Design. Three primers were initially mapped out using the *D. melanogaster* mitochondrial genome sequence found in the GenBank Database (5). Primers were selected using the guidelines listed by Sharrocks (13). In all, ten primers were analyzed (Table 1). The first

Table 1. PCR Primers

Primer Name[1]	Primer Sequence[2]	MW[3]	GenBank Sequence[4]
Primer 1 (Fred-A)	GAACATAAACCATGAGCA	5495	8613 to 8630
Primer 2 (Edward-B)	GTTGAGGTTATCAGCCAG	5543	9142 to 9159
Primer 3 (Maggie-C)	GGAACTTTACCTCGATTTC	6071	11826 to 11845
Primer 4 (Sue-D)	CTGGAGCTTCAACATGAGC	5799	8908 to 8926
Primer 5 (Deb-E)	CTTCAACTGGTCGAGCTTCC	5722	11514 to 11533
Primer 6 (Jim-F)	GAACAGGATGAACTGTTTATCC	6737	1828 to 1856
Primer 7 (Vicky-G)	TTCTCGTGATACATCTCGTCATC	6919	4907 to 4930
Primer 8 (Cristina-H)	GACCTCCAAAATATTCTGATC	6329	6528 to 6552

[1] Primer name given below each primer.
[2] Given in the 5′→3′ direction.
[3] Given in Daltons (Da).
[4] Using the GenBank numbering system (5).

three primers targeted two overlapping mtDNA sequences, one 512 ntp in length and the other 3196 ntp in length. After several PCR reactions, a fourth primer was designed to complement

one of the primers and target a 216 ntp sequence. Then, a fifth primer was ordered to replace a previous primer and target a 2588 ntp sequence. Three more primers were later ordered to target two new sequences. Finally, a pair of primers were selected with the aid of two computer programs, Primer3 and NetPrimer (10, 9). This new primer pair targeted another sequence 1267 ntp in length. Each of the ten primers arrived from Genosys (The Woodlands, TX) lyophilized and desalted. Each primer was resuspended in 100 µl and diluted to make a 20.0 µM stock primer suspension.

Polymerase Chain Reaction. Four different PCR programs were used to amplify *Drosophila* mtDNA (Table 2). All PCR reactions were performed in a Thermojet™ temperature cycler from EquiBio (Belgium). Each program cycled 30 times and ended with a 10-minute post-cycle at 72°C. Following the post-cycle, the thermocycler incubated the tubes at a final resting temperature of 4°C.

Table 2. PCR Program Parameters

Program #	Trials	Denaturation[1]	Time[2]	Annealing[1]	Time[2]	Elongation[1]	Time[2]
Program 1	1-8,10-12,25-26	94	1	62	1	72	2
Program 2	9,13,19,23,24	94	1	62	1	72	4
Program 3	14-18,21,22	94	1	55	1	72	4
Program 4	20	94	1	65	1	72	4

[1] Measured in °C
[2] Measured in min.

Each microcentrifuge reaction tube contained varying amounts of reagents according to this general formula: 2 µl of DNA, 2 µl of each of the two 20 µM stock primers, 4 µl of deoxynucleotide triphosphates (1.25 µM each), 0.375 µl of DNA polymerase (Table 2), 2.5 µl of 10X TAE buffer, and variable concentrations of $MgCl_2$. Glass distilled, deionized water (d^2H_2O) was added to increase the final tube volume to 25 µl. A drop of sterile mineral oil was used to maintain reagent concentration and to prevent evaporation during PCR reactions.

Table 2. Thermostable DNA polymerases

Polymerase	Isolated from	Company
Taq	*Thermus aquaticus*	FisherBiotech
Taq-A	*Thermus aquaticus*	Promega
Taq-B	*Thermus aquaticus*	Promega
Tbr	*Thermus brokianus*	Amresco
Tth	*Thermus thermophilus*	Epicentre Technologies
Amplitherm	Proprietary	Epicentre Technologies

