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VALIDATION OF FOUR MULTIPLEX SNP PANELS

FOR FORENSIC DNA TESTING: AN ASSESSMENT

OF THE SENSITIVITY AND REPRODUCIBILITY

OF THE SNaPshot™ PRIMER EXTENSION ASSAY

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VALIDATION OF FOUR MULTIPLEX SNP PANELS FOR FORENSIC DNA TESTING: AN ASSESSMENT OF THE SENSITIVITY AND REPRODUCIBILITY OF THE SNaPshot™ PRIMER EXTENSION ASSAY

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CHAPTER I

INTRODUCTION

Deoxyribonucleic acid (DNA), the genetic blueprint of life, is present in every cell of the body, with the exception of red blood cells. Individuals are 99.9% genetically identical [6]. The remaining 0.1% of DNA is composed of variations in the human genome differentiating individuals from one another. Forensic DNA analysts examine these variations, or polymorphisms, in order to identify the source of a biological sample found at a crime scene. STRs (Short Tandem Repeats) are length polymorphisms in DNA that have become the standard for identifying evidence. Mitochondrial DNA (mtDNA), which is maternally inherited, is frequently used to determine identity when biological samples are too highly degraded for STR analysis. Another type of polymorphism found in the human genome is the single nucleotide polymorphism (SNP). SNPs are biallelic markers in the human genome considered to represent the majority of genetic variability differentiating individuals [14]. In order to be useful in human identification, a SNP, a polymorphic nucleotide at a specific locus, must occur with a frequency exceeding 1% in a given population [14]. While SNPs have been studied for many years in fields such as pharmacogenetics and molecular epidemiology, they are currently being evaluated for use in forensic DNA analysis. SNPs have an advantage over STRs and mtDNA because they require shorter fragments of DNA for detection. However, because SNPs have only

2 alleles compared to 5 or more alleles per STR loci, many more SNPs (>50) are required to provide a significant level of discrimination between individuals [16].

SNPs are the most basic and common unit of genetic variation, accounting for 85% of the genetic variability in humans [15]. An individual may have a cytosine (C) at a specific position in the genome where another individual has a thymine (T). It is believed that SNPs occur once every 1000 bases, however one report suggests they occur every 100-300 base pairs (bp) [6, 7]. With approximately 3 billion base pairs in a single copy of the human genome [4], it is estimated each individual has between 3 and 10 million SNPs [13]. Often when examining biological evidence from crime scenes, the DNA is too degraded for successful forensic analysis using STRs. STR analysis requires fragments of DNA approximately 300 to 400bp in length [12], and mtDNA sequencing involves the initial amplification of fragments approximately 150 to 300bp. Due to harsh environmental conditions for prolonged periods of time DNA may be degraded below these fragment sizes. SNP detection involves the analysis of sequence polymorphisms rather than length polymorphisms. Therefore, shorter fragments of DNA can be utilized for SNP analysis as opposed to STR typing. SNP analysis can be done on pieces of DNA as small as 60-80bp [8]. In addition to working with smaller pieces of DNA, SNPs have a potential forensic value because numerous markers are being identified that are population specific and have a low mutation rate [4]. It is believed SNPs will aid forensic DNA analysts in the identification process of highly degraded and minute quantities of human biological remains.

Orchid Cellmark provided four proprietary multiplex SNP primer panels, each of which has been developed to identify 10-12 SNPs using a modified version of the SNaPshot™ Multiplex protocol. The commercially available STR typing kits routinely used in forensic testing require an input of between 0.5 to 2ng of DNA. Orchid Cellmark has suggested using 2ng of DNA with each of their multiplex SNP primer panels. However, preliminary data has indicated that as little as 100pg of DNA can yield results with many of the SNP markers. Several methods of SNP detection exist. This project relied on the use of multiplex SNP extension primers in conjunction with the ABI® SNaPshot™ Multiplex Kit (Applied Biosystems, Foster City, CA) to identify SNPs in the nuclear genome. Analysis of 50 or more SNPs would be very laborious if single-tube polymerase chain reaction (PCR) was used for sample testing. Multiplexing SNP primers to include 10-12 per reaction tube will increase the throughput of SNP analysis. The cost of the analysis can also be reduced using multiplexes since the amount of reagents per SNP is decreased. This project determined if the SNaPshot™ extension assay used in conjunction with the Orchid Cellmark multiplex panels could accurately detect SNPs at quantities less than 2ng DNA on a capillary electrophoresis (CE) platform.

Orchid Cellmark developed a total of 45 SNP markers for their initial analysis. SNP markers were chosen based upon their distribution throughout the human genome and because they have either a cytosine or thymine allelic variation. Table 1 shows the 45 SNP markers and their chromosomal locations. The amplification and analysis of 45 individual SNP loci would be extremely time consuming and laborious. Therefore, the 45 SNP markers have been grouped into four multiplex reactions, which allows the

simultaneous amplification of 10-12 SNP loci per panel. Following amplification and cleanup, a cocktail of extension primers is added which allows the simultaneous detection of the allelic variation present at each of the SNP markers in a panel. Detection of SNPs using this method is accomplished by using primers which anneal to single stranded DNA immediately 5' to the SNP site of interest. The primers included in each multiplex extension panel are composed of two distinct regions. A sequence specific segment (21 to 27 nucleotides) which anneals to the template DNA to identify the SNP position, and a segment attached to the 5' end composed of a poly-T tail of varying length (3 to 68 nucleotides). The poly-T tail enables the extension products within a multiplex to vary in length between 23 and 91 bases. Once a primer binds to the appropriate sequence, Taq polymerase adds a fluorescently labeled dideoxynucleotide (ddNTP) to the 3' end of the primer. Addition of a specific ddNTP depends on which of the four bases is present at the particular SNP site in question on the template strand. Chain elongation of the sequence is terminated when a ddNTP is incorporated. ddNTPs do not have a 3' hydroxyl group required for chain elongation [3]. The fluorescently labeled extension products are then separated by capillary electrophoresis. The results of the multiplex SNaPshot™ extension assay are visualized using the GeneScan® software (Applied Biosystems, Foster City, CA) on the data collected by the sequencing instrument. The length of the extension product identifies the SNP marker within the multiplex, and the color (fluor) indicates the specific base found at the SNP position.

Multiplexing of the extension assay will greatly increase the throughput and decrease the cost of SNP analysis in forensic identity testing. Validation of this

technology is required by the National Standards in order for forensic laboratories to utilize it for casework analysis [4]. This project provided some of the validation data utilizing the multiplex SNP primer panels with the SNaPshot™ Multiplex Kit by assessing the sensitivity and reproducibility of each panel on the CE platform.

The first objective of this project was to evaluate six DNA samples to assess the sensitivity of each multiplex SNP panel provided by Orchid Cellmark. Each of the four SNP multiplex panels were analyzed with the DNA from six individuals. The DNA from these six individuals was serially diluted in order to determine the minimum quantity of DNA capable of producing accurate genotyping results with all SNP markers for each of the four panels. The protocol provided by Orchid Cellmark calls for a standardized input DNA quantity of 2ng, however this project involved the amplification and evaluation of significantly lower quantities of DNA with each panel. Eight dilutions ranging from 2ng to 10pg were prepared for each of the six DNA samples. Following amplification, the SNP markers were analyzed using a primer extension assay in conjunction with the ABI® SNaPshot™ Multiplex Kit. GeneScan® analysis of each sample followed capillary electrophoresis. The allelic variation at each SNP marker was identified based upon the incorporation of either a fluorescently-labeled dideoxycytosine triphosphate (ddCTP) or dideoxythymidine triphosphate (ddTTP). Analyses consisted of reporting the SNP genotypes and respective relative fluorescence unit (RFU) values designating peak heights of the fragments.

The second goal of this project was to determine the accuracy and reproducibility of the SNP typing process. The quantity of DNA used for the reproducibility assays was

selected based on the amount of DNA needed to yield correct typing of approximately 85% of SNPs at all markers within each panel. At lower DNA concentrations, it was expected that some SNP markers would show a loss of one of the two alleles at a heterozygous locus (loss of heterozygosity) or no detectable alleles. The appropriate amount of DNA in conjunction with the SNaPshot™ multiplex kit was repeated a total of 10 times for each of the six DNA samples using Panels 12, 15, and 17. Due to a lack of reagents, Panel 41 could only be repeated a total of three times with each of the six samples. Each of the four SNP panels were tested independently. Therefore, the amount of DNA utilized for the reproducibility assays differed between each of the four panels. This project was designed to provide some of the validation data required to demonstrate the utility of the four multiplex SNP primer panels for forensic DNA testing.

Although primer extension is very robust and a highly flexible allelic discrimination method [9], one of the biggest challenges is to reliably construct multiplex PCR amplification mixtures and a pool of extension primers capable of detecting large numbers of SNP loci [4]. The interpretation of the results from a multiplex primer extension assay may be complicated by the observation of low peak or no peak signals, overlapping peak signals, and extraneous peaks. Low peak or no peak signals of one or more of the primers included in the multiplex may be caused by several factors. These include the concentration of the specific PCR or extension primers in the multiplex mixes, the rate at which the primer anneals to the template, inappropriate injection time during capillary electrophoresis, or problems with the initial amplification of the template DNA [1]. The optimization of the amplification, extension, and CE analysis parameters

must be completed prior to use in forensic casework. A second observation, which could limit the interpretation of results, involves the inability to discriminate the length of the extension products. The inability to effectively separate the SNP markers within a panel could result from the length of the individual primer sequences within the multiplex panel. In addition to the length, the base composition and secondary structure of the extension primer may play a role. Extraneous peaks may also pose a problem in interpreting the final SNaPshot™ extension results. Potential explanations for extra peaks include both contamination and the incomplete removal of primers and/or dNTPs. Extra peaks may also result from two primers annealing to one another and subsequently participating in the extension reaction. Primers in a multiplex amplification/extension system must be carefully designed to avoid this problem.

Guidelines set forth by the DNA Advisory Board require forensic DNA labs to attempt to quantify human DNA found in biological evidence collected from crime scenes. Quantification is essential in forensic testing so that the optimal amount of DNA is amplified in order to obtain successful STR profiles. Determining the quantity of DNA in a biological sample is also important so that a portion of the sample may be retained for retesting. However, as a result of degradation, the ability to obtain a DNA profile with conventional STR testing or mtDNA sequencing may prove unsuccessful. Highly degraded samples or those with only trace amounts of DNA may result in either a partial profile or no profile at all. This has been observed with many of the samples collected from the World Trade Center disaster. In many cases where conventional forensic DNA testing has failed to produce a result, SNP analysis may prove successful. SNP analysis

can utilize shorter fragments of DNA and potentially smaller quantities of DNA. Thus, determining the sensitivity and reproducibility of each of these multiplex SNP extension primer panels is important when considering the quantity and quality of DNA in a biological crime scene sample. Although limitations may exist with this technology, SNP analysis could become an extremely valuable tool for forensic DNA testing.

CHAPTER II

MATERIALS AND METHODS

Samples

Blood samples from six individuals were obtained to assess the sensitivity and reproducibility of the four multiplex SNP primer panels. A clinician at UNT-HSC collected approximately 5ml fresh blood from each volunteer in EDTA vacutainer tubes.

Organic Phenol/Chloroform Extraction of DNA from Fresh Blood

The Phenol/Chloroform Organic Extraction method was utilized to extract DNA from the six blood samples. Organic extraction produces highly pure DNA of sufficient quantity and quality. The process begins with lysis of red blood cells by treating the sample with cell lysis buffer (CLB). After centrifuging the CLB treated sample, the pellet is treated with protein lysis buffer (PLB) to break open the white blood cells. PLB includes sodium dodecyl sulfate (SDS) and Proteinase K, which together digest proteinaceous material present in the solution. DNA in the sample is then extracted with phenol, which removes the proteinaceous material and chloroform to remove residual phenol. Cold absolute ethanol is then used to precipitate DNA out of the aqueous solution by centrifugation. Residual ethanol is removed by subjecting the samples to a speed vacuum while centrifugation. The pellet is then resuspended in TE^{-4} buffer. The following protocol for the Phenol/Chloroform organic extraction of DNA was utilized during this project.

Organic Phenol/Chloroform Extraction Protocol

1. Aliquot ~500ul blood into 2ml tubes.
2. Add 1ml ice cold Cell Lysis Buffer, vortex, and centrifuge 5 minutes at 5000rpm.
3. Pour off supernatant and repeat step #2. Pour off supernatant.
4. Add 300ul Protein Lysis Buffer/0.5% SDS/Proteinase K to tubes and vortex vigorously.
5. Incubate 2 hours at 65°C…vortexing every 30 minutes.
6. Add 300ul Phenol/Chloroform/Isoamyl alcohol to tubes, vortex 20 seconds, and centrifuge at high speed for 3 minutes.
7. Transfer aqueous DNA top layers into new tubes.
8. Repeat step 7 and 8.
9. Add 1ml cold absolute ethanol to each tube.
10. Mix by inverting tubes ~20-50 times.
11. Store at 4°C for 10 minutes.
12. Centrifuge 10 minutes at maximum speed. Decant alcohol.
13. Wash pellet with 1ml room temperature 70% ethanol.
14. Centrifuge 15 minutes at maximum speed. Decant alcohol.
15. Centrifuge tubes in a speed vac 15-30 minutes at high temperature.
16. Reconstitute with 500ul TE buffer at 65°C at least 2 hours.

Quantification of DNA

Determining the quantity of DNA in these samples was essential in this research project since serial dilutions were necessary to determine the minimum amount of DNA required for SNP detection using the multiplex primer panels. The quantity of DNA in

the samples was first estimated by spectrophotometry. Spectrophotometry measures the quantity of soluble DNA in a sample by absorbance at 260nm [4]. A 1:50 dilution of each sample was prepared by adding 10µl DNA to 490µl nanopure water. The samples were then quantified with the spectrophotometer at a DNA setting of 260/280. The values obtained from spectrophotometry were considered to be only an estimate of the quantity of DNA present in each sample because ribonucleic acid (RNA) and proteins may be present in the samples and quantified along with the DNA. The DNA for each sample was adjusted to 10ng/µl with TE buffer. The samples were then requantified using a real-time PCR system developed by Applied Biosystems and analyzed with their ABI® 7000 Sequence Detection System. The system can be used to determine the quantity of a target nucleic acid sequence in a sample by analyzing the cycle-to-cycle change in fluorescence signal as a result of PCR amplification [2]. The six DNA solutions were adjusted to a final concentration of 2ng/µl.

Serial Dilutions of DNA Samples

Once the DNA samples were successfully quantified to 2ng/µl using real-time PCR, serial dilutions were carried out for all six samples. Each sample was diluted with TE^{-4} buffer producing concentrations starting with 2ng/µl down to 1ng/µl, 500pg/µl, 250pg/µl, 100pg/µl, 50pg/µl, 25pg/µl, and 10pg/µl.

PCR Amplification

Orchid Cellmark provided our laboratory with multiplex mixes of amplification and extension primers for the analysis of 45 SNP markers. The 45 SNP markers have been divided into four multiplex panels (Panels 12, 15, 17, and 41). Each panel consists

of 10-12 SNP markers. The analysis of each SNP marker requires a pair of amplification primers and an extension primer. Amplification mixes for each panel consist of a multiplex between 20 and 24 amplification primers. All DNA extract samples were amplified with primers provided by Orchid Cellmark in a single reaction tube using an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA). A final volume of 5μl was used for amplifying the target DNA with PCR. The PCR reaction for each sample was set up as follows: 0.5μl 10X PCR Buffer II (100mM Tris-HCl, pH=8.3, 500mM KCl), 1.0μl $MgCl_2$ (25mM), 0.15μl dNTPs (2.5mM), 0.03μl Primer Pool (10μM each), 0.10μl AmpliTaq® Gold DNA Polymerase (5U/μl, Applied Biosytems, Foster City, CA), and 2.23μl dH2O. A master mix was produced with all the reagents for sample preparation. To obtain 2.5mM dNTPs, a 1:4 dilution was carried out by adding 25μl 10mM dNTPs to 75μl dH2O for a final volume of 100μl dNTPs (2.5mM). Dilutions of DNA samples were performed for each concentration at a 1:1 ratio with dH2O to allow 2μl of each DNA sample concentration to be added to the PCR. For each reaction, 3μl master mix was mixed with 2μl DNA dilution. Beginning 02/11/03, I increased the volume of dH2O to 2.23μl from 1.23μl in the PCR master mix. This eliminated the DNA dilution step and decreased the risk of contamination caused by pipetting error. The final reaction volume then included 4μl master mix and 1μl DNA. Positive and negative controls as well as three blank reactions containing TE buffer accompanied each PCR amplification procedure. The thermal cycling conditions were carried out as follows.

Step	Temperature	Time
1	95°C	5 minutes
2	95°C	30 seconds
3	50°C	55 seconds
4	72°C	30 seconds
5	Loop steps 2-4, 2 times	-
Step	**Temperature**	**Time**
6	95°C	30 seconds
7	50°C, Increase 0.2°C each cycle	55 seconds
8	72°C	30 seconds
9	Loop steps 6-8, 18 times	-
10	95°C	30 seconds
11	55°C	55 seconds
12	72°C	30 seconds
13	Loop steps 10-12, 8 times	-
14	72°C	7 minutes
15	4°C	hold

Purification of PCR Product

Excess primers and dNTPs from the PCR reaction were eliminated to prevent interference with the final SNaPshot™ product. PCR products were purified with 8.2µl of a master mix of dH2O and ExoSAP-IT™ (Exonuclease I/Shrimp Alkaline Phosphatase, USB Corporation, Cleveland, Ohio). The master mix consisted of 2.1µl ExoSAP-IT™ and 6.1µl dH2O. Following addition of 8.2µl master mix, the samples were mixed thoroughly by pipetting. The thermal cycling conditions included 60 minutes at 37°C, 15 minutes at 72°C, and a 4°C hold.

SNaPshot™ Extension

Figure 1 illustrates an overview of the SNaPshot™ extension assay. Again, the analysis of each SNP marker requires a pair of amplification primers and an extension

primer. SNP extension mixes for each panel consists of a multiplex of between 10 and 12 extension primers. The purified PCR templates were extended with the multiplex SNP extension primer pool using a modified version of the SNaPshot™ extension protocol. The SNaPshot™ Ready Reaction Mix includes fluorescently labeled ddNTPs and Taq polymerase. A master mix was prepared as follows, with volumes indicated per reaction: 0.5µl SNP Primer Pool (10µM stock concentration) for Panel 17 and 0.6µl SNP Primer Pool (10µM stock concentration) for Panels 12, 15, and 41, 2.5µl SNaPshot™ Ready Reaction Mix, 3.5µl 5X Sequencing Buffer (400mM Tris-HCl, pH=9, 10mM $MgCl_2$), and 3.5µl dH2O for Panel 17 and 3.4µl dH2O for Panels 12, 15, and 41. Both positive and negative SNaPshot™ controls were made for each extension procedure. The positive control included 5µl SNaPshot™ Ready Reaction Mix, 2µl pGEM+ 3Zf(+) control template, 1µl SNaPshot™ Multiplex Control Primer, and 2µl dH2O. The negative control included 5µl SNaPshot™ Ready Reaction Mix, 1µl SNaPshot™ Multiplex Control Primer, and 4µl dH2O. All reactions included 7µl SNP extension master mix and 3µl ExoSAP-IT™ product. The thermal cycling conditions involved 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 30 seconds at 60°C, and a final hold at 4°C.

Purification of the SNaPshot™ Extension Product

Excess fluorescently labeled ddNTPs were removed from the extension products to prevent interference with the final analysis. Shrimp Alkaline Phosphatase (SAP, USB Corporation, Cleveland, Ohio) was used to purify the extension products by eliminating excess ddNTPs. A mix of 1µl SAP (1U/µl) and 5µl dH2O were prepared for each

reaction. Following addition of 6µl SAP mix, the samples were mixed by pipetting. The thermal cycling conditions included 30 minutes at 37°C, 20 minutes at 85°C, and a hold at 4°C.

Capillary Electrophoresis and GeneScan® Analysis of Final Products

Sensitivity assays for all six samples utilizing Panel 15 primers were carried out with the ABI Prism® 310 (Applied Biosystems, Foster City, CA). The ABI Prism® 310 was also employed to analyze samples KD, XA, and JW with Panel 17 primers and samples DH, SS, and MM with Panel 12 primers. In addition to Panels 17 and 41 sensitivity assays for all samples and all reproducibility assays, samples KD, XA, and JW assessed with Panel 12 primers were analyzed with the ABI Prism® 3100 (Applied Biosystems, Foster City, CA). The methods for using each genetic analyzer are different and are described below.

1. Samples subjected to CE on the ABI Prism® 310 were prepared by first making a master mix of 120 LIZ™ (Applied Biosystems, Foster City, CA) size standard and formamide. For each reaction, 14µl formamide was added to 0.5µl 120 LIZ™. After vortexing the master mix, 14.5µl was added to 0.9µl SNaPshot™ extension product. The samples were mixed by pipetting then denatured at 95°C for 3 minutes. Following denaturation, the samples were snap-cooled on ice while the ABI Prism® 310 was set up. Once the ABI Prism® 310 was ready, the samples were loaded and the instrument set to run.

2. Samples subjected to CE on the ABI Prism® 3100 were prepared by first making a master mix of 120 LIZ™ size standard and formamide. For each reaction, 8.5µl formamide was added to 0.5µl 120 LIZ™. After vortexing the master mix, 9µl was added to 1µl SNaPshot™ extension product in a Micro Amp® Optical 96-well plate. The samples were centrifuged then denatured at 95°C for 5 minutes. Following denaturation, the samples were snap-cooled on ice while the ABI Prism® 3100 was set up. Once the ABI Prism® 3100 was ready, the sample tray was loaded and the instrument set to run.

The data collected from either CE instrument was analyzed with GeneScan® software. The RFU threshold was set at 75 during this project. SNP genotypes and RFU values were collected and reported for each sample.

CHAPTER III

RESULTS AND DISCUSSION

Four multiplex SNP extension primer panels were evaluated for sensitivity by testing DNA from six individuals at eight different quantities in triplicate. The electropherogram results from CE of all samples indicated that C/T SNPs were detected at all markers present in each panel. Figure 2 shows an example of all 45 SNP markers detected in sample KD. Data obtained from samples were plotted as a function of the relative fluorescence intensity observed from the fluorescence emission of the dyes as they passed a laser detector [4]. In several instances, the 120LIZ™ size standard (orange peaks) was observed to pull-up the dROX™ (Applied Biosystems, Foster City, CA) dye (red peaks). This did not interfere with SNP detection since analysis of the sample was done with the size standard peaks present in the electropherograms. If the size standard peaks were absent, a red peak resulting from pull-up in the orange channel may have appeared to be a T/T SNP. Although the peak height values of C/T SNPs did not significantly differ between the two CE instruments, the ABI Prism® 3100 was more sensitive in detecting SNP markers at lower quantities of DNA. Table 2 shows the results of the dilution series for all four SNP panels. The dilution series results with each of the four panels demonstrated that as the quantity of DNA decreased, fewer SNPs were detected. Therefore, the sensitivity of SNP detection with all four primer panels decreased as the quantity of DNA decreased. The reproducibility of each panel was

examined using the quantity of DNA in which approximately 85% of SNP markers were detected without a loss of heterozygosity. A quantity of 100pg DNA was used to evaluate the reproducibility of Panel 17, 250pg for Panels 15 and 41, and 500pg for Panel 12. The reproducibility analyses with Panels 12, 15, and 17 were repeated a total of ten times with each of the six samples and Panel 41 was repeated a total of three times. The reproducibility results for each panel are shown in Table 7. A summary of the findings for each of the tested SNP panels follows.

Panel 17

Panel 17 was originally designed to interrogate a total 10 SNP markers. Midway through this project, Orchid Cellmark provided additional reagents for Panel 17 that included only nine of the 10 SNP markers. The primers designed to detect SNP 211324 were omitted from the second batch of primers. The original amplification and extension primer mixes for Panel 17 were used to assess the sensitivity with samples KD, XA, and JW. The second set of amplification and extension mixes were used with samples DH, SS, and MM. The second set of primer mixes were also used with all six samples for the subsequent reproducibility assays.

Samples KD, XA, and JW were tested with the first set of PCR and SNP primer pools and analyzed on both the ABI Prism® 310 and ABI Prism® 3100 to compare the sensitivity of the two CE instruments since both were used for this project. The RFU values obtained for the samples reported by both instruments were not significantly different, however the ABI Prism® 3100 detected more SNPs in reactions containing smaller quantities of DNA. For example, in all samples analyzed, SNP marker 63404 was

detected on both instruments equally well, however below 250pg, the marker could only be detected on the ABI Prism® 3100. This may be due to a higher signal to noise ratio observed in the ABI Prism® 3100. Figures 3 and 4 show electropherograms for sample XA with 2ng, 100pg, and 50pg reactions analyzed with both the ABI Prism® 310 and the 3100. These figures illustrate a loss of heterozygosity was detected with larger DNA quantities utilizing the ABI Prism® 310.

Panel 17, SNP 207160 showed a loss of heterozygosity below 1ng DNA. All other SNP markers showed a loss of heterozygosity occurring below 250pg of DNA (Table 3). SNP analysis of markers 211324 and 228468 detected at least one allele with only 10pg of DNA. The dilution series revealed that with 100pg DNA, 81% of SNPs were detected in all six samples at all markers present. At 50pg DNA, only 46% of SNPs were detected for all markers in all six samples. Figure 5 shows dilution series reactions carried out on the ABI Prism® 3100 for sample JW with quantities of 2ng, 100pg, and 50pg DNA. A loss of heterozygosity occurred at SNP markers 214373, 214674, and 207160 below 100pg DNA.

Assessment of the reproducibility of Panel 17 was carried out with a quantity of 100pg DNA in 10 repeat reactions. Reproducibility can be defined as the ability to obtain the same SNP results in each repeat reaction. Panel 17 was found to be reproducible in 96% of the reactions using 100pg DNA. Four percent of the reactions resulted in no SNPs detected or a loss of heterozygosity in C/T SNPs. Figure 14 shows the electropherogram results for all 44 SNPs detected in sample MM, including the 9 SNPs detected with Panel 17 multiplex SNP pool.

Analysis of the positive and negative amplification controls using the initial amplification and extension primer mixes revealed two extraneous peaks having sizes of approximately 48bp and 58bp. Results of the PCR controls using the second batch of primer mixes showed two extra peaks as well, with sizes of 58bp and 71bp. Figure 6 shows electropherogram results for these reactions. One plausible explanation for the extra peaks is contamination of a PCR reagent with DNA from an individual. Since extra peaks were not observed in the control reactions carried out with all SNP panels, any contamination would be limited to the amplification and/or extension primer mixes. It is possible that one or both primer mixes were contaminated, either when they were first produced or at some point after the project began. Table 4 shows at least one allele was detected at SNP markers 211324 and 228468 with only 10pg DNA. Therefore, a sample diluted to 25pg or 10pg may have contaminated one or both primer mixes resulting in the observation of extra peaks in the samples. Another possible explanation for the spurious peaks is extension of primers that have annealed to other primers present in the PCR or SNP pools. Since the peak sizes are approximately the same size as SNPs at markers 211324, 228468, and 207160, the primers designed to detect these SNPs could have annealed to other primers in the PCR or SNP pool. Two primers that have annealed to one another would act as a primer bound to template DNA during the SNaPshot™ reaction and thus get extended with a fluorescent ddNTP. The 46bp primer possibly annealed to another primer, which extended it with a ddCTP, resulting in a black peak. As for the 55bp primer designed for detecting SNP 228468, it is possible that during the extension reaction a ddCTP and a ddATP were added, resulting in both a black and a

green peak. Therefore, this primer could have annealed to two other primers in the SNP pool. The 70bp black peak indicates the primer detecting SNPs at marker 207160 may have annealed to another primer resulting in the subsequent elongation with a ddCTP. Presence of the 58bp green peak does not interfere with the interpretation of the results because the primers are specifically designed for C/T, rather than A/G, SNP detection in human DNA. However, the 48bp, 58bp, and 71bp black peaks can hamper the results since they are labeled with dTAMRA™ (Applied Biosystems, Foster City, CA), which is attached to ddCTPs in the SNaPshot™ Ready Reaction Mix, and are the same sizes as true SNP alleles. If SNP 211324, 228468, or 207160 are detected as C/C or C/T in a sample, the extra peaks will be masked by the true C allele. However, if a T/T SNP is present, these black peaks may lead to the false interpretation of a C/T SNP. Evaluation of all samples showed SNPs at markers 228468 and 207160 were detected at all quantities of DNA tested, including 10pg. All samples were identified as C/C homozygotes for SNP 228468 except XA, which was detected as a C/T. Due to the extra 58bp peak seen in the control reactions, XA may actually be a T/T SNP at this marker. Likewise, samples KD, XA, and JW were all detected as heterozygotes for SNP 211324, however, one or more of these individuals may actually be a T/T. Samples XA, JW, DH, SS, and MM were called a C/T heterozygote for SNP 207160, but in reality may be a T/T SNP.

Panel 12

The genetic profiles produced with Panel 12 were distinct for each of the six individuals. Samples KD, XA, and JW were analyzed with the ABI Prism® 3100 and

samples DH, SS, and MM were analyzed with the ABI Prism® 310. No significant difference in RFU values was observed between the two instruments at the higher quantities of DNA, however, the ABI Prism® 3100 was more sensitive in detecting SNPs with quantities less than 100pg of DNA as seen with Panel 17.

The results of the dilution series tested with Panel 12 are shown in Table 2. At 500pg, 93% of the extension assays accurately detected SNPs in all samples. The dilution series showed that with 250pg of DNA, 81% of SNPs were detected in all six samples at all markers present. Figures 7 and 8 illustrate the results for samples SS and MM using Panel 12 to detect SNPs at DNA quantities of 2ng, 500pg, and 250pg DNA. Observation of these two samples, as well as the other four, indicates SNP 241554 showed loss of heterozygosity with 2ng DNA. All other SNP markers showed a loss of heterozygosity occurring below 1ng DNA. All six samples on average revealed that SNP 81917 began losing both alleles at quantities below 500pg of DNA. Table 5 shows the loss of heterozygosity at each SNP marker as detected with the dilution series reactions for Panel 12.

At 500pg of DNA, 93% of SNPs were detected in all samples at all 12 markers and only two SNP markers, 81917 and 241554 showed a loss of heterozygosity. Thus, 500pg of DNA was chosen to evaluate the reproducibility of Panel 12 by carrying out 10 reactions for each of the six samples. On average, Panel 12 was found to be 100% reproducible. Sample MM was 97% reproducible with Panel 12. This demonstrates that 3% of the time, at least one allele was lost at a SNP marker with sample MM. One

reaction performed for sample MM is illustrated in Figure 14 along with the other three panels.

Controls carried out with Panel 12 sensitivity assays did not reveal the same type of extraneous peaks as detected with Panel 17. However, analyses of samples with Panel 12 revealed small extra peaks labeled with both the dROX™ and dTAMRA™ dyes, having sizes of 24-29bp as seen in Figures 7 and 8. One possible explanation for these small peaks is that amplification primers may have been carried over into the extension reaction. The amplification primers have sizes ranging from 20-25bp. The ExoI/SAP purification process may not have completely eliminated excess primers, therefore they may have participated in the extension reaction and were subsequently detected during capillary electrophoresis. These peaks did not interfere with SNP typing of any sample because they were smaller than the first SNP marker, 230299, having an approximate size of 32bp.

Panel 15

The results showed each of the six samples had distinct genetic profiles with Panel 15. The dilution series experiments were analyzed with the ABI Prism® 310 and the reproducibility reactions were carried out with the ABI Prism® 3100. Table 2 shows the results of the sensitivity study for Panel 15 determined from the dilution series experiments. Evaluation of all six samples tested at a quantity of 250pg revealed 85% of SNPs were detected at all 12 markers. At 100pg DNA, only 61% of SNPs were successfully interrogated at all markers. Table 5 shows the loss of heterozygosity for all markers in Panel 15. SNP 85187 consistently revealed peaks with low or no peak signal.

Increasing the concentration of primer in either the PCR mix or SNP extension mix or both may improve peak signal at this marker. A loss of heterozygosity at all other SNP markers occurred with quantities below 500pg DNA. Figures 9 and 10 show results for samples XA and DH evaluating quantities of 2ng, 250pg, and 100pg. In sample XA, SNP marker 85187 was not even detected with 2ng DNA. However, a T/T SNP was detected at this marker in sample DH at quantities of 2ng and 250pg DNA.

Reproducibility of Panel 15 was examined by performing 10 repeat reactions with a quantity of 250pg of DNA for all six samples since the dilution series experiments revealed 85% of SNPs were detected at this quantity. In addition, this quantity showed SNPs at markers 168115 and 221499 began showing a loss of heterozygosity. Panel 15 was 99% reproducible for all samples, implying 1% of the time, at least one allele was lost at a SNP marker (Table 7). Results for one reaction using sample MM is shown in Figure 14.

Analysis of PCR controls carried out with Panel 15 show three extraneous peaks. These peaks were observed in controls using both the initial primer mixes and the second primer mixes obtained from Orchid Cellmark. One 29bp peak was detected with the fluorescent dR6G dye, producing a green signal. As with Panel 17, this peak may be due to a primer that has annealed to another primer and participated in the extension reaction. This peak is seen in the positive control electropherogram at approximately the same position as a C/C SNP at marker 231480 having a size of 28bp. Since the green peak has a ddATP attached, it did not interfere directly with C/T SNP typing. However, in the negative control, this large peak with a RFU value of 3938, pulls up a small black peak

with a RFU value of 279. This is likely the result of dye overlap in the spectral matrix. Since all six samples were either C/C or C/T at this SNP marker, this small black peak blended into the C peaks. Another occurrence of extra peaks was observed in both PCR controls. A blue and green peak, labeled with the fluorescent dyes dR110 and dR6G respectively, were detected as having a size of 72bp. Again, one possibility for this observation is that the extension primer designed to detect SNP marker 82031 annealed to another primer in the panel and subsequently participated in the extension reaction. These peaks had no effect on C/T SNP typing since they were labeled with dR110 and dR6G dyes, which identify ddATPs and ddGTPs, respectively.

An overlap of peaks at SNP markers 177589 and 231480 occurred in samples DH and MM. This poses problems in SNP typing, especially with lower quantities of DNA (See Figure 10). The overlap of peaks is a result of the mobility of fragments, determined not only by the fluorescent dye and nucleotide composition, but the size of the fragments. The primers detecting these SNPs at markers 177589 and 231480 are 22 and 27 nucleotides long, respectively. The effect of nucleotide composition on mobility can be important when the primer is short [1]. Therefore, the size and/or mobility shift in dyes caused C/T SNPs at marker 177589 to overlap with C/C SNPs at marker 231480.

Panel 41

The dilution series experiments evaluating Panel 41 revealed all six samples had distinct genotypes at all 12 markers. All testing involving Panel 41 was carried out with the ABI Prism® 3100. Table 2 shows the results of Panel 41 dilution assays. At 250pg DNA, Panel 41 revealed 80% of reactions resulted in accurate SNP detection in all

samples. At 100pg of DNA, only 63% of SNPs were interrogated at all 12 markers. Figures 11 and 12 show SNP typing results for samples KD and JW using 2ng, 250pg, and 100pg DNA. These figures demonstrate the primer designed to detect SNPs at marker 89614 consistently gave low or no peak signals, resulting in a loss of heterozygosity occurring at quantities below 2ng DNA (See Table 6). At marker 76268, a loss of heterozygosity occurred with quantities of DNA below 1ng. All other SNP markers revealed a loss of heterozygosity with approximately 50-100pg DNA. Reproducibility assays were carried out using 250pg of DNA with triplicate reactions. This quantity was selected to assess the reproducibility since 80% of SNPs were detected at all 12 markers and only 3 markers revealed a loss of heterozygosity with this amount of DNA. Table 7 shows that Panel 41 is 86% reproducible in detecting SNPs at all 12 markers in all six samples. However this may not be reliable since only triplicate reactions were used compared to 10 reactions carried out with the other three panels. The highest level of reproducibility is seen with sample MM, revealing that 97% of SNPs were detected at all 12 markers (See Figure 14).