Agarose Gel Electrophoresis. Following amplification, all PCR products were visualized via agarose gel electrophoresis in a Bio-Rad MiniSub™ DNA cell (Hercules, CA) which was connected to a FisherBiotech Electrophoresis System (Pittsburgh, PA). All PCR products were electrophoresed in a 1% agarose gel with a standard 1X TAE buffer (11). All wells were filled with 10 µl of PCR product and 2 µl of tracking dye except the wells containing the standards (100 and 200 ntp ladders) which were filled with 1 µl of standard and 2 µl of tracking dye. The tracking dye consisted of 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in a solution of 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA. The gels were usually run at 105±2 volts for five minutes before decreasing to 72±2 volts for 80-90 minutes. Ethidium bromide (1.25 µg/ml) was used to stain the gels, and DNA was visualized with an ultraviolet light. Photographs were taken on Polaroid film #667 with a Polaroid Direct Screen Instant Camera (DS 34) using a FisherBiotech Photo-Documentation Hood (Pittsburgh, PA).

RESULTS

To determine if previously extracted *Drosophila* DNA and other components of the PCR reaction mixture were still reliable, Trial 1 was run using primers Sue and Ed with the thermostable DNA polymerase Taq from Promega (Madison, WI). Distinct bands at the correct length of approximately 250 ntp were visible in all three lanes. The first lane which corresponded to a reaction mixture with 2.5 mM of Mg^{2+} stained most intensely (Figure 1). Primer-dimers and

the 100 and 200 ntp standard ladders were clearly visible. For all subsequent trials, unless indicated otherwise, electrophoretic analysis confirmed the presence of primers with visible primer-dimers and successful electrophoresis with well-separated standard bands.

In order to investigate two other primer pairs, trials 2 and 3 using primers Fred and Ed, and Fred and Maggie respectively, were run, but they did not amplify any mtDNA. No bands were observed.

Trial 4, which used primers Sue and Ed, compared four different DNA extractions in order to evaluate DNA integrity. Distinct bands were observed in lanes corresponding to two larval DNA extractions. Larval DNA in the first lane was considered to be optimal for use in PCR reactions due to a slightly higher banding intensity. Curiously, bands approximately 50 ntp in length were observed in the two lanes with amplification (Figure 2).

For trials 5 and 6, which used Fred and Ed, and Fred and Maggie combinations respectively, FisherBiotech Taq A was substituted for Promega Taq A, but the buffer remained the same. No amplification was noted for either trial.

Trial 7 used Sue and Ed primers to determine the effect of buffers from Promega and FisherBiotech. Using the Promega 10X buffer A with the FisherBiotech Taq A polymerase seemed to work better than a FisherBiotech buffer/Taq A combination (Figure 3).

Another primer pair was tested in trial 8. Primers Sue and Maggie were supposed to amplify a 2900 ntp sequence, but when PCR was done and the products were electrophoresed, no amplification was apparent.

Trial 9 doubled the PCR elongation time used in trial 8 from two minutes to four minutes to allow ample time for the primers to be elongated. No bands were seen after electrophoresis.

Trial 10 was run with primers Sue and Ed to check the status of the primers. No amplification was apparent even after the PCR product was rerun as trial 11.

A new solution of Sue was prepared and tested in Trial 12. Primers Sue and Ed were used with three Mg^{2+} concentrations. Gel electrophoresis of the PCR product showed significant amplification of the expected size (~250 ntp) in all lanes. As can be seen in Figure 4, the last two bands corresponding to 2.5 mM and 3.0 mM respectively stained more intensely than the first.

Trial 13 combined Maggie with the newly verified Sue. The PCR elongation time was kept at four minutes. After electrophoresis and staining, no bands were observed.

The next variable changed was the annealing temperature. Trial 14 changed the annealing temperature from 62°C to 55°C. However, no bands were present.

In trial 15, the primers Sue and Maggie were once again used. Tbr, a recombinant thermostable DNA polymerase isolated from *Thermus brokianus* by Amresco (Solon, OH), was substituted for Taq polymerase. Once again no amplification was observed.

Trials 16 and 17 used a new primer named Deb with Sue to attempt to amplify a 2588 ntp segment and to explore several thermostable DNA polymerases. Trial 16 used Tth from Epicentre Technologies (Madison, WI), and trial 17 used Amplitherm, also from Epicentre. Neither trial showed any evidence of DNA amplification.

Trial 18 used the recombinant Tbr with Sue and Deb. Amplification was observed in lanes corresponding to Mg^{2+} concentrations of 2.5 mM and 3.5 mM respectively (Figure 5). The DNA smeared over approximately a 4000 ntp range (between 25 and 4000 ntp). This range encompassed the 2588 ntp amplification expected from primers Sue and Deb.

Trial 19 imposed more stringent conditions by increasing the PCR annealing temperature back to 62°C. When the PCR products were electrophoresed, long streaks were visible in lanes 5 and 6 corresponding to 3.0 mM and 3.5 mM of $MgCl_2$ respectively.

Trial 20 attempted to make conditions for Tbr even harsher. The annealing temperature was increased to 65°C. When the gel was visualized, DNA smears appeared in all five lanes. The most amplification was seen at higher Mg^{2+} concentrations with a range of approximately 3500 ntp (between 25 and 3500 ntp).

In order to amplify a 2590 ntp sequence, trial 21 used the new primer Cristina with Ed while trial 22 used two new primers, Jim and Vicky, to attempt to amplify a 3051 ntp sequence. Trial 22's primer-dimers were more concentrated than the primer-dimers from trial 21.

Trials 23 and 24 substituted the polymerase Tbr for the Taq that had been used before. Trial 23 used a Jim and Vicky primer combination and trial 24 used a Cristina and Ed primer combination. The results were similar to trials 21 and 22 with differentially staining primer-dimers.

Trial 25 tested two newly-ordered primers, Dave and Norman, with both Taq and Tbr polymerases. Neither was able to amplify mtDNA.

To check the status of the reagents, Trial 26 used a Sue/Ed combination with Taq-B polymerase. Also, a cheek epithelial cell PCR run was conducted with Tbr and Taq-B polymerase to act as a control for the enzymes, buffers, and dNTPs. The cheek epithelial primers had been shown to work in previous experiments. When the gel was visualized after electrophoresis, the cheek epithelial DNA had been amplified, but the Sue and Ed primer pair had not amplified any mtDNA.

DISCUSSION

The results of this research project illustrate the complex nature of the polymerase chain reaction. Much fine-tuning of the reaction conditions is often necessary in order to amplify DNA successfully. Many experiments were conducted to adjust variables, verify the reliability of reagents, and optimize amplification. For instance, trial 9 increased PCR elongation time to allow for a longer mtDNA sequence. Trials 14, 21, and 23 altered the PCR annealing temperatures to make conditions more stringent. Even different DNA polymerases were tested in trials 15, 18, and 19. Of course, different Mg^{2+} concentrations were tested in almost all PCR reactions.

Working with previously determined protocols, the Sue/Ed primer pair consistently amplified a 216 ntp sequence. The first trial confirmed the optimum concentration of Mg^{2+} needed for successful PCR of a 216 ntp sequence. Trial 4, which also used Sue and Ed, was used to determine the best of four DNA extracts for amplification. In trial 7, Sue and Ed were used to discover the effect of two buffers on PCR.

Many of the trials did not exhibit any amplification although the standard ladders and primer-dimers were observed. The function of the two standards was twofold. First, any distinct bands could be measured against the known standard DNA ladder to estimate approximate length. Just as important, the existence of visible standards (and primer-dimers) verified that electrophoresis was successful.

Primer design was found to be one of the most challenging aspects of PCR technique. Some of the variables to consider when selecting primers are G/C ratio, secondary structure, self-complementarity, dimerization, and GC clamps. If the G/C ratio is low, the oligonucleotide will not bind strongly to its complementary strand. If secondary structure or self-complementarity

occurs, the primers will not be able to bind to DNA due to their conformation. GC clamps are needed to increase the likelihood of primer-binding and the polymerization of the targeted sequence.