Several extra peaks were detected in PCR controls performed with Panel 41 sensitivity and reproducibility assays. Four blue peaks were observed with sizes of 41bp, 72bp, 79bp, and 91bp. Three green peaks were observed with sizes of 42bp, 48bp, and 53bp. Two black peaks were detected with sizes of 42bp and 53bp. All of these peaks can be seen in the controls illustrated in Figure 13. As with Panels 15 and 17, these may be due to primers that have annealed to other primers in the panel, thus participating in the extension reaction and getting labeled with a fluorescent ddNTP. The 41-42bp peaks may

be a result from the primer designed to detect the SNP at marker 63184 annealing to other primers and being extended with dR110, dR6G, or dTAMRA™. Although the green and blue peaks did not interfere with SNP detection, the black peak signifying that a ddCTP has been added did interfere with C/T SNP typing. Therefore, T/T SNPs may have falsely been interpreted as C/T SNPs (See Figure 12). The 48bp and 53bp green peaks may be due to the primers designed for detecting SNPs at markers 182622 and 66567 annealing to another primer and getting extended with a ddATP. The 53bp black peak seems to be pull-up of the dTAMRA™ (black) dye in the dR6G (green) dye and may result in T/T SNPs being falsely typed as C/T SNPs. The 72bp, 79bp, and 91bp blue peaks could result from primers detecting SNPs at markers 66683, 63979, and 76268 annealing to other primers and participating in the extension reaction. These did not interfere with C/T SNP typing since they are labeled with the dR110 blue dye. Another possible explanation for the 41bp and 53bp black peaks is a low quantity of DNA may have contaminated the PCR or SNP primer mixes. This could have occurred during the production of the multiplex mixes or at some point during the project. At least one allele was detected at marker 63184 with 10pg DNA and at marker 66567 with 50pg DNA. This observation may be due to the detection of partial SNPs from a low level contaminant.

CHAPTER IV

CONCLUSIONS

Four multiplex panels designed to detect a total of 45 SNP markers within the human genome were evaluated for use in forensic DNA testing. The SNP markers were detected utilizing the SNaPshot™ primer extension assay on a CE platform. The sensitivity of the detection assay was evaluated using a dilution series with six DNA samples. Each panel was evaluated independently using serial dilutions of DNA from 2ng down to 10pg. The loss of heterozygosity at an individual SNP locus was used to detect the limits of sensitivity of the assay. Within each panel, the individual SNP markers displayed varying levels of sensitivity as can be seen in Tables 3 through 6. The reproducibility of the CE detection system was evaluated using a DNA quantity which resulted in the correct typing of 85% of the SNP markers within all samples. Table 7 shows the reproducibility results obtained using each DNA sample to evaluate all four panels.

Based upon the results obtained, it does not appear that the amplification and/or extension primers within a given panel have been optimized to produce consistent and reliable results. Orchid Cellmark originally developed the system to identify 10-12 SNP loci per panel. The analysis of each SNP marker requires a pair of amplification primers and an extension primer. Their original reaction mixes for both amplification and extension were designed with equal molar concentrations of all the primers. The results

obtained during this project revealed individual primers within each panel need to be optimized in order to achieve balanced peak heights. For example, the concentration of the amplification and/or extension primers in Panel 17 designed to detect SNP 207160 must be increased to improve peak signal. All other markers within this panel appeared to be relatively well balanced. Increasing peak signal at this marker will provide better balance of peak heights at all ten markers in Panel 17. Some primer concentrations could be decreased in the mixes in order to balance peak heights at all SNP markers. Three markers within Panel 41 (70371, 105677, and 63184) consistently gave higher signals (See Table 6 and Figures 11 and 12). Decreasing the concentration of the primers associated with these three markers would improve peak height balance among all 12 markers in Panel 41.

Reduced signal in the detection assay may also be caused by low efficiency in the removal of dNTPs and primers from the initial template amplification. If the clean-up step following amplification does not completely eliminate excess dNTPs, they will be carried over to the extension reaction. The fluorescent ddNTPs will have to compete with these dNTPs in elongation of the primers. Therefore, if only half of the available primers are elongated with a ddNTP, a low peak signal may result. Optimization of the ExoI/SAP procedure may be required to completely eliminate excess dNTPs and primers following amplification.

Peak height imbalance was observed frequently among heterozygous SNPs at individual markers with all four panels. This is potentially due to the differential incorporation of ddNTPs during the extension reaction. It appears the C allele was

usually lost at heterozygous loci as the quantity of DNA decreased. Therefore, the polymerase may have a greater affinity for ddTTP and preferentially incorporate it as opposed to ddCTP [18]. Alternative forms of Taq polymerase could be explored with the SNaPshot™ assay. The selection of markers in a panel should include those that consistently produce heterozygotes with balanced peak heights. These SNP panels can be improved by grouping together markers with the same sensitivity. Each marker within a panel should show a loss of heterozygosity occurring at the same quantity of DNA as with the other markers in the panel. For example, marker 241554 in Panel 12 showed an average loss of heterozygosity in all samples at 2ng DNA (See Table 4). The poor sensitivity of this marker may be the result of sub-optimum amplification and/or extension parameters. A loss of heterozygosity at the other markers was detected with approximately 50-250pg DNA. Therefore, the sensitivity of Panel 12 can be improved by replacing marker 241554 with one that shows a loss of heterozygosity occurring between 50pg and 250pg DNA.

The inability to adequately separate SNP markers can be observed in the electropherogram results by the appearance of overlapping or poorly-spaced peaks. Problems with overlapping peaks can only be resolved by changing the length of individual primers within the panel. Figure 10 shows overlapping peaks at markers 177589 and 231480 in Panel 15. The number of thymidine residues can be increased or decreased in the poly-T tail to alter the length of one or both of these primers and therefore increase the spacing between these two peaks. Although no overlap was observed with the markers in Panel 41, several extension primers may need to be changed

in length in order to clearly distinguish the SNP markers in the electropherogram. At SNP markers 66683, 89614, and 63979 in Panel 41, peaks were observed very close together in the electropherograms and made interpretation difficult.

Several extraneous peaks appeared in the results using Panels 17 and 41. One potential explanation for the extra peaks is contamination. Since these SNP assays are very sensitive and can detect small quantities of DNA, extreme caution must be taken in all steps of the protocol to avoid contamination, as with any forensic DNA test. Due to the numerous pipetting steps during this procedure, contaminants could be introduced throughout the process, including during the production of the primer mixtures. During the manufacturing of the primer mixes at Orchid Cellmark, a small quantity of DNA or amplified product may have contaminated the primer pools and subsequently showed up as peaks in the 10pg reactions as observed in Panel 17 at markers 211324 and 228468 (See Table 3). It is not unexpected to see contaminant peaks at these SNP markers due to their high sensitivity. At both markers, at least one allele was detected in all six samples at all DNA dilutions, including 10pg. The frequent observation of extra peaks seen in the PCR controls may also be due to primers annealing to other primers and then participating in the extension reaction. Extension primers may have annealed to each other or to excess primers from the initial amplification, and formed false templates. The extra peaks observed were usually 1-2bp longer than the primer sequence designed for SNP interrogation. For example, Figure 13 shows extra peaks with a size of 41-42bp at the same location where SNP marker 63184 appears. The primers having approximately the same base pair size as the extra fragments need to be evaluated using a single-plex

reaction with template DNA. If the extra peak is still detected, then something else is occurring in the reactions such as a transversion at a SNP locus that has been interrogated with a ddATP or ddGTP. This observation would indicate that the extra peak is due to contamination. If the peak is not seen in the single-plex reaction, then the primer needs to be examined with duplex reactions using other primers in the panel and template DNA. If a duplex reaction produces one of these extra peaks, then both primer sequences need to be examined for complimentary regions. The solution may be as simple as raising the annealing temperature during the primer extension assay to increase the stringency of primer annealing. If this fails, then the extension primers for those markers may need to be redesigned.

These four SNP panels were successful in giving distinct profiles for all six samples, however, they are not ready to be used in forensic DNA testing. All four SNP panels need to be optimized. Within each panel, the individual primers can be optimized by increasing or decreasing their concentrations to improve the balance of peak heights. In addition, several markers within a panel may need to be replaced with those that share the same limits of detection. The observation of overlapping peaks in Panel 12 would indicate that the lengths of the extension primers should be modified to avoid falsely interpreting SNP markers. Further testing also needs to be carried out to determine the source of the extra peaks seen in Panels 17 and 41. Primer sequences may need to be altered or specific primers replaced if extraneous peaks are consistently observed. In conclusion, this project shows that all four SNP panels need to be optimized before they can be successfully used for SNP testing of forensic casework.

CHAPTER V

TABLES AND FIGURES

CHROMOSOMAL LOCATION OF 45 SNPS			
Panel 12	Chromosome	Panel 15	Chromosome
230299	10	177589	8
81917	13	231480	1
66561	6	85187	2
231735	6	216327	8
62684	15	248075	12
224922	17	60409	7
119770	7	168115	17
56593	7	225225	10
217856	18	60188	19
241554	6	82031	6
126264	22	221499	11
63799	2	173632	18
Panel 17	Chromosome	Panel 41	Chromosome
214373	5	70371	22
214674	20	105677	9
63404	13	63184	20
211324	15	182622	2
126243	22	66567	22
228468	11	219561	2
66683	11	58388	2
72171	20	66683	11
207160	19	89614	17
234217	1	63979	6
		130240	22
		76268	15

Table 1 – Chromosomal location of all 45 SNP markers in the human genome. Note that Panels 17 and 41 both contain SNP marker 66683 located on chromosome 11.

DILUTION SERIES RESULTS OF THE FOUR MULTIPLEX SNP PANELS				
Quantity	Panel 12	Panel 15	Panel 17	Panel 41
2ng	99%	95%	97%	100%
1ng	96%	89%	95%*	99%
500pg	93%	90%	96%	88%
250pg	81%	85%	91%	80%
100pg	57%	61%	81%	63%
50pg	33%	38%	46%	47%
25pg	18%	9%	25%	18%
10pg	6%	3%	18%	10%

Table 2 – Dilution series results for all four multiplex SNP panels tested with eight quantities of DNA. An (*) indicates results for sample JW were not accounted for due to pipetting error in the initial PCR amplification reactions.

LOSS OF HETEROZYGOSITY IN PANEL 17 SNPS								
SNP	**2ng**	**1ng**	**500pg**	**250pg**	**100pg**	**50pg**	**25pg**	**10pg**
214373	+	+	+	+	+/-	-	-	-
214674	+	+	+	+	+	+/-	-	-
63404	+	+	+	+	+/-	-	-	-
211324	+	+	+	+	+	+/-	+/-	+/-
126243	+	+	+	+	+/-	-	-	-
228468	+	+	+	+	+	+/-	+/-	+/-
66683	+	+	+	+	+	+/-	-	-
72171	+	+	+	+	+	+/-	-	-
207160	+	+/-	+/-	+/-	+/-	+/-	-	-
234217	+	+	+	+	+	-	-	-

Table 3 – Loss of heterozygosity detected in Panel 17 SNPs. A [+] signifies that on average, complete C/T SNPs were obtained for all samples, [+/-] indicates a loss of one allele, and [-] represents both alleles dropped out or fell below the detection threshold of 75 RFU.

LOSS OF HETEROZYGOSITY IN PANEL 12 SNPS								
SNP	**2ng**	**1ng**	**500pg**	**250pg**	**100pg**	**50pg**	**25pg**	**10pg**
230299	+	+	+	+	+	+/-	+/-	-
81917	+	+	+/-	-	-	-	-	-
66561	+	+	+	+/-	+/-	-	-	-
231735	+	+	+	+	+/-	-	-	-
62684	+	+	+	+/-	-	-	-	-
224922	+	+	+	+	+	+/-	-	-
119770	+	+	+	+	+/-	+/-	-	-
56593	+	+	+	+	+	+/-	-	-
217856	+	+	+	+	-	-	-	-
241554	+/-	+/-	+/-	+/-	-	-	-	-
126264	+	+	+	+	+/-	-	-	-
63799	+	+	+	+	+	+/-	-	-

Table 4 - Loss of heterozygosity detected in Panel 12 SNPs. A [+] signifies that on average, complete C/T SNPs were obtained for all samples, [+/-] indicates a loss of one allele, and [-] represents both alleles dropped out or fell below the detection threshold of 75 RFU.

LOSS OF HETEROZYGOSITY IN PANEL 15 SNPS								
SNP	2ng	1ng	500pg	250pg	100pg	50pg	25pg	10pg
177589	+	+	+	+	+	+	+/-	-
231480	+	+	+	+	+	+/-	-	-
85187	+	+	+/-	-	-	-	-	-
216327	+	+	+	+	+	+/-	-	-
248075	+	+	+	+	-	-	-	-
60409	+	+	+	+	+	+/-	-	-
168115	+	+	+	+/-	-	-	-	-
225225	+	+	+	+	+/-	-	-	-
60188	+	+	+	+	+/-	+/-	-	-
82031	+	+	+	+	-	-	-	-
221499	+	+	+	+/-	-	-	-	-
173632	+	+	+	+	+	+/-	-	-

Table 5 - Loss of heterozygosity detected in Panel 15 SNPs. A [+] signifies that on average, complete C/T SNPs were obtained for all samples, [+/-] indicates a loss of one allele, and [-] represents both alleles dropped out or fell below the detection threshold of 75 RFU.

LOSS OF HETEROZYGOSITY IN PANEL 41 SNPS								
SNP	**2ng**	**1ng**	**500pg**	**250pg**	**100pg**	**50pg**	**25pg**	**10pg**
70371	+	+	+	+	+	+	+/-	+/-
105677	+	+	+	+	+	+/-	+/-	-
63184	+	+	+	+	+	+	+/-	+/-
182622	+	+	+	+	+/-	-	-	-
66567	+	+	+	+	+/-	+/-	-	-
219561	+	+	+	+	+	+	+/-	-
58388	+	+	+	+/-	-	-	-	-
66683	+	+	+	+	+/-	+/-	-	-
89614	+	+/-	-	-	-	-	-	-
63979	+	+	+	+	+/-	-	-	-
130240	+	+	+	+	+	+/-	+/-	-
76268	+	+	+/-	-	-	-	-	-

Table 6 - Loss of heterozygosity detected in Panel 17 SNPs. A [+] signifies that on average, complete C/T SNPs were obtained for all samples, [+/-] indicates a loss of one allele, and [-] represents both alleles dropped out or fell below the detection threshold of 75 RFU.

REPRODUCIBILITY OF THE MULTIPLEX SNP EXTENSION PRIMER PANELS				
Sample	Panel 12	Panel 15	Panel 17	Panel 41
KD	100%	100%	100%	89%
XA	100%	100%	93%	83%
JW	100%	100%	100%	97%
DH	100%	97%	94%	83%
SS	100%	99%	98%	67%
MM	97%	96%	90%	97%
AVERAGE	100%	99%	96%	86%

Table 7 - Reproducibility of each panel was determined as the ability to obtain the same SNP results in each repeat reaction carried out for each sample. Ten repeat reactions per sample were carried out with Panels 12, 15, and 17. Only 3 reactions per sample were performed with Panel 41 due to an insufficient amount of SNP primer pool.

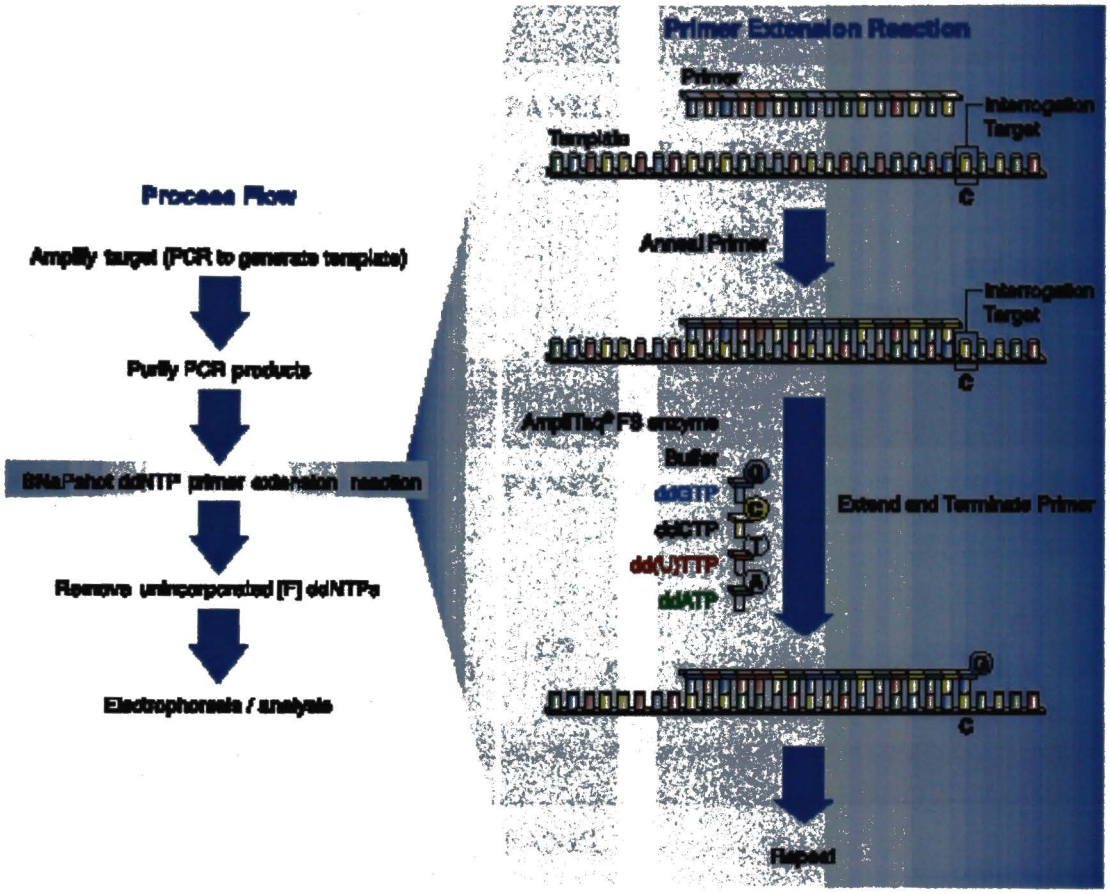

Figure 1 – SNaPshot multiplex extension assay and process flow. (Adapted from the ABI Prism® SNaPshot™ Multiplex System Product Bulletin, P/N 107PB06-0L.)