Sue and Ed were the only primers that consistently amplified mtDNA. Fred was eliminated after it was noticed that all PCR reactions with that primer present were not amplifying DNA. Likewise, Maggie was eliminated after a series of unproductive experiments. Jim, Vicky, and Cristina showed increased secondary structure and dimerization (Table 3) and thus were also retired after PCR and gel electrophoresis evaluation. For unknown reasons, Deb,

Table 3. Primer Design Variables

Primer	Secondary Structure[1]	Dimerization[1]
Primer 1 (Fred-A)	Very weak	No
Primer 2 (Edward-B)	Very weak	No
Primer 3 (Maggie-C)	Very weak	No
Primer 4 (Sue-D)	Weak	No
Primer 5 (Deb-E)	None	No
Primer 6 (Jim-F)	Moderate	No
Primer 7 (Vicky-G)	Very weak	No
Primer 8 (Cristina-H)	None	Yes
Primer 9 (Dave)	None	No
Primer 10 (Norman)	None	No

[1]As tested by Genosys (The Woodlands, TX)

Norman, and Dave never successfully amplified DNA. It is possible that secondary structure in the mtDNA such as tRNA gene regions prevented the primers from binding or the DNA polymerases from replicating.

The cause of the smears observed in trials 18 and 20 could be attributed to many factors. Unspecific annealing exhibits DNA smears similar to the pattern seen in Figure 5. The wide range was not characteristic of amplification in the reverse direction. It is possible that endonucleases contaminated the reaction mixture during the pipetting of reagents. It should also be noted that the smearing was not uniform at different Mg^{2+} concentrations. This leads to the conclusion that the tubes containing the PCR reaction mixture for those lanes were contaminated as a result of laboratory procedure. However, these characteristic smears were detected consecutively, and no other gels had the same difficulty.

The "extra" bands seen on the gel in trial 4 were another unusual situation (Figure 1). These bands, observed below 100 ntps but before the primer-dimers, were visible in lanes 2 and 4 corresponding to larval DNA 1 and 3. The cause of these unidentified bands is not clear. It is possible that the two DNA samples exhibiting the extra bands had low levels of endonuclease activity that partially digested the DNA; however, the unexpected bands seem to be associated with DNA amplification. Since the bands were not consistent over all four lanes, the problem does not seem to lie with the primers or the Mg^{2+} concentration. It is likely that they are a consequence of additional dimerization of the primers although the cause is unknown.

CONCLUSION

The difficulties inherent in modifying PCR amplification protocol for a particular sequence prevented the full accomplishment of the original goals. However, many primers were eliminated, and the conditions for a shorter sequence were confirmed. Primer design proved to be

the most complex problem. The next step in this project would be to design effective primers. Two primers that were designed by computer software and ordered from Genosys (The Woodlands, TX) were not able to be fully examined. These primers would a good place to start. Once localized smears or diffuse bands are observed, the adjustment of annealing temperature and other variables could elucidate reaction parameters relatively quickly.

Much more research remains to be done on this project. This study has been limited to larval mtDNA. Adult mtDNA especially in senescent fruitflies should experience more mutations and fragmentation. Once reaction parameters are established for several lengths of DNA, *Drosophila* can be evaluated for deletion mutations throughout the life cycle. Another logical step would be to raise the fruitflies at different temperatures and test for deletions. The increased temperature should raise their metabolic rate, more ROS should be produced, and mutations should accumulate with increasing temperature.

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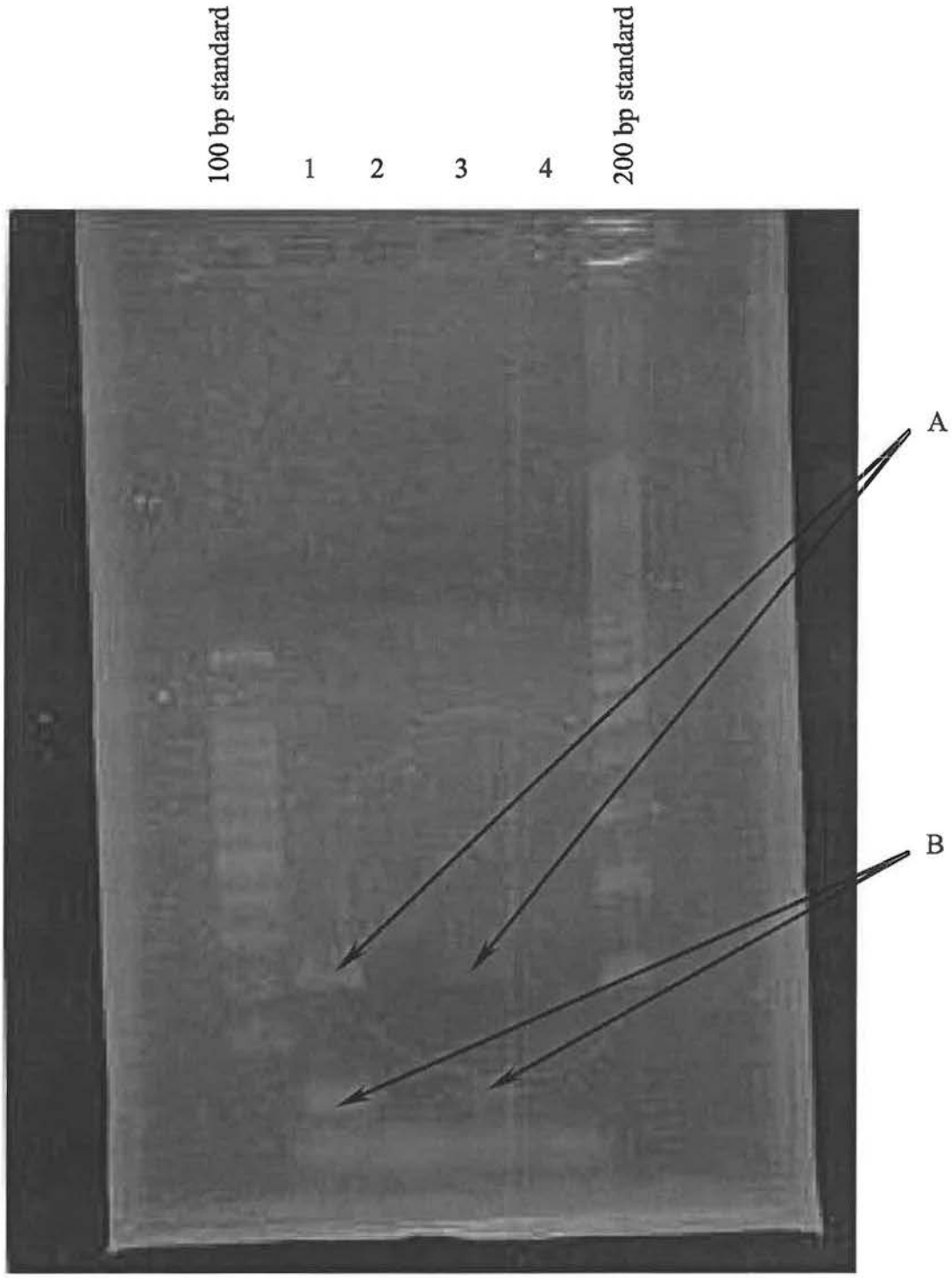

Figure 2. Trial 4 agarose gel electrophoresis. This trial compared four extracts of *Drosophila* DNA. Note the 100 and 200 ntp standard ladders and the presence of bands in the first and third lanes. A) Expected 216 ntp bands B) Unexpected bands