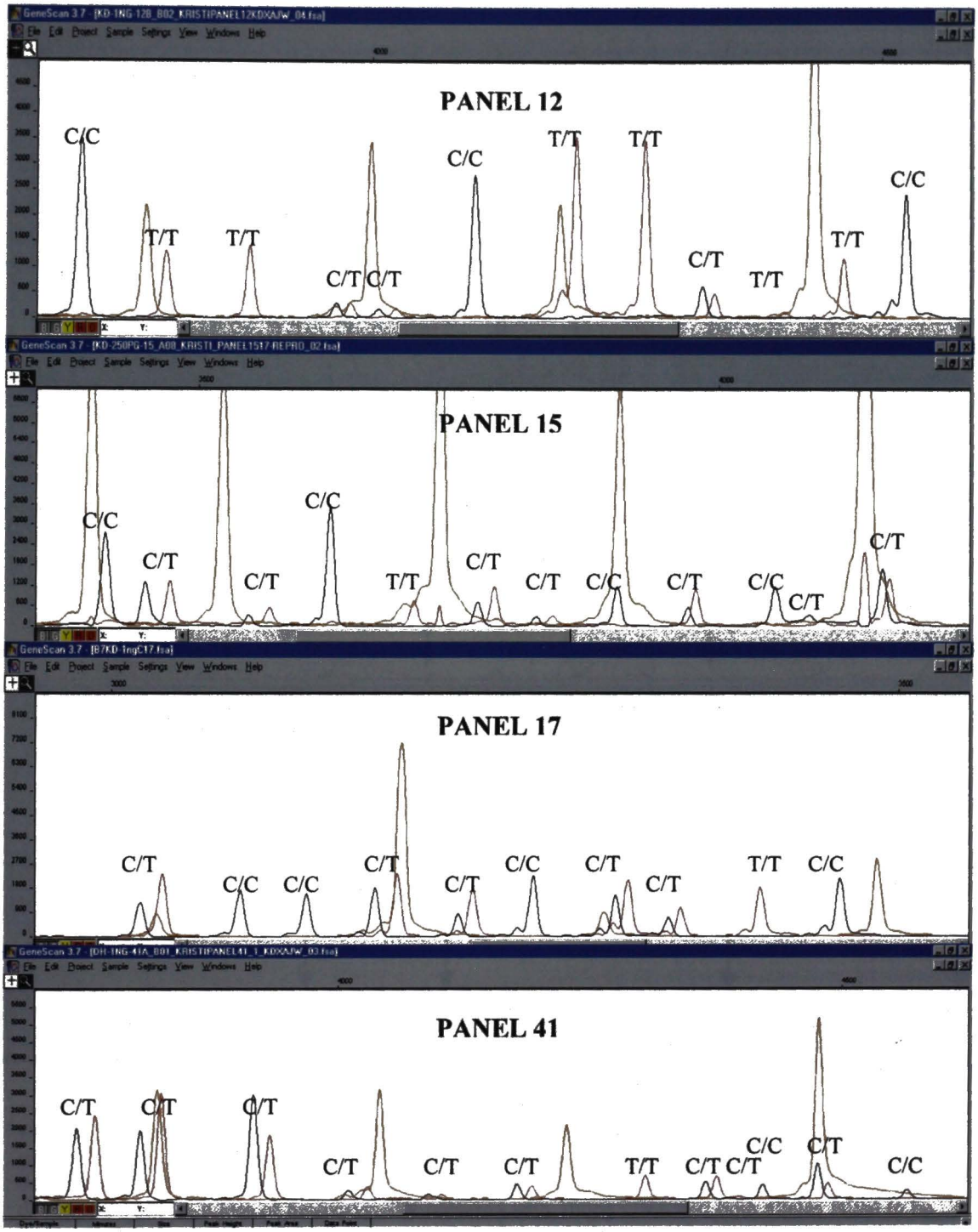

Figure 2 - SNP profile for sample KD showing all 45 SNP markers detected with the four multiplex panels. Panel 17 interrogates 10 SNP markers while Panels 12, 15, and 41 all identify 12 SNP markers.

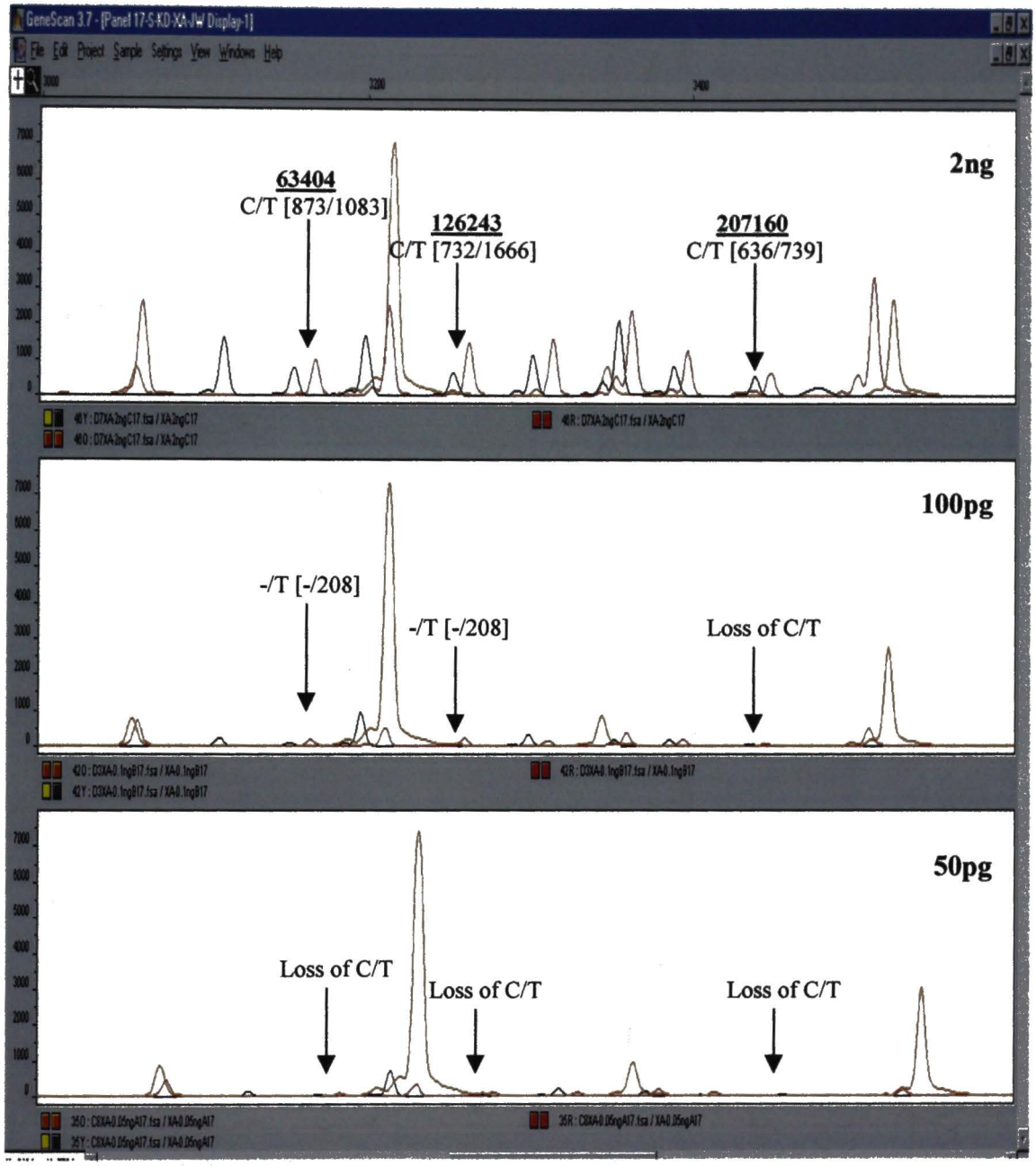

Figure 3 - Electropherogram results from analysis on the ABI 310 for Panel 17 SNP detection of sample XA at three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. At SNP markers 63404 and 126243, a loss of heterozygosity occurred with 100pg DNA and both alleles failed to be detected with 50pg DNA. A SNP at marker 207160 was not detected at 100pg and 50pg.

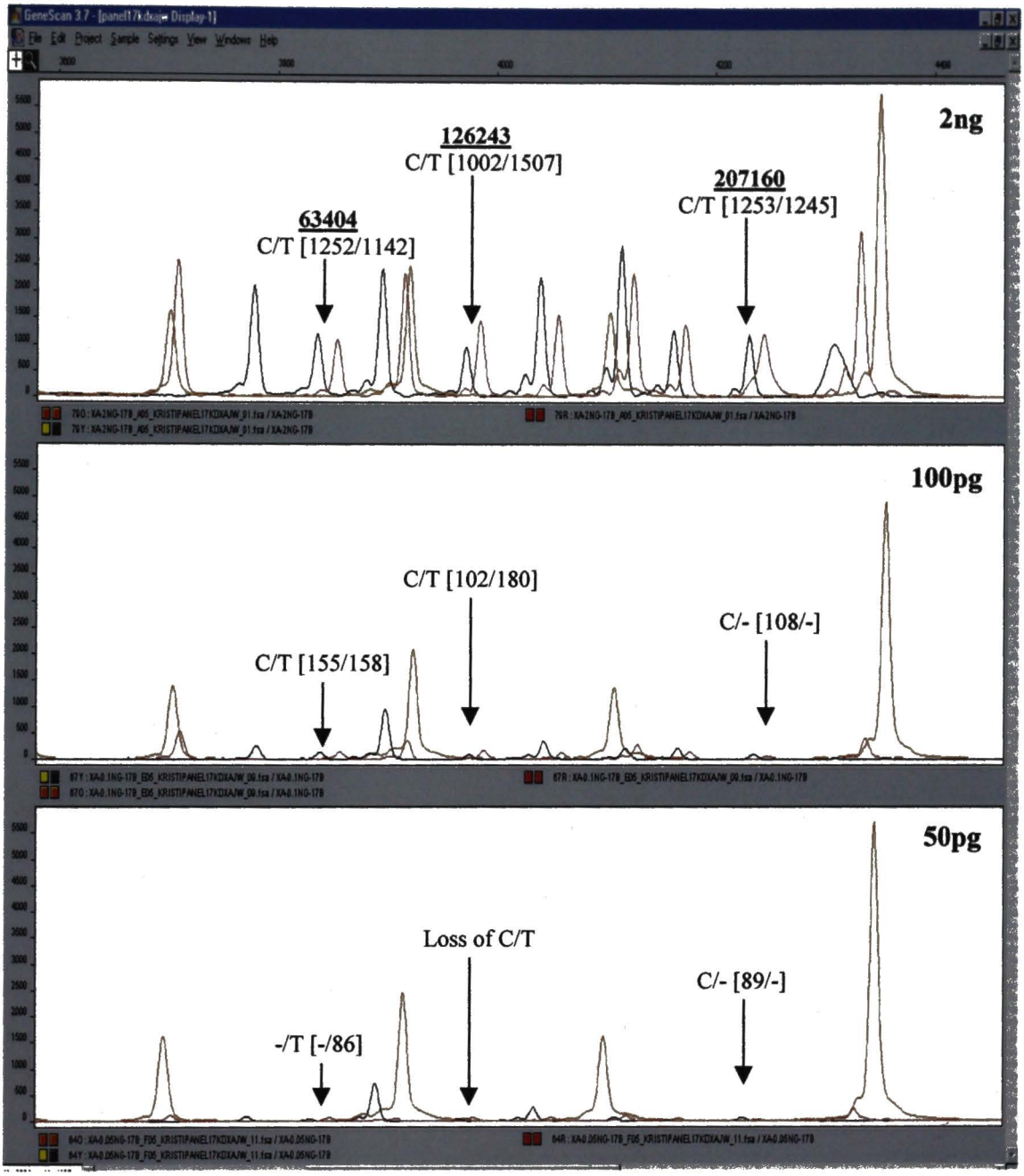

Figure 4 - Electropherogram results from analysis on the ABI 3100 for Panel 17 SNP detection of sample XA at three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. A loss of herozygosity occurred at marker 63404 below 100pg DNA. A SNP at marker 126243 was undetected with 50pg DNA. Loss of heterozygosity occurred at marker 207160 at quantities of 100pg and 50pg.

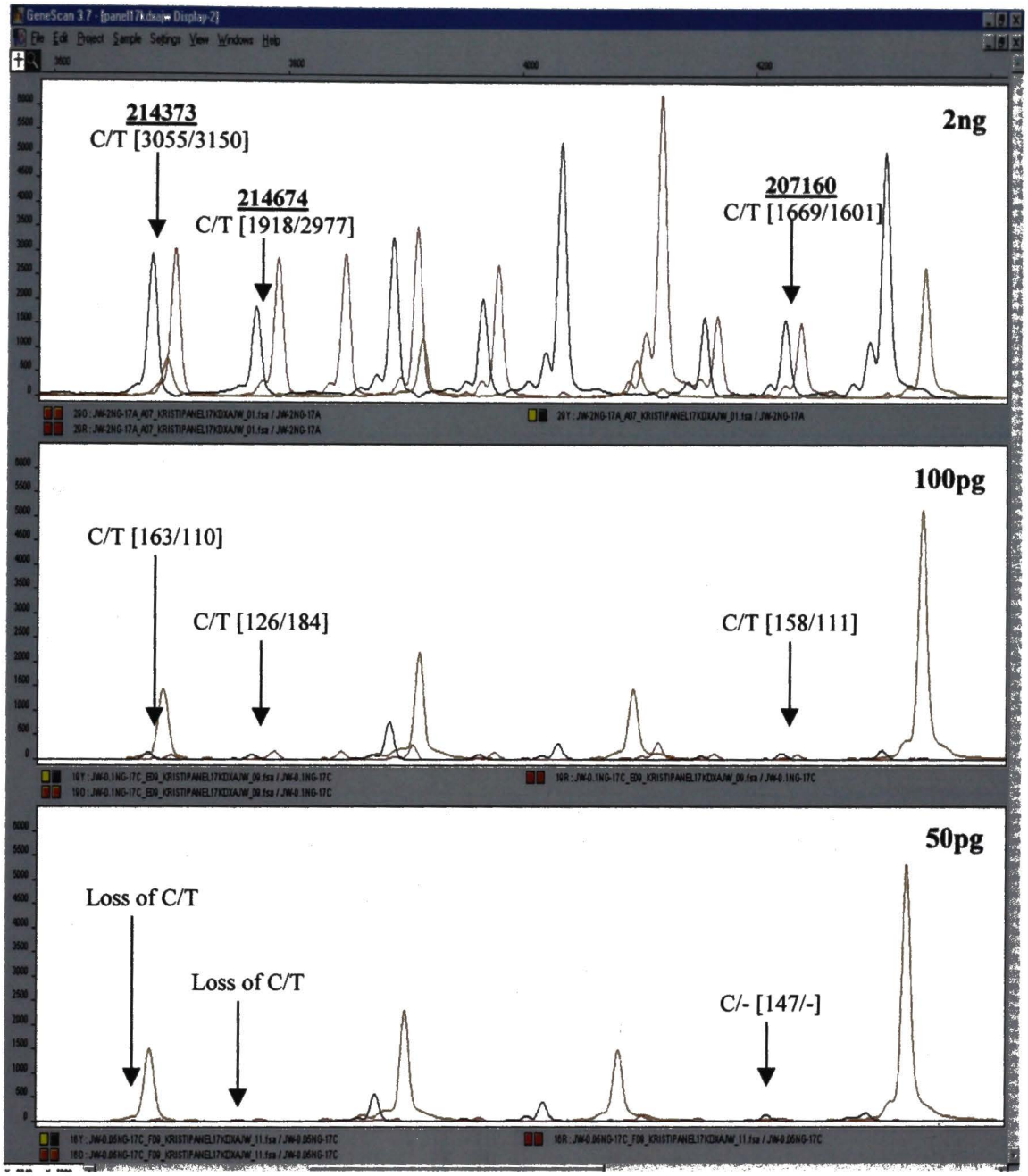

Figure 5 - Electropherogram results from analysis on the ABI 3100 for Panel 17 SNP detection of sample JW at three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. At 50pg, SNPs at markers 214373 and 214674 were not detected or fall below the detection threshold. At marker 207160, a loss of heterozygosity occurred below 100pg DNA.

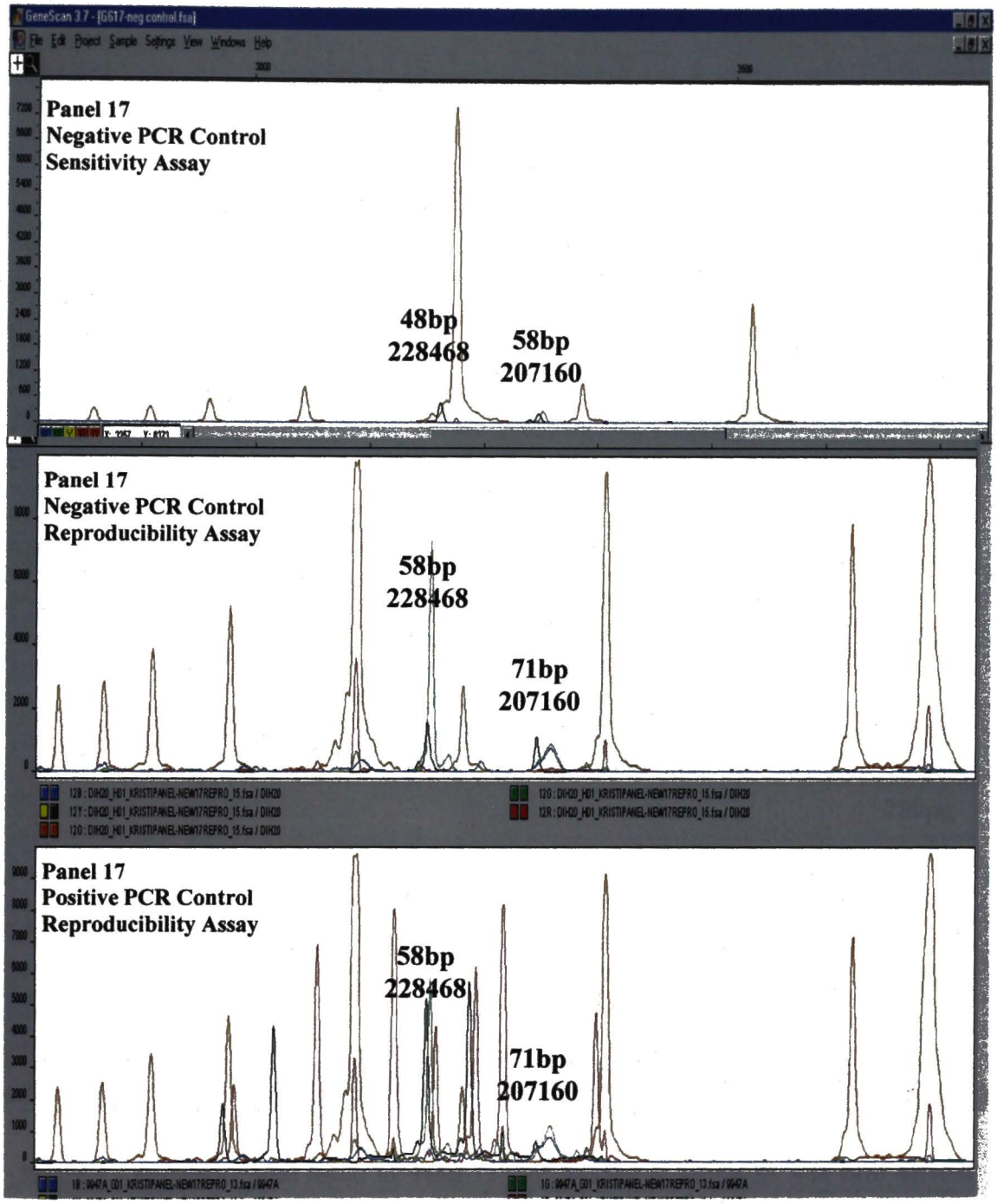

Figure 6 - Electropherograms of the negative PCR control carried out with the original set of multiplex primer mixes (top panel) and negative and positive PCR controls carried out with the second batch of primer mixes (middle and bottom panels). The extraneous peaks may be caused by a low quantity contaminant or two primers may have annealed together and participated in the extension reaction.