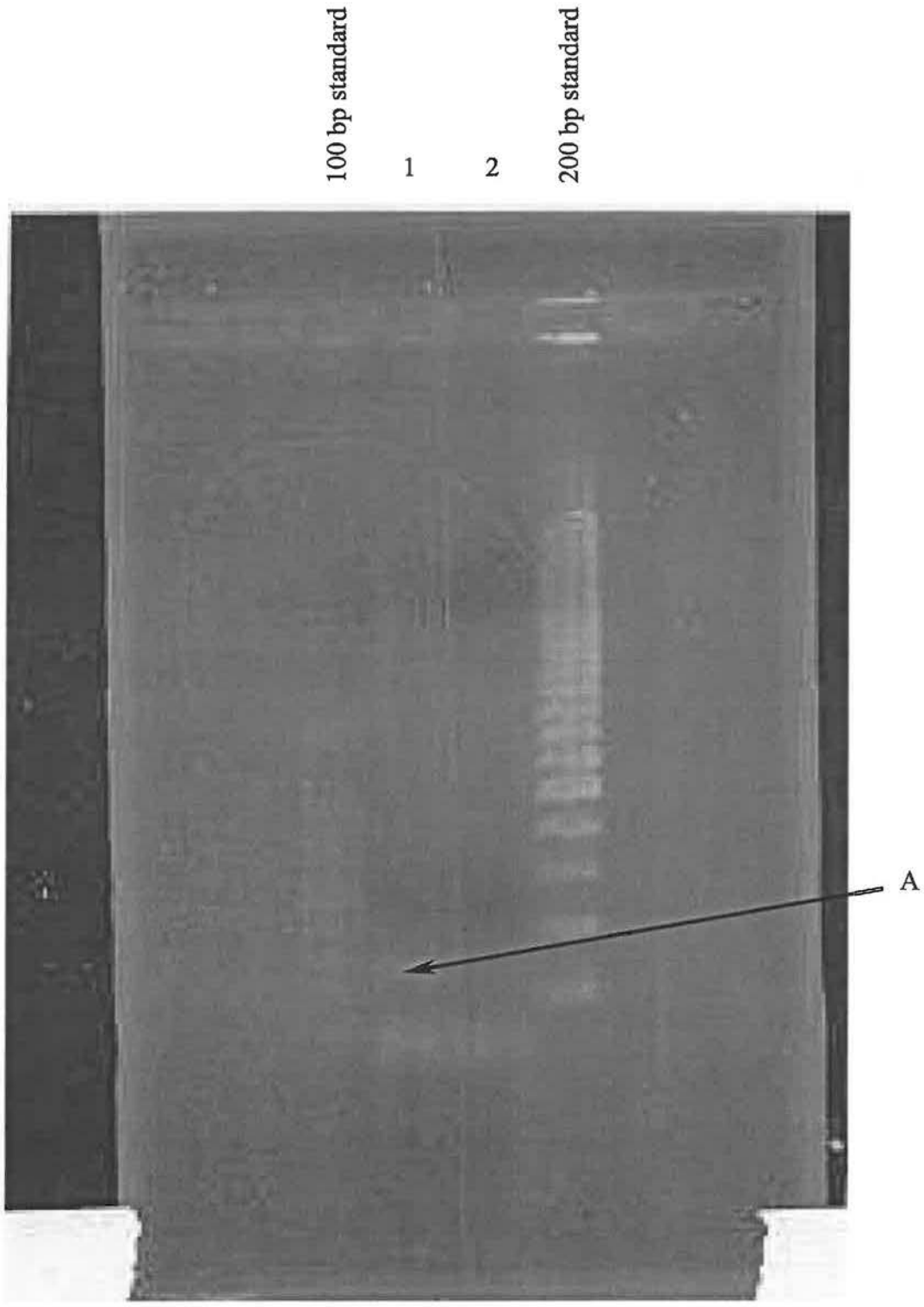

Figure 3. Trial 7 agarose gel electrophoresis. This trial determined the effect of buffers from Promega (lane 1)and FisherBiotech (lane 2). Note the 100 and 200 ntp standard ladders and the presence of a band in the first lane. A) 216 ntp band

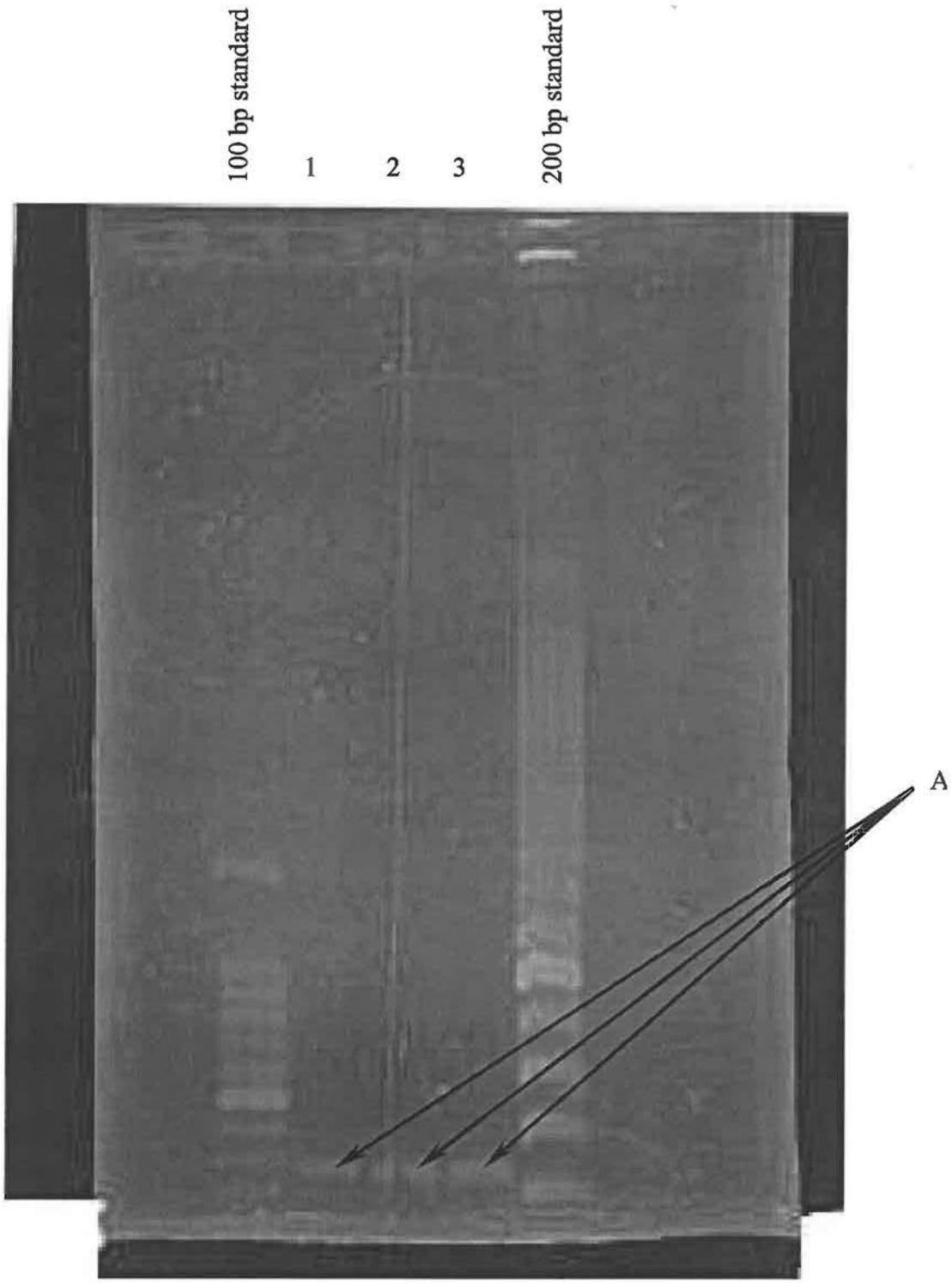

Figure 4. Trial 12 agarose gel electrophoresis. This trial tested primers Sue and Ed while varying Mg^{2+} concentration. Note the 100 and 200 ntp standard ladders and the presence of bands in amplification lanes 1-3. A) 216 ntp bands