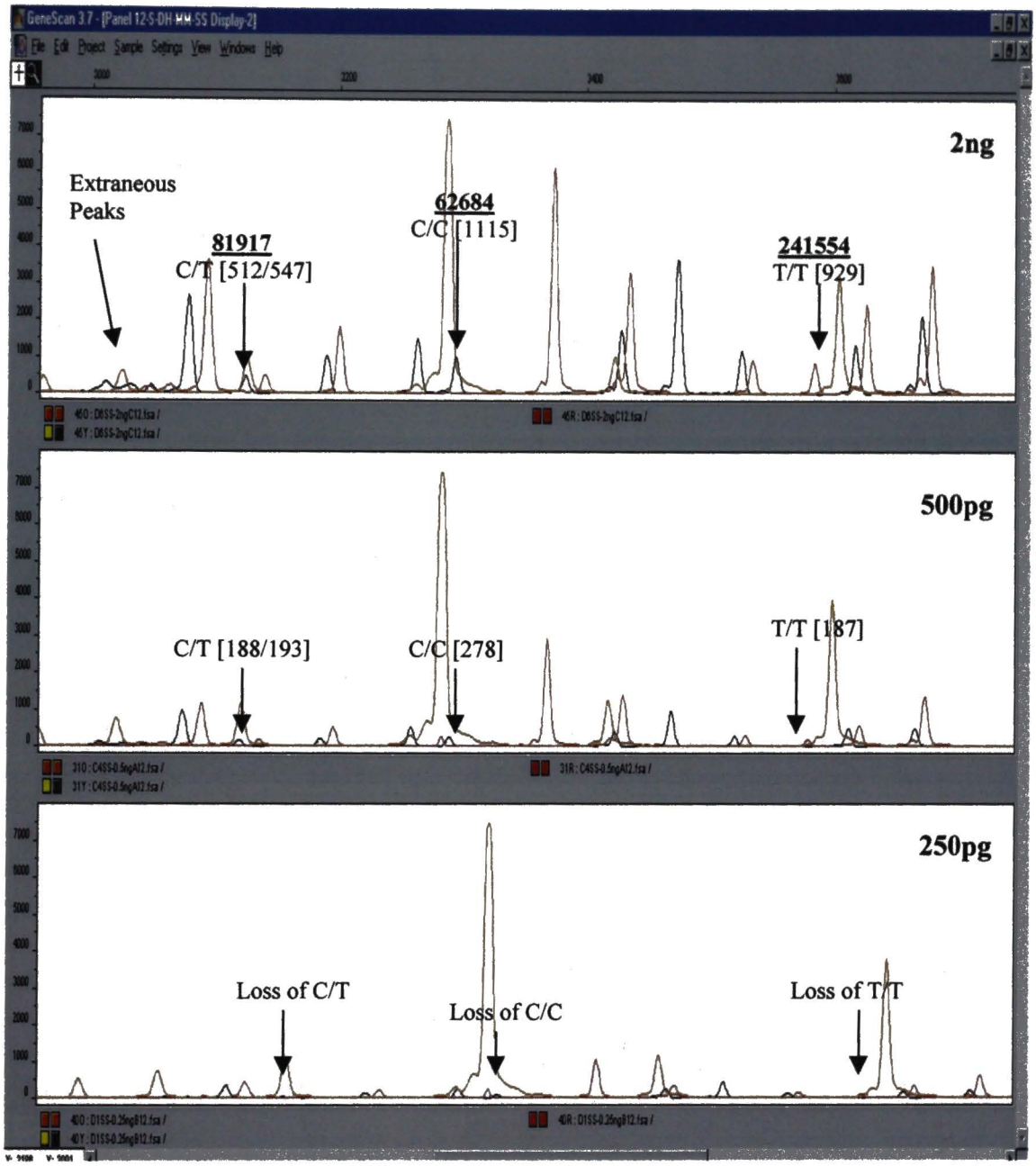

Figure 7 - Electropherogram results for Panel 12 SNP detection of sample SS with three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. SNPs at markers 81917, 62684, and 241554 were not detected below a quantity of 500pg DNA. Extraneous peaks were detected having approximate sizes of 24-29bp, but did not interfere with SNP typing since the shortest true SNP marker was 32bp.

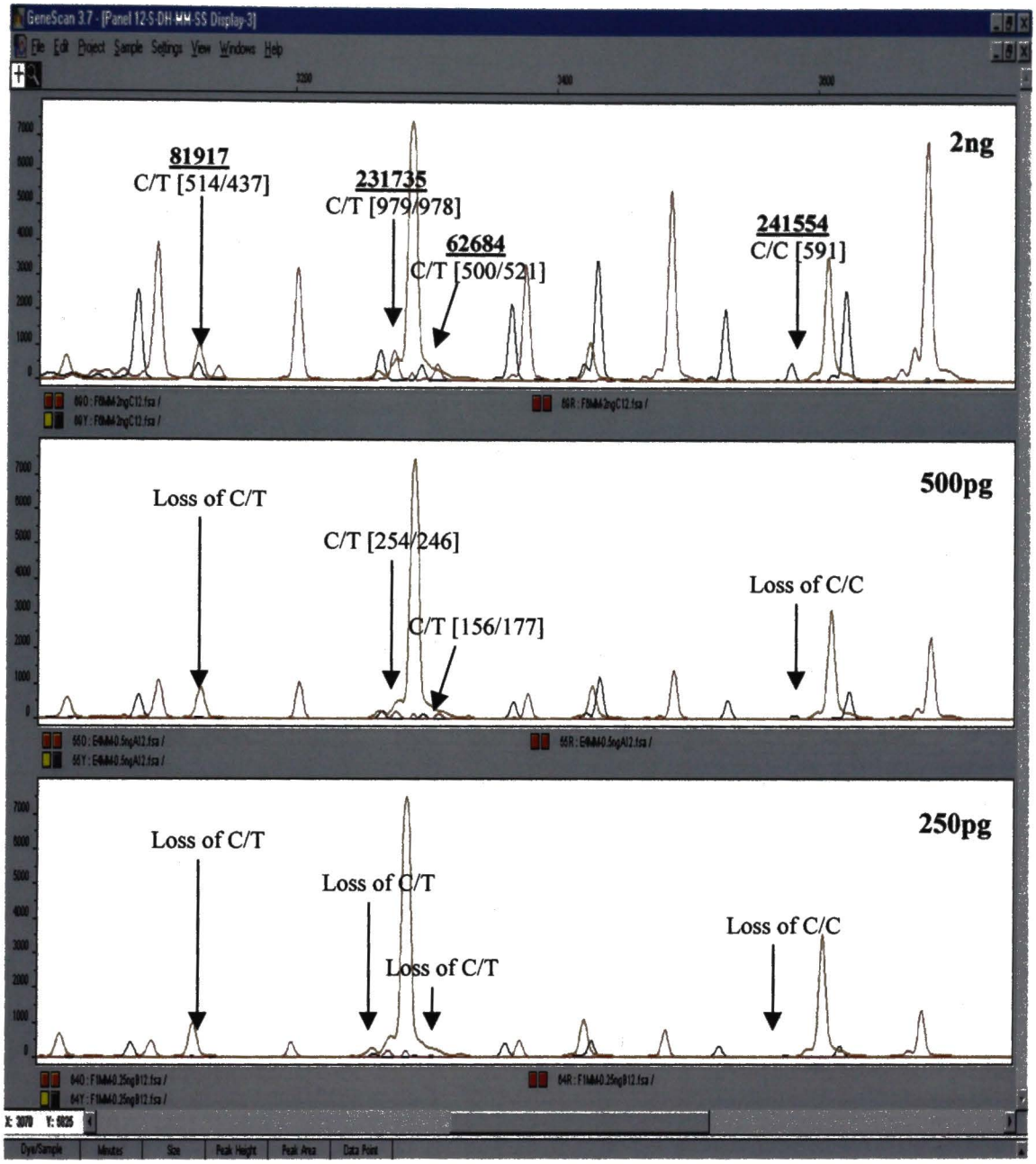

Figure 8 - Electropherogram results for Panel 12 SNP detection of sample MM at three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. A SNP at marker 81917 was not identified at quantities below 500pg DNA. Two SNPs detected at markers 231735 and 62684 fell below detection threshold with 250pg DNA. A C/C SNP marker 241554 was not detected below 500pg DNA.

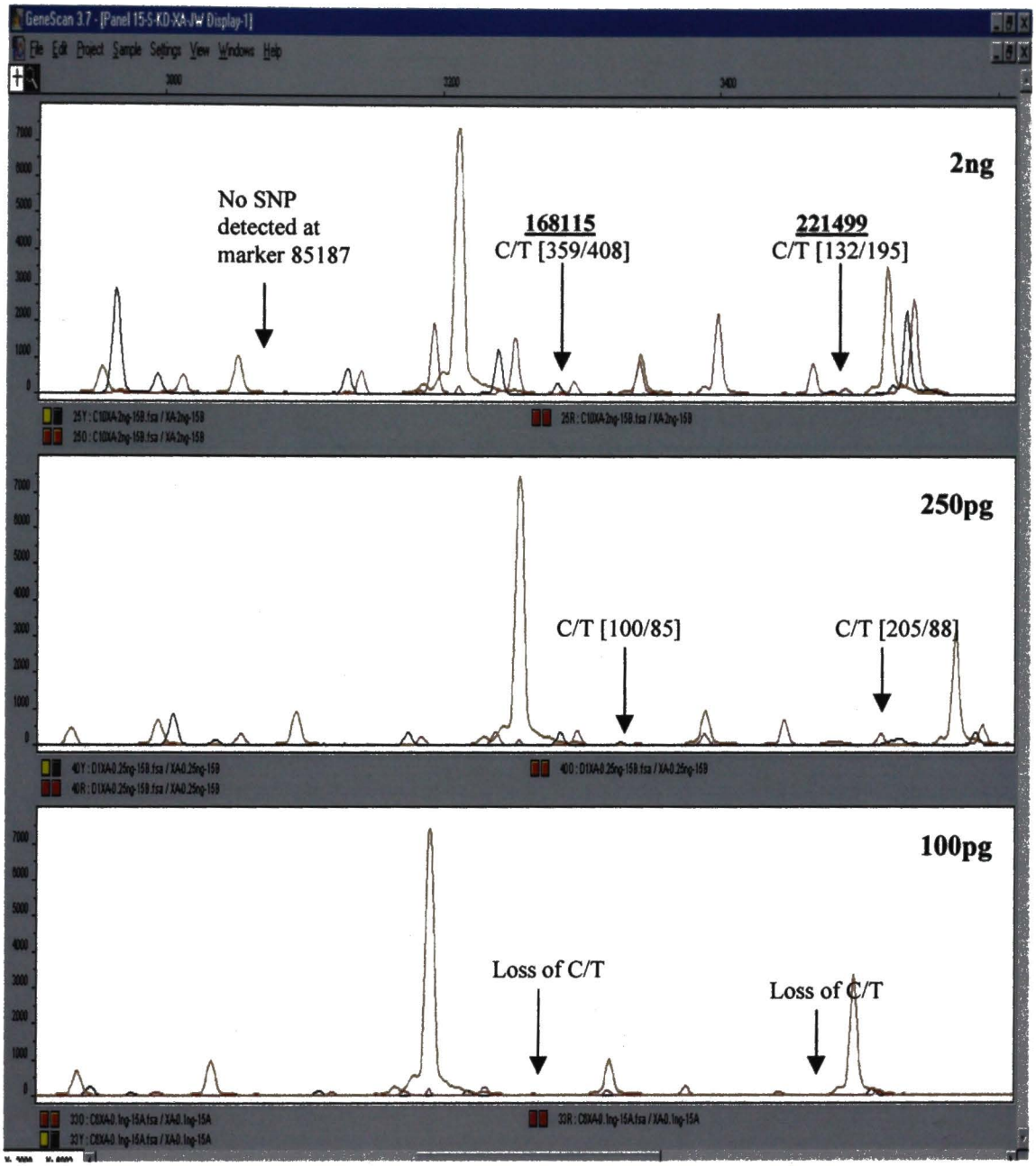

Figure 9 - Electropherogram results for Panel 15 SNP detection of sample XA with three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. C/T SNPs at markers 168115 and 221499 were not detected with 100pg DNA. No SNP was detected at marker 85187 even with 2ng DNA.

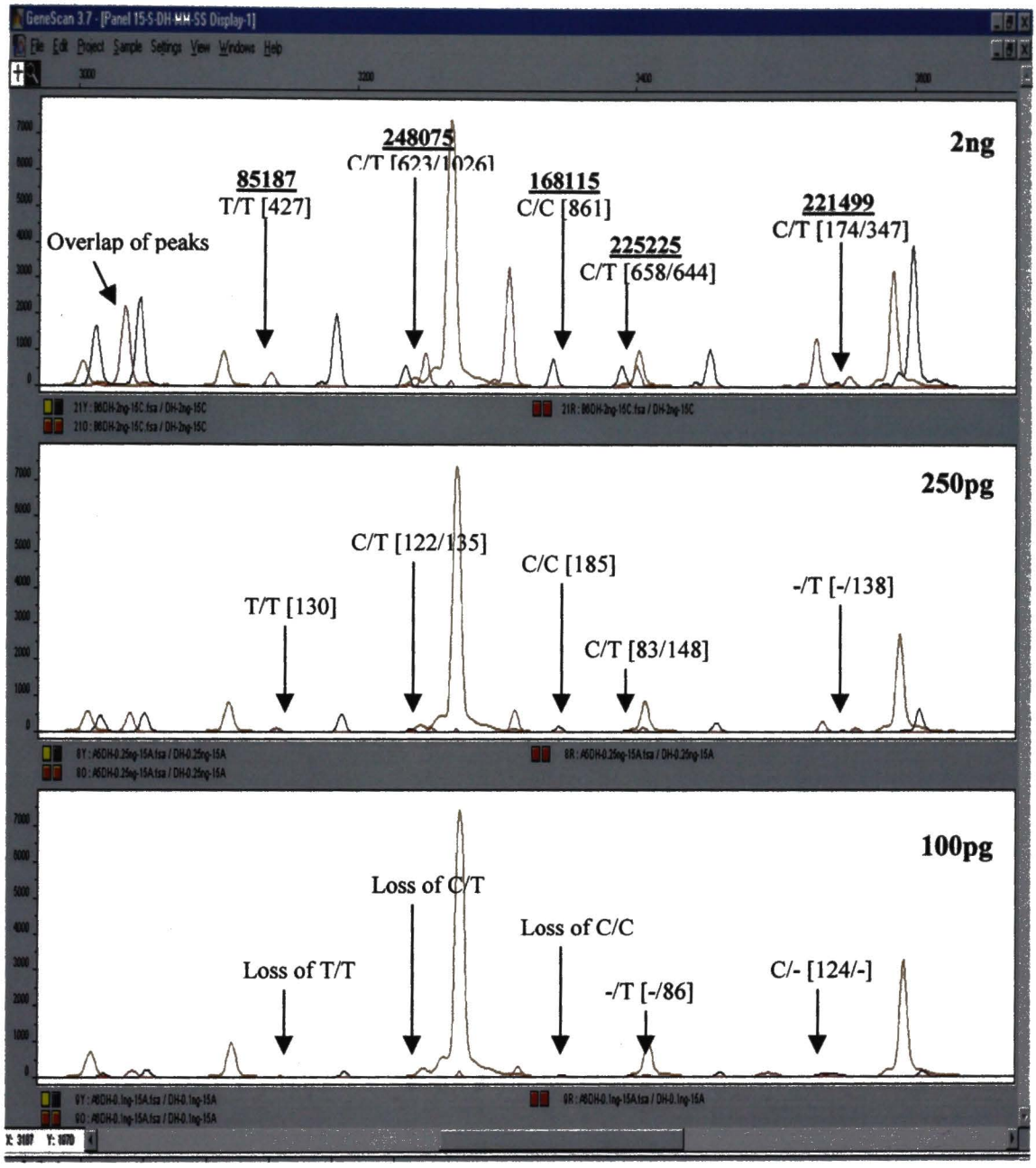

Figure 10 - Electropherogram results for Panel 15 SNP detection of sample DH at three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. An overlap of peaks was seen with markers 177589 and 231480. SNPs at markers 85187, 248075, and 168115 were not detected with 100pg DNA. A loss of heterozygosity occurred at marker 225225 with 100pg DNA, and at marker 221499 with 250pg DNA.

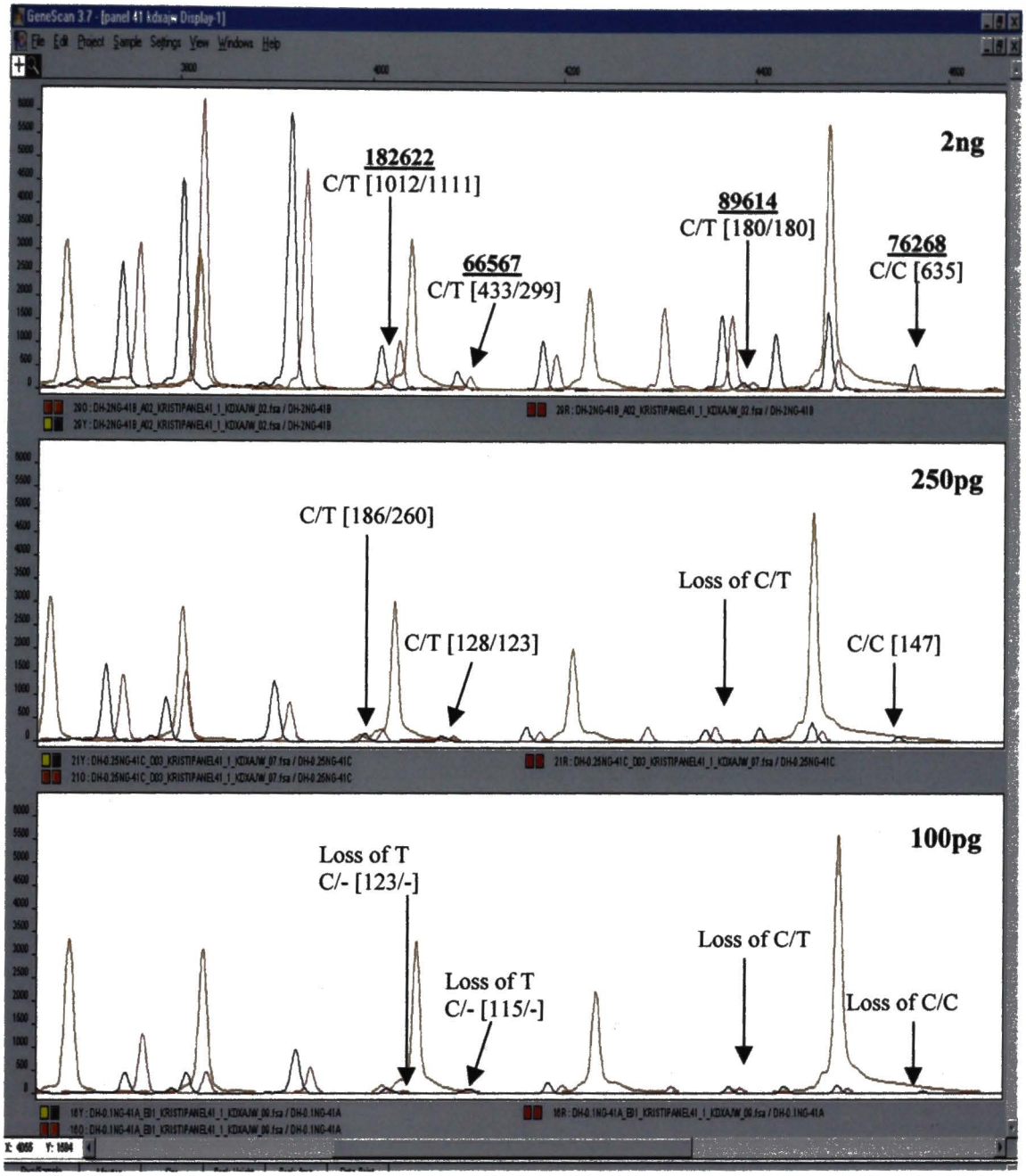

Figure 11 - Electropherogram results for Panel 41 SNP detection of sample KD at three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. A loss of heterozygosity occured at markers 182622 and 66567 with 100pg DNA. A SNP detected at marker 89614 was not detected with 250pg DNA. A SNP at marker 76268 was not detected with a quantity of 100pg DNA.

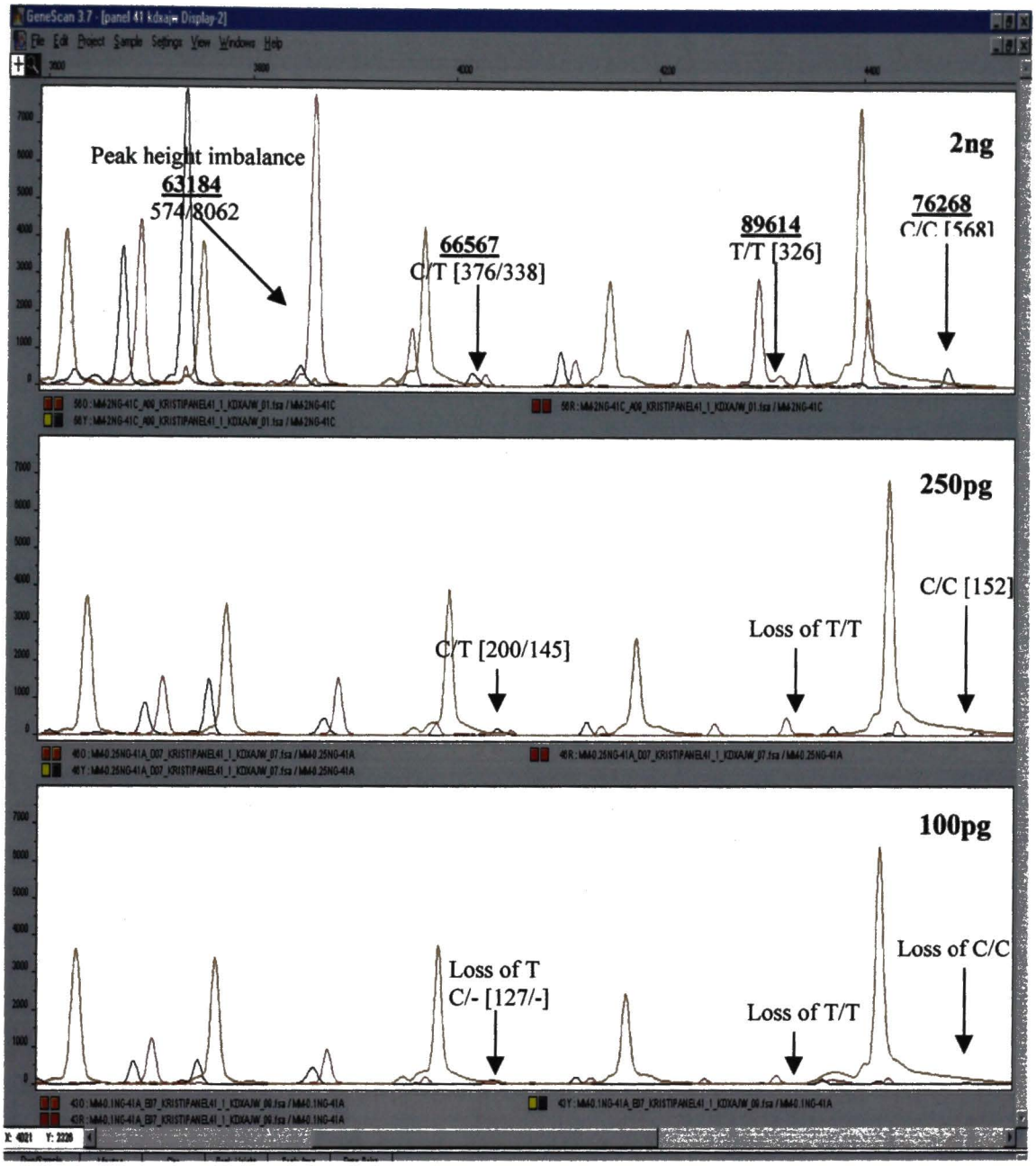

Figure 12 - Electropherogram results for Panel 41 SNP detection of sample JW at three different quantities of DNA. C alleles are designated by red peaks and T alleles designated by black peaks. The orange peaks represent the Liz120 size standard. A loss of heterozygosity occurred at SNP marker 66567 with 100pg DNA. At 250pg and 100pg, a TT SNP was not detected at marker 89614. A CC SNP at marker 76268 fell below detection threshold with 100pg DNA. A large peak height imbalance occurred at marker 63184.

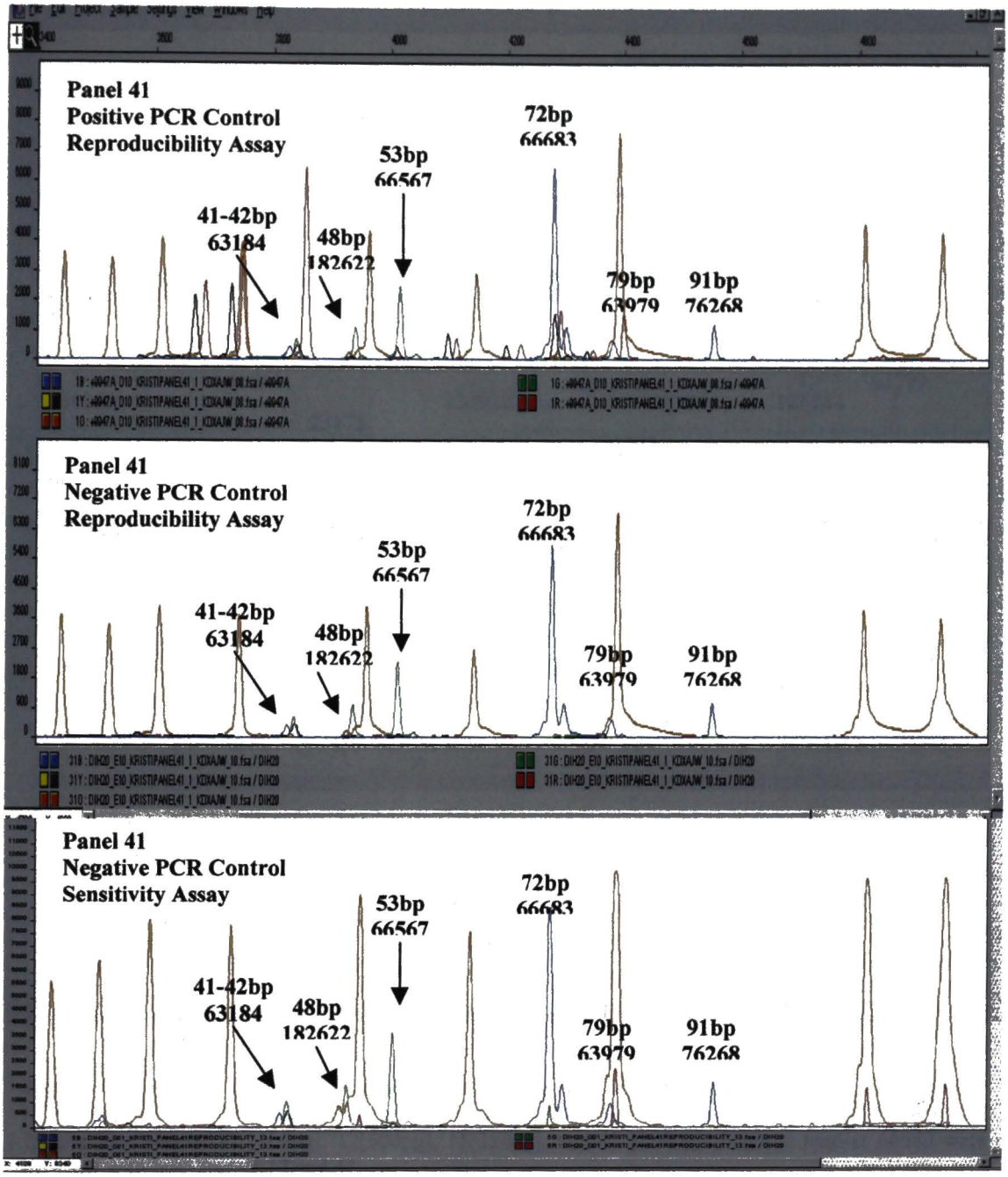

Figure 13 - Electropherograms of the positive and negative PCR controls carried out with Panel 41 reproducibility assays (top and middle panels) and the negative PCR control carried out with Panel 41 sensitivity reactions of samples KD, XA, and JW (bottom panel). These extra peaks may be a result of contamination or primers in one or both multiplex mixes may have annealed to one another and participated in the extension reacion.

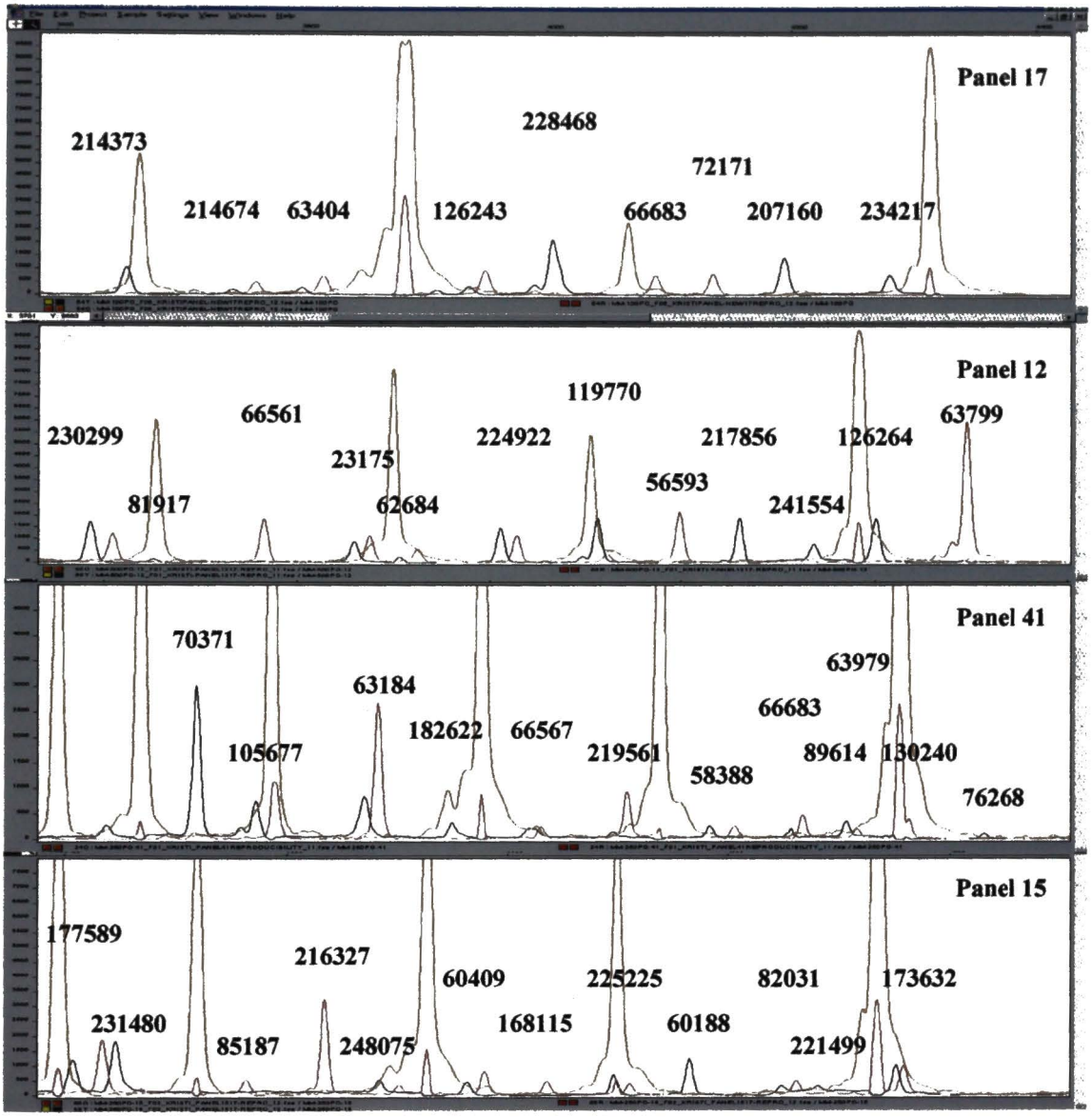

Figure 14 - Electropherograms from the reproducibility reactions show 44 SNPs detected in sample MM using the four multiplex SNP extension primer panels. The following quantities of DNA were used for the four panels: 17 [100pg], 12[500pg], 15[250pg], and 41 [250pg]. SNPs at marker 66683 were detected with both Panels 17 and 41.

APPENDIX

LABORATORY NOTEBOOK/DAILY LOG

Laboratory Notebook/Daily Log

The goal of this project was to validate four multiplex SNP extension primer panels by looking at sensitivity and reproducibility assays. Throughout the course of the project, I kept a daily log of all the assays and procedures I carried out. In addition, I recorded all the lot numbers and expiration dates when available for the reagents used during the project. The following is a list of this information. When more than one lot was used for a particular reagent during the course of the project, I mentioned it in the daily log for each day it was used. I have listed those reagents where only one lot was used for the entire project in the following table instead of noting it for each day.

REAGENT	LOT NUMBER	EXPIRATION DATE
Cell Lysis Buffer	N/A	12/27/00
Protein Lysis Buffer	N/A	09/25/03
PCIA (Phenol/Chloroform/Isoamyl)	121340	06/05/03
100% Ethanol	L7148	09/05/03
70% Ethanol	N/A	08/01/03
TE Buffer	N/A	07/27/03
TE^{-4} Buffer	N/A	03/11/03
Proprietary RT-PCR Kit from ABI®	010603 DO7578	08/31/03
10X PCR Buffer	DO3434	09/30/03
$MgCl_2$	DO3123	10/31/03
10mM dNTPs	36227505044	N/A
9947A (1.25ng/µl) Control DNA	DD100A 15230001	01/21/04
5X Sequencing Buffer	N/A	10/22/03
pGEM+3Zf(+)	36085110054	N/A
SNaPshot Multiplex Control Primer	4323293	N/A
SAP (1U/µl - Shrimp Alkaline Phosphatase)	112550	N/A
POP-4™ Polymer for ABI 310	0209050	03/17/03
POP-4™ Polymer for ABI 3100	0211057	08/12/03
10X buffer with EDTA for ABI® Prism 310	0207142	03/17/03
10X buffer with EDTA for ABI® Prism 3100	0209145	03/11/03

01/22/03

- **Organic DNA Extraction from 3 Fresh Blood Samples** – Clinician in UNT-HSC Paternity Clinic collected fresh blood (~5ml) in EDTA vacutainer tubes from three volunteers identified as: KD, XA, and JW. I did a phenol/chloroform organic extraction according to the protocol described in the Materials and Methods for all 5ml of blood from each volunteer. After addition of PCIA and centrifugation, I discovered the PCIA was expired. I recovered solution into new tubes and added fresh PCIA. Proceeded with extraction protocol. I ethanol precipitated and resuspended the DNA samples in 500µl TE^{-4} buffer.

01/23/03

- **Organic DNA Extraction from 3 Fresh Blood Samples** – Clinician collected fresh blood (~5ml) in EDTA vacutainer tubes from three more volunteers identified as: DH, SS, and MM. I did a phenol/chloroform organic extraction, ethanol precipitation, and resuspended the DNA samples in TE^{-4} buffer as with the samples on 1/22/03.

01/27/03

- **Pooling of DNA Extracts** – I pooled extracts together for each sample into 15ml conical tubes. Centrifuged tubes in clinic and aliquot 1ml of each into new 2ml tubes. Aliquot remainder of each sample into new tubes as well for a total volume of about 2-2.5ml of each sample
- **Spectrophotometer Quantification of DNA Extracts** – Made 1:50 dilution of each sample extract by adding 10µl DNA to 490µl nanopure water. I used the

spectrophotometer in the Lyme Disease lab to quantify the samples at the DNA 260/280 setting.

- **Prepared TE Buffer for Dilutions** – To a glass bottle, I added 10ml Tris-HCl (pH = 7.5), 2ml EDTA, and 988ml nanopure H_2O. Autoclaved for 20 minutes and cooled to room temperature overnight. TE buffer will be used to dilute the DNA stock for long-term storage. TE^{-4} buffer will be used to make dilutions required for this project.

01/28/03

- **Prepared 2.5ml of 10ng/ul DNA solution from samples** - Used estimated quantitation values obtained from spectrophotometer measurements to determine the volume of each sample needed to be diluted with TE buffer in order to make 2.5ml of 10ng/µl DNA solution.

01/31/03

- **Real Time PCR Quantification of 10ng/ul DNA samples** – Used proprietary quantification kit from ABI™ to quantify samples via real-time PCR on the ABI® Prism 7000 Sequence Detection System. Made master mix of primer/probe (10µl/sample) and universal master mix (12.5µl/sample) for 6 samples in duplicate. I added 22.5µl master mix to designated wells in plate. Added 2.5µl DNA to respective wells. I pipetted to mix and centrifuged the plate ~2 minutes at 3000rpm. Placed in ABI® Prism 7000 for real-time PCR program. Analyzed results of RT-PCR. I then calculated more precise quantities of DNA needed for each sample to make new 10ng/µl dilutions with TE buffer. Used the average

value of duplicate reaction quantities to figure new dilution calculations. I made each new sample in 15ml conical tubes.

02/03/03

- **RT-PCR Quantification of New 10ng/ul Samples** – Prepared master mix as before: 10µl primer/probe mix per sample plus 12.5µl universal master mix per sample. I added 22.5µl master mix into wells and 2.5µl DNA sample into respective wells and pipetted to mix. Centrifuged the plate 2 minutes at 3000rpm. Placed in ABI® Prism 7000 and started program. Analysis of the RT-PCR results reveals quantities range from 10.27ng/µl to 16.04ng/µl for the 6 samples. Calculated new dilution factors to refigure how much more TE buffer is needed to bring concentration to 10ng/µl. After bringing each sample to 10ng/µl, I made 1:5 dilution of each sample to obtain concentrations of 2ng/µl
- **RT-PCR Quantification of 2ng/µl DNA Samples** – Made master mix as before: 10µl primer/probe per sample plus 12.5µl Universal master mix per sample. Vortexed and added 22.5µl to wells. I added 2.5µl DNA sample to respective wells and centrifuged 2 minutes at 3000rpm and placed plate in the ABI® Prism 7000 for RT-PCR run. Analysis of RT-PCR results reveal the average quantity of DNA for each sample ranged from 2.21-2.51ng/µl
- **Serial Dilutions** – Calculated amount of DNA and TE^{-4} buffer needed to make the following concentrations for each sample (beginning with 2ng/µl): 1ng/µl,

500pg/µl, 250pg/µl, 100pg/µl, 50pg/µl, 25pg/µl, and 10pg/µl. Performed serial dilutions for each sample beginning with the 2ng/µl concentrated sample.

02/05/03

- **Panel 17: Sensitivity Assay Samples KD, XA, JW – PCR Amplification –** Prepare master mix for 85 reactions by adding 42.5µl 10X PCR buffer, 85µl $MgCl_2$, 12.75µl 2.5mM dNTPs, 2.55µl panel 17 amplification primer pool, 8.5µl AmpliTaq® Gold (Lot #DO4942), and 104.55µl dH_2O to tube. I designated the 8 dilutions made earlier with numbers 1-8 for ease in labeling tubes [1) 2ng/µl, 2) 1ng/µl, 3) 500pg/µl, 4) 250pg/µl, 5) 100pg/µl, 6) 50pg/µl, 7) 25pg/µl, and 8) 10pg/µl]. Labeled 24 tubes for each of the 3 samples for each dilution in triplicate, 3 tubes for the 0ng/µl TE buffer, 1 negative control tube with dH_2O, and 1 positive control tube with 1.25ng/µl 9947A. Made 1:1 dilutions of each of the 3 DNA sample concentrations since the protocol requires adding 2µl DNA to the PCR reaction. Added 3µl PCR master mix to tubes and 2µl DNA, TE, dH_2O, and 9947A to respective tubes. Pipet to mix. Place on thermal cycler program SNP-PCR-Amp.

02/06/03

- **Panel 17: Sensitivity Assay Samples KD, XA, JW – ExoI/SAP –** Made master mix for 85 reactions by adding 178.5µl ExoI/SAP per sample and 518.5µl dH_2O to 1.5mL tube. Vortexed and added 8.2µl mix to each PCR product and pipet mixed. Placed on thermal cycler SNP-Exo-Sap program.

- **SNP Extension** – Prepare master mix for 90 reactions in a 1.5mL tube by adding 45µl Panel 17 SNP extension primer pool, 225µl SNaPshot Ready Reaction mix (Lot #431218207014), 45µl 5X sequencing buffer, and 315µl dH_2O. Vortex. Made positive and negative SNaPshot™ controls. Added 7µl master mix to new labeled tubes. Added 3µl ExoI/SAP product to respective tubes and pipetted to mix. Placed on thermal cycler program SNP-Ext. Store ExoI/SAP products at 4°C.
- **SAP** – Made master mix for 90 reactions by adding 90µl SAP and 450µl dH_2O to 1.5mL tube. Vortex. Added 6µl to each product and pipet mixed. Place on thermal cycler program SNP-Sap.

02/07/03

- **Panel 17: Sensitivity Assay Samples KD, XA, JW – Sample Preparation for ABI Prism® 310 CE** – Made 120LIZ™/formamide mix for 90 reactions. Added 45µl 120LIZ™ (Lot #025009) and 1260µl formamide to 1.5ml tube. Vortexed and added 14.5µl mix to new labeled tubes. I added 0.9µl DNA product to respective tubes. Pipet mixed and centrifuged 1 minute at 3000rpm. Heat denatured 3 minutes at 95°C and snap cooled on ice. I set up the 310 and loaded my samples. Stored remaining SAP products at 4°C.
- **Panel 17: Sensitivity Assay Samples DH, SS, MM – PCR Amplification** – Made master mix for 90 samples as before by adding to 1.5ml tube 45µl 10X PCR tube, 90µl $MgCl_2$, 13.5µl 2.5mM dNTPs, 2.7µl Panel 17 PCR primer pool,

9µl AmpliTaq® Gold (Lot #DO4942), and 110.7µl dH_2O. Vortexed master mix. I made a 1:1 dilution of DNA for each sample dilution to add 2µl DNA to the PCR reaction. Added 3µl master mix to labeled tubes and 2µl DNA to respective tubes. Added 2µl TE, dH_2O, or 9947A to control tubes. Place on thermal cycler SNP-PCR Amp program.

- **ExoI/SAP** – Made master mix for 85 reactions by adding 178.5µl ExoI/SAP and 518.5µl dH_2O to tube. Vortex and add 8.2µl to each DNA product. Pipet to mix. Place on thermal cycler SNP-Exo-Sap program. Store samples at 4°C until receive more Panel 17 SNP Extension primer pool from Orchid-Cellmark to continue with extension step.

02/08/03

- **Panel 12: Sensitivity Assay Samples DH, SS, MM – PCR Amplification** – Made master mix for 170 samples (enough for all 6 samples and dilutions). Add to 1.5ml tube: 85µl 10X PCR buffer, 170µl $MgCl_2$, 25.5µl 2.5mM dNTPs, 5.1µl Panel 12 PCR primer pool, 17µl AmpliTaq® Gold (Lot #DO4942), and 209.1µl dH_2O. Vortex. Prepared 1:1 dilutions of DNA samples. Added 3µl PCR master mix to 77 labeled tubes (72 for 3 samples, 8 dilutions, in triplicate, 3 – 0ng/µl TE buffer, 1 positive control and 1 negative control). Added 2µl DNA, TE, 9947A, or dH_2O to respective tubes. Pipet mix and place on thermal cycler program SNP-PCR-Amp.

02/09/03

- **Panel 12: Sensitivity Assay Samples DH, SS, MM – ExoI/SAP** – Made ExoI/SAP mix for 100 samples (mine and a few of Xavier's) by adding 210µl ExoI/SAP and 610µl dH_2O to 1.5ml tube. Vortex. Add 8.2µl to PCR product, mix. Place on thermal cycler SNP-Exo-Sap program.
- **SNP Extension** – Made master mix for 90 samples by adding 54µl Panel 12 SNP extension primer pool, 225µl SNaPshot™ Ready Reaction Mix (Lot #431218207014), 45µl 5X buffer, and 306µl dH_2O to 1.5mL tube. Vortex and made positive and negative controls. Label new tubes and added 7µl master mix to each. Added 3µl ExoI/SAP product to respective tubes. Mixed and placed on thermal cycler SNP-Ext program. Stored remaining product at 4°C.
- **SAP** – Made SAP mix for 90 reactions by adding 90µl SAP to 450µl dH_2O. Vortexed and added 6µl to SNP-Ext products. Pipet mixed and placed on thermal cycler SNP-Sap program.
- **Prepare samples for ABI Prism® 310 CE** – Made 120LIZ™/formamide mix for 85 reactions by adding 42.5µl 120LIZ™ (Lot #025009) to 1190µl formamide in a tube and vortexing. Labeled new tubes and added 14.5µl mix to each. Added 0.9µl SAP product to respective tubes. Pipet mixed and centrifuged at 3000rpm for 1 minute. Heat denatured at 95°C for 3 minutes and snap cooled on ice. I placed the samples on the ABI Prism® 310 and started the run. Stored SAP products at 4°C.

- **Analyze Panel 17 – Sensitivity – KD, XA, JW results** – Stored ABI Prism® 310 samples at 4°C.
- **Panel 12: Sensitivity Assay Samples KD, XA, JW – PCR Amplification** – Made 1:1 dilution of each DNA sample. Label 77 tubes as before. Added 3µl master mix (made 02/08/03) to each tube. Added 2µl DNA, TE buffer, dH_2O, or 9947A to respective tubes and pipet mixed. Placed on thermal cycler SNP-PCR-Amp.
- **ExoI/SAP** – Made mix for 80 reactions. Added 168µl ExoI/SAP to 488µl dH_2O in a tube and vortexed. I added 8.2µl to PCR products and mix. Put on thermal cycler SNP-Exo-Sap program.

02/10/03

- **Panel 12: Sensitivity Assay Samples KD, XA, JW – SNP Extension** – Made mix for 90 samples as before and made controls. Labeled new tubes and added 7µl mix to all tubes. I added 3µl ExoI/SAP products to respective tubes and mixed. Place on thermal cycler SNP-Ext. Store ExoI/SAP products at 4°C.
- **SAP** – Made mix incorrectly [used ExoI/SAP recipe and added to JW samples]. I then prepared SAP mix using the incorrect volumes [used ExoI/SAP volumes] and added to remaining samples. Place on thermal cycler SNP-Sap. Will continue on with protocol for samples.

02/11/03

- **Panel 12: Sensitivity Assay Samples KD, XA, JW – Prepare Samples for ABI Prism 310® CE** – Made master mix of 120LIZ™/formamide for 85 samples by adding 42.5µl 120LIZ™ (Lot #025009) and 1190µl formamide to 1.5ml tube. Vortexed. Labeled new tubes and added 14.5µl mix to each. I added 0.9µl DNA sample to respective tubes and pipet mixed. Centrifuged samples 1 minute at 3000rpm. Heat denatured 3 minutes at 95°C and snap cooled on ice. I added POP-4™ polymer to syringe and prepared sample sheet and injection list on the ABI Prism® 310. Loaded samples and started run. Stored SAP products at 4°C.
- **Analyze results from Panel 12: Sensitivity Assay Samples DH, SS, MM** – Stored samples at 4°C.
- **Panel 15: Sensitivity Assay Samples KD, XA, JW – PCR Amplification** – Made master mix for 170 samples (enough for all 6 blood extracts) in a 1.5ml tube. Added 85µl 10X buffer, 170µl $MgCl_2$, 25.5µl dNTPs, 5.1µl Panel 15 amplification primers, 379.1µl dH_2O and only 8.5µl AmpliTaq® Gold (Lot #DO4942). (Ran out of AmpliTaq® Gold so before adding it, I vortexed other reagents and aliquoted ½ mixture into a second tube. Then I added the 8.5µl Taq to one tube to use for these samples. Will add 8.5µl Taq to second tube when needed). Also, I increased the amount of dH_2O in the master mix to eliminate the dilution of DNA samples in this step. This will decrease the chance for pipetting errors. Added 4µl master mix to the labeled tubes and 1µl DNA, TE, dH_2O, or

9947A to respective tubes. Vortexed to mix. Placed on thermal cycler program SNP-PCR-Amp.

- **ExoI/SAP** – Made mix in a 1.5ml tube for 85 reactions by adding 178.5µl ExoI/SAP to 518.5µl dH_2O. Vortexed. Added 8.2µl mix to each PCR product. Pipet mixed and placed on thermal cycler SNP-Exo-Sap program.
- **Panel 15: Sensitivity Assay Samples KD, XA, JW – SNP Extension** – Prepared mix for 90 reactions in a 1.5ml tube by adding 54µl panel 15 SNP extension primer pool, 225µl SNaPshot™ Ready Reaction mix (Lot #431218207014), 45µl 5X sequencing buffer, and 306µl dH_2O. Vortex. Label new tubes. Make SNaPshot™ positive and negative controls. Added 7µl master mix to new tubes and 3µl ExoI/SAP product to respective tubes and pipet mixed. Put on thermal cycler program SNP-Ext program. Store ExoI/SAP products at 4°C.
- **Panel 15: Sensitivity Assay Samples KD, XA, JW – SAP** – Made mix for 90 reactions in a 1.5ml tube by adding 90µl SAP to 450µl dH_2O. Vortex. Added 6µl mix to each SNP-extension product and mix. Place on thermal cycler program SNP-SAP.

02/12/03

- **Panel 15: Sensitivity Assay Samples KD, XA, JW – Prepare Samples for ABI Prism® 310 CE** – Changed buffer in 310: Made 1X buffer by adding 1.5ml 10X buffer to 13.5ml dH_2O in a 15ml conical tube. Mix and add to vials. Added POP-4™ polymer to syringe and set temperature to 60°C. Did a sequence fill capillary.

Made 120LIZ™/formamide mix for 85 reactions. Added 42.5µl 120LIZ™ (Lot #025009) to 1190µl formamide. Vortexed. Label new tubes. Added 14.5µl mix to each tube. Added 0.9µl DNA sample to respective tubes and pipetted to mix. Centrifuged samples 1 minute at 3000rpm. Heat denatured at 95°C 3 minutes and snap cooled on ice. Set up sample sheet and injection list on ABI Prism® 310. Loaded samples and start run. Store SAP products at 4°C.

- **Panel 15: Sensitivity Assay Samples DH, SS, MM – PCR Amplification** – Made master mix for 77 samples in a 1.5ml tube. Retrieve mix made 02/11/03 and added 8.5µl AmpliTaq® Gold (Lot #CO3741) and vortexed. Added 4µl mix to each tube. Added 1µl DNA, 9947A, TE, or dH_2O to respective tubes. Place on thermal cycler program SNP-PCR-Amp.
- **ExoI/SAP** – Made mix for 85 reactions in a 1.5ml tube by adding 178.5µl ExoI/SAP and 518.5µl dH_2O to tube. Vortex. Added 8.2µl mix to each product and mix. Place on thermal cycler SNP-Exo-Sap program.
- **Analyze Sensitivity Panel 12 – KD, XA, JW Samples from 310** – Results were poor. I will redo SNP extension and SAP reactions when I receive more SNP primer pool. Stored samples at 4°C.

02/13/03

- **Panel 15: Sensitivity Assay Samples DH, SS, MM – SNP Extension** – Made mix for 90 reactions in a 1.5ml tube. Add 54µl Panel 15 SNP primer pool, 225µl SNaPshot™ Ready Reaction Mix (Lot #431218207014), 45µl 5X sequencing

buffer, and 306µl dH_2O to tube. Made positive and negative controls. Added 7µl mix to new labeled tubes. Added 3µl ExoI/SAP products to respective tubes. Pipetted to mix and placed on thermal cycler SNP-Ext program. Stored ExoI/SAP products at 4°C.

- **SAP** – Made mix in a 1.5ml tube for 85 reactions by adding 85µl SAP to 425µl dH_2O. Vortexed. Add 6µl mix to each SNP-Ext product. Pipet to mix. Put on thermal cycler SNP-SAP program.

02/14/03

- **Panel 15: Sensitivity Assay Samples DH, SS, MM – Prepare Samples for ABI Prism 310® CE** – Add POP-4™ polymer to syringe, set up sample sheet and injection list, set temperature to 60°C and do sequence fill capillary. Labeled new tubes for samples and also for 8 samples that failed to produce data from Panel 12 DH, SS, MM. Make 120LIZ™/formamide mix for 100 reactions in a 1.5ml tube. Added 50µl 120LIZ™ (Lot #025009) to 1400µl formamide. Vortexed. Add 14.5µl mix to all tubes. Add 0.9µl DNA sample to respective tubes. Centrifuged 1 minute at 3000rpm. Heat denatured at 95°C for 3 minutes and snap cool on ice. Loaded samples on ABI Prism® 310 and started run. Stored SAP products at 4°C.
- **Analyze results for Panel 15: Sensitivity Assay Samples KD, XA, JW**

02/16/03

- **Analyze results for Panel 15: Sensitivity Assay Samples DH, SS, MM and 8 samples from Panel 12 – DH, SS, MM** – Store samples at 4°C.

03/06/03

- Spoke with Dr. Eisenberg about lack of primer pools remaining to finish project. He spoke with Janine at Orchid-Cellmark to schedule a meeting and obtain more panel primer pools.

03/07/03

- **Panel 41: Sensitivity Assay Samples KD, XA, JW, DH, MM – PCR Amplification** – I determined there is enough PCR primer pool to amplify all 6 samples, 8 dilutions, in triplicate. Labeled 154 PCR tubes for samples, 4 controls, and 6 TE buffer 0ng tubes (3 for each PCR plate). Made PCR master mix for 165 reactions in a 1.5ml tube. Vortex reagents first. Add 82.5µl 10X PCR buffer, 165µl $MgCl_2$, 24.75µl 25mM dNTPs, 16.5µl AmpliTaq® Gold (Lot #CO3741), 4.95µl Panel 41 PCR primer pool, and 367.95µl dH_2O. Vortexed. Added 4µl mix to each tube. Added 1µl DNA, TE buffer, 9947A, or dH_2O to respective tubes. Pipetted to mix. Put on thermal cycler program SNP-Amp.
- **ExoI/SAP** – Made mix for 165 reactions in a 1.5ml tube. Vortexed ExoI/SAP first. Added 346.5µl ExoI/SAP to 1006.5µl dH_2O to tube and vortex. Added 8.2µl mix to each PCR product. Pipetted to mix. Placed on thermal cycler program SNP-Exo-SAP. Stored samples at 4°C until more primers are received from Orchid Cellmark to continue protocol.

03/17/03

- Spoke with Dr. Eisenberg about using the ABI Prism® 3100 to finish project. This will allow others in the lab to use the ABI Prism® 310 for their projects and provide all of us to finish our projects by the set deadlines. Per Dr. Eisenberg, Xavier will need to be present when I use the ABI Prism® 3100 for sample analysis.

03/18/03

- Dr. Eisenberg, Dr. Planz, and Xavier met with Janine at Orchid Cellmark to discuss SNP project and obtain more primer panels. Orchid will call when primers are ready to pick up.

03/19/03

- **120LIZ™/Panel 17 Sample Test Run on ABI Prism® 3100** – Need to determine optimum reaction volume for running samples on ABI Prism® 3100. Added 1µl sample KD-2ng-17A to 6 wells in a Micro-Amp® Optical 96-well plate. Made master mix of formamide/120LIZ™ (Lot #0302016-new) for 4 reactions by adding 34µl formamide (8.5µl/sample) to 4µl 120LIZ™ (0.5µl/sample) to PCR tube. Mix by pipetting. Added 9µl to 3 wells containing samples plus one blank well. Made master mix of formamide/120LIZ™ (Lot #0205009) for 4 reactions by adding 34µl formamide (8.5µl/sample) to 4µl 120LIZ™ (0.5µl/sample) to PCR tube. Mixed and added 9µl to other 3 wells containing sample plus to one blank well. Added 9µl plain formamide to 8

adjacent wells for 16 total wells. Centrifuged plate at 3000rpm for 1 minute. Heat denatured 5 minutes at 95°C and snap cool on ice 5 minutes. Set up ABI Prism® 3100 plate record and run samples. Analysis shows this is a good reaction volume and concentration of reagents so this will be used for remaining sample SNP analysis on the 3100.

- **Panel 17 KD-XA-JW 3100 Run for Comparison to 310** – Need to run the samples previously examined on the ABI Prism 310® on the ABI Prism® 3100 to compare the sensitivity of the instruments. Retrieved SAP products for samples 17-KD-XA-JW from storage and centrifuged 1 minute at 3000rpm. To a Micro-Amp® Optical 96-well plate, add 1µl sample to designated wells (79 total). Made 120LIZ™/formamide (Vortex 120LIZ™) mix for 85 samples by adding 42.5µl 120LIZ™ (Lot #0302016) to 722.5µl formamide in a 1.5ml tube. Vortexed and added 9µl to each well containing sample, plus one additional well to serve as a 120LIZ™ blank. Put septum cover on plate and centrifuged 1 minute at 3000rpm. Heat denatured at 95°C for 5 minutes then snap cooled on ice 5 minutes. Store at room temperature overnight. Replace SAP products at 4°C storage.

03/20/03

- **Panel 17 KD-XA-JW 3100 Run for Comparison to ABI Prism® 310** – Make plate record on ABI Prism® 3100 and replace 1X buffer. Set tray in ABI Prism® 3100 and run. Received new PCR pool for each panel and SNP primer pools for panels 12, 15, and 41 from Orchid-Cellmark. Received 9 out of 10 primers to

produce stock solution for panel 17. Need to find out concentration of each primer from Orchid in order to make solution. Need to do a test run for each panel on ABI Prism® 310 first using each of the 6 samples at 2ng/μl to ensure the new primer pools work properly before using the ABI Prism® 3100.

- **ABI Prism® 310 Test Run with New Primer Panels – PCR Amplification –** Labeled 32 tubes: 8 per panel for all 6 samples and positive and negative controls. Made PCR master mix for each panel for a total of 20 reactions. Vortexed reagents first. In 0.2ml PCR tubes (4), made mix by adding 10μl 10X buffer, 20μl $MgCl_2$, 3μl dNTPs, 0.6μl primer pool, 2μl AmpliTaq® Gold (Lot #CO3741), 44.6μl dH_2O. Vortexed to mix. Add 4μl PCR mix to respective 8 tubes. Stored remaining mix. Added 1μl DNA, 9947A, or dH_2O to corresponding tubes. Pipetted to mix. Placed on thermal cycler SNP-PCR-Amp.
- **ExoI/SAP** – Made ExoI/SAP mix for 35 reactions. Vortexed ExoI/SAP and add 73.5μl to 213.5μl dH_2O in a 1.5ml tube. Vortexed. Added 8.2μl to each PCR product and pipetted to mix. Placed back on thermal cycler for SNP-Exo-Sap program. Stored panel 17 samples until SNP extension primer pool is made.
- **SNP Extension** – Make SNP extension master mix for each panel 12, 15, and 41 for a total of 20 reactions. Vortexed reagents first and added 12μl primer pool, 50μl SNaPshot™ Ready Reaction Mix (Lot #43121820714), 10μl 5X sequencing buffer, and 68μl dH_2O to three separate 0.2ml PCR tube. Made positive and negative controls for SNaPshot™ reaction. Vortexed. Labeled new tubes and add

7µl SNP extension mix to respective tubes for each panel. Added 3µl ExoI/SAP product to respective tubes and pipet to mix. Stored remaining ExoI/SAP product. Placed tubes on thermal cycler for SNP-Ext program.

- **SAP** – Made SAP mix for 30 reactions. Vortexed SAP and added 30µl to 150µl dH_2O to a 1.5ml tube. Vortexed and added 6µl to each SNP extension product. Pipet mixed and placed on thermal cycler SNP-SAP program.
- **ABI Prism® 310 Run** – Labeled new tubes, plus one for a 120LIZ™ blank. Made 120LIZ™/formamide mix for 30 reactions. Vortexed first and added 15µl 120LIZ™ (Lot #0302016) to 420µl dH_2O (14µl/sample). Vortexed. Added 14.5µl to each new tube. Added 0.9µl SAP product to respective tubes and pipet mixed. Stored remaining SAP product. Centrifuged tubes 1 minute at 3000rpm. Heat denatured 3 minutes at 95°C and snap cooled on ice. Placed tray in ABI Prism® 310 and started run after setting up sample sheet, injection list, and executing sequence fill capillary.
- **Analyze Panel 17-KD, XA, JW results from ABI Prism® 3100 comparison run.** Stored ABI Prism® 310 samples at 4°C.

03/21/03

- Obtained concentration of primer #72171 from Orchid-Cellmark, 120uM. I calculated how much of each of the nine primers to add together to obtain 100µl of stock solution with a10uM final concentration of each primer.

PRIMER	214373	214674	63404	126243	228468	66683	72171	207160	234217
CONCENTRATION	100uM	100uM	100uM	120uM	120uM	120uM	120uM	120uM	120uM

- **Panel 12: Sensitivity Assay Samples KD, XA, JW – SNP Extension with new SNP pool** – Made SNP extension mix for 90 reactions in 1.5ml tube. Vortexed reagents first and added 54µl primer pool, 225µl SNaPshot™ Ready Reaction Mix (Lot #0206008), 45µl 5X sequencing buffer and 306µl dH_2O to tube. Vortexed and add 7µl to new labeled tubes. Add 3µl ExoI/SAP product to respective tubes and pipet mix. Made SNaPshot™ controls as before. Placed on thermal cycler SNP-Ext program. Stored remaining ExoI/SAP products.
- **SAP** – Made SAP mix for 90 reactions by first vortexing and adding 90µl SAP to 450µl dH_2O in a 1.5ml tube. Vortex and add 6µl to each SNP extension product. Placed on thermal cycler SNP-Sap program.
- **ABI® Prism 3100 Run** – Made 120LIZ™/formamide mix for 90 reactions by vortexing 120LIZ™ (Lot #0302016) and adding 45µl to 765µl dH_2O in 1.5ml tube. Vortexed. Add 1µl SAP product to designated wells of 96-well plate. Add 9µl 120LIZ™/formamide mix to each well with sample plus one blank well. Covered plate with septum and centrifuged 1 minute at 3000rpm. Heat denatured 5 minutes at 95°C and snap cooled on ice 5 minutes. Set up plate record on ABI Prism® 3100 and loaded tray. Ran samples.
- **Panel 17 – Test Assay New Panels Samples DH, SS, MM, KD, XA, JW** – Made SNP extension mix for 10 reactions, 1 of each sample at 2ng plus controls.

Added 5µl primer pool, 25µl SNaPshot™ Ready Reaction mix (Lot #0206008), 5µl 5X sequencing buffer, and 35µl dH_2O to tube and vortex. Made SNaPshot™ controls. Labeled 8 tubes and add 7µl mix to each. Retrieved ExoI/SAP samples from storage and add 3µl to respective tubes. Stored SAP products and placed tubes on thermal cycler SNP-ext program.

- **SAP** – Made mix for 12 reactions. Vortexed SAP and added 12µl to 60µl dH_2O in tube. Vortexed and added 6µl to extension products. Placed on thermal cycler SNP-Sap program. Also need to run 6 Panel 12 samples at increased sample volume and injection time to determine if this gives better peak results. Did 6 samples and positive 9947A with 1.5µul sample and 6 samples and 9947A with 1.5µl sample and an increased injection time of 7 seconds. Made 120LIZ™ (Lot #0302016) mix for 28 reactions by adding 14µl 120LIZ™ to 392µl formamide in tube. Vortexed. Labeled new tubes and add 14.5µl 120LIZ™/formamide mix to each including one blank. Add 0.9µl or 1.5µl sample to respective tubes and pipet to mix. Stored all remaining products. Centrifuged tubes 1 minute at 3000rpm, heat denatured 3 minutes at 95°C, and snap cooled on ice. Add POP-4™ polymer to syringe, set up sample sheet and injection list, and loaded tray and started run.
- **Panel 41: Sensitivity Assay Samples DH, SS, MM – SNP Extension** – Made SNP extension mix for 90 reactions. Vortexed reagents and add 54µl primer pool, 225µl SNaPshot™ ready reaction mix (Lot #0206008), 45µl 5X sequencing buffer, and 306µl dH_2O to tube and vortex. Made SNaPshot™ controls as before.

Labeled new tubes and added 7µl mix to each. Retrieved Panel 41 samples from storage and added 3µl to respective tubes and pipetted to mix. Placed on thermal cycler SNP-Ext program and stored remaining samples.

- **SAP** - Made SAP mix for 90 reactions. Vortex SAP and add 90µl to 450µl dH_2O in tube and vortexed. Added 6µl to each product. Placed on thermal cycler SNP-Sap program.

03/22/03

- **Panel 41: Sensitivity Assay Samples DH, SS, MM – Prepare Samples for ABI Prism® 3100 CE** - Made 120LIZ™/formamide mix for 90 reactions. Vortexed 120LIZ™ (Lot #0302016) and added 45µl to 765µl formamide in tube. Vortexed. Added 1µl sample to designated wells of a Micro-Amp® Optical 96-well plate. Added 9µl 120LIZ™ mix to each well plus one blank. Covered plate with septum and centrifuged 1 minute at 3000rpm. Heat denatured 5 minutes at 95°C and snap cooled on ice 5 minutes. Set up ABI Prism® 3100 instrument by changing buffer, adding polymer, and changing capillary. Set up plate record and loaded tray to run samples.
- **Panel 17: Sensitivity Assay Samples DH, SS, MM – SNP Extension** – Made SNP extension mix for 90 reactions. Vortexed reagents and added 45µl primer pool, 225µl SNaPshot™ Ready Reaction mix (Lot #0206008), 45µl 5X sequencing buffer, and 315µl dH_2O to tube. Vortexed. Made SNaPshot™ controls as before. Labeled new tubes and added 7µl extension mix to each. Retrieved

ExoI/SAP products and added 3µl to respective tubes. Pipet mixed. Placed on thermal cycler SNP-Ext. Stored remaining ExoI/SAP samples.

- **SAP** – Made mix for 90 reactions. Vortexed SAP and added 90µl to 450µl dH_2O in tube. Vortexed. Added 6µl to extension products. Placed on thermal cycler SNP-Sap program.
- **Analyze Results for Panel 12: Sensitivity Assay Samples KD, XA, JW**

03/23/03

- **Panel 17: Sensitivity Assay Samples DH, SS, MM – Prepare Samples for ABI Prism® 3100 CE** – Made 120LIZ™/formamide mix for 90 reactions. Vortexed 120LIZ™ (Lot #0302016) and added 45µl to 765µl formamide. Vortexed. Added 1µl sample to designated wells of a Micro-Amp® Optical 96-well plate. Added 9µl 120LIZ™ mix to each well with sample plus one additional blank well. Covered plate with septum and centrifuged 1 minute at 3000rpm. Heat denatured 5 minutes at 95°C and snap cooled on ice 5 minutes. Changed buffer in ABI Prism® 3100 and set up plate record. Loaded tray and started run. Stored remaining SAP samples at 4°C.

03/24/03

- **Panel 41: Sensitivity Assay Samples KD, XA, JW – SNP Extension** – Determined not enough of panel 41 primer pool to make enough for 80 samples. I will make enough for 65 reactions and combine with previously made SNP extension mix made by Xavier or myself. Vortexed reagents and added 39µl

primer pool, 162.5µl SNaPshot™ Ready Reaction mix (Lot #0206008), 32.5µl 5X sequencing buffer, and 221µl dH_2O to tube. Vortexed. Added previously made mixes to same tube and vortexed final mix. Labeled new tubes. Added 7µl mix to each. Retrieved ExoI/SAP samples and add 3µl to respective tubes and mix. Made SNaPshot™ controls as before. Placed tubes on thermal cycler SNP-Ext program. Stored remaining products.

- **SAP** – Made mix for 90 reactions. Vortexed SAP and added 90µl to 450µl dH_2O in tube. Vortexed. Added 6µl to each SNP-Ext product and mixed. Placed tubes on thermal cycler SNP-Sap program.
- **Analyze Panel 12-KD, XA, JW data on ABI Prism® 3100.** Stored ABI® Prism 3100 samples at 4°C.
- **Panel 41 – DH, SS, MM – SNP Extension** – Labeled new tubes. Added 7µl SNP extension mix made 03/23/03 to each. Retrieved samples and added 3µl to respective tubes. Made SNaPshot™ controls as before. Placed tubes on thermal cycler SNP-Ext program. Stored remaining ExoI/SAP products.
- **SAP** – Made SAP mix for 90 reactions. Vortexed SAP and added 90µl to 450µl dH_2O in tube. Vortexed and added 6µl to each SNP extension product. Placed on thermal cycler SNP-Sap program.
- **Analyze Panel 12-KD, XA, JW results from ABI Prism® 3100 run.** Store samples at 4°C.

03/25/03

- **Analyze Panel 17-DH, SS, MM and Panel 41-DH, SS, MM results from ABI Prism® 3100 run.** Tried increasing sample volume to 2µl for panel 17 to obtain higher/better peak results. Tested Panel 17 only using samples KD, XA, DH at 2ng and 100pg in duplicate plus 9947A. Results show no great improvement of peak height data at #9 primer, 207160.

03/26/03

- **Panel 41: Sensitivity Assay Samples KD, XA, JW – Prepare for Run on ABI Prism® 3100** – Made 120LIZ™/formamide master mix for 90 reactions. Vortexed 120LIZ™ (Lot #0302016) and added 45µl to 765µl formamide in tube. Vortexed. Retrieved samples and added 1µl to designated wells of 96-well plate. Added 9µl 120LIZ™ mix to each well with sample plus one additional blank well. Centrifuged plate 1 minute at 3000rpm. Heat denatured at 95°C for 5 minutes and snap cooled on ice 5 minutes. Set up ABI Prism® 3100 by changing buffer and setting up plate record. Loaded tray and ran samples. Stored remaining SAP products.
- **Analyze Panel 41 KD, XA, JW data from ABI Prism® 3100 run.** Store samples at 4°C.
- **Reproducibility Assays** – Determined for each panel which DNA quantity will be used for reproducibility assays. Counted the number of NRs (no results obtained) from sensitivity assays for each sample at each dilution in triplicate.

Added the number of NRs for each panel and subtracted from the total possible number of sites and calculated a percentage. Used the percentage values just when the success rate dropped 10% or more to determine the quantity of DNA to use for each reproducibility assay. For Panel 17 – 100pg [average: 87% - 58%], Panel 12 – 500pg [97% - 86%], Panel 15 – 250pg [86% - 65%], Panel 41 – 250pg [81% - 69%].

- **Reproducibility Assays for Panels 12, 15, 17** – Made PCR master mix for each panel for 70 reactions each in separate tubes. Vortexed reagents. Added 35µl 10X PCR buffer, 70µl $MgCl_2$, 10.5µl 25uM dNTPs, 7µl AmpliTaq® Gold (Lot #CO3741), 2.1µl respective primer pool, and 156.1µl dH_2O to tubes. Labeled tubes for each panel, each sample 10 times, plus controls. Added 1µl DNA [17 = 100pg, 12 = 