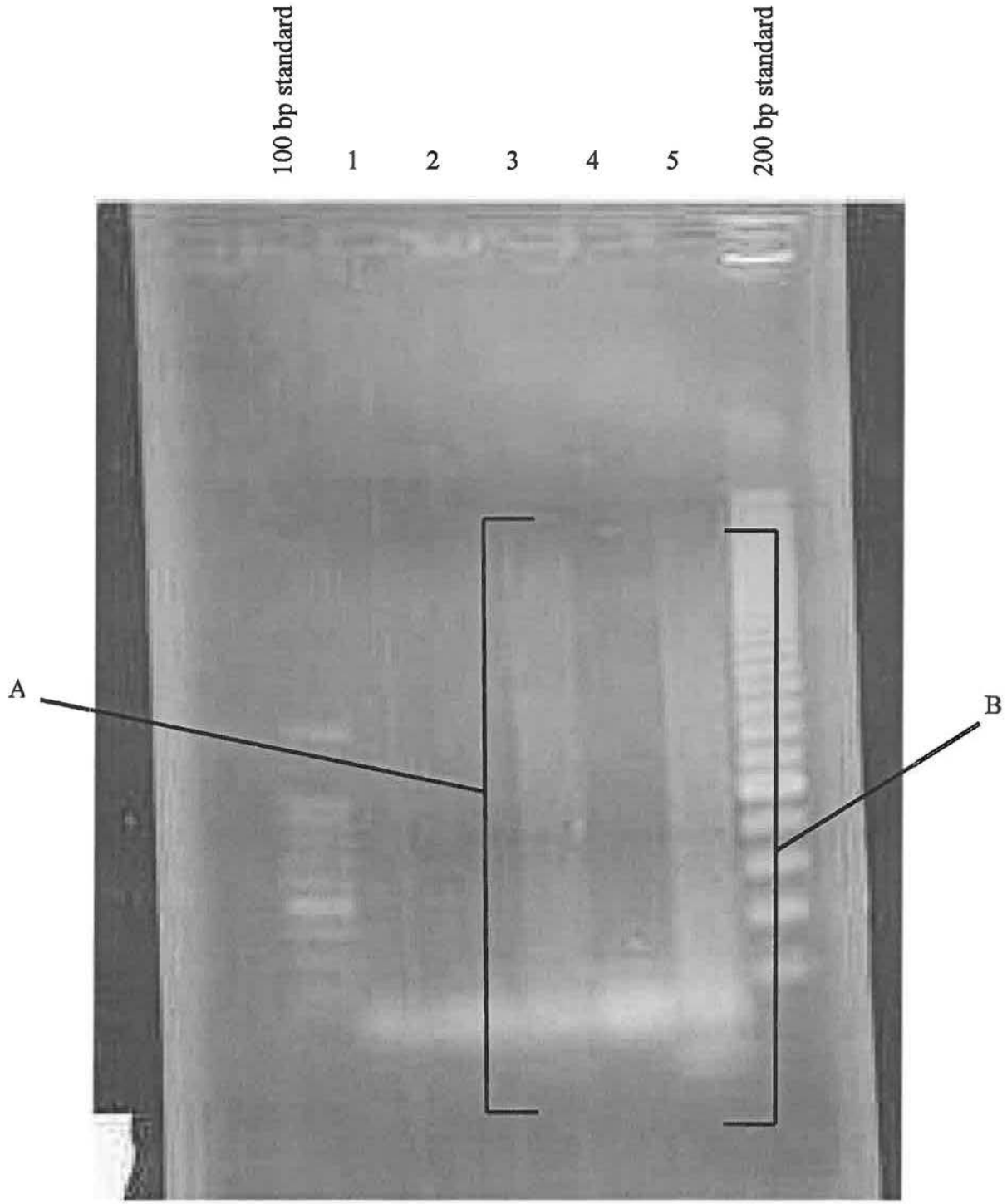

Figure 5. Trial 20 agarose gel electrophoresis. This trial made conditions for primers Sue and Deb more stringent by annealing at 65°C. Note the 100 and 200 ntp standard ladders and the presence of heavy smears in the third and fifth lanes. A) Lane 3 smear B) Lane 5 smear

SOUTHERN SCHOLARS SENIOR PROJECT

Name: Tim Arakawa Date: 10/29/98

Major: Biology

Senior Project

A significant scholarly project, involving research, writing, or special performance, appropriate to the major in question, is ordinarily completed the senior year. Ideally, this project will demonstrate an understanding of the relationship between the student's major field and some other discipline. The project is expected to be of sufficiently high quality to warrant a grade of A and to justify public presentation. The completed project, to be turned in in duplicate, must be approved by the Honors Committee in consultation with the student's supervising professor three weeks prior to graduation. The 2-3 hours of credit for this project is done as directed study or in a research class.

Keeping in mind the above senior project description, please describe in as much detail as you can the project you will undertake:

This senior research project will study the effects of aging on fruitflies. Using the polymerase chain reaction (PCR) and agarose gel electrophoresis, I am determining the reaction conditions necessary to amplify large (3196 bp) and small (512 bp) segments of the mitochondrial genome. I will then search for "hot spots" on Drosophila mitochondrial DNA (mtDNA), areas where mtDNA are most likely to occur. Once these hot spots are located, I will be able to study how variations in mtDNA correlate with age.

Expected date of completion April 1999

Signature of faculty advisor JAzevedo

Approval to be signed by faculty advisor when project is completed:

This project has been completed as planned: yes

This is an "A" project: yes (A-)

The project is worth 2-3 hours of credit: 2

Advisor's Final Signature JAzevedo

Chair, Honors Committee [illegible] Date Approved: 4/15/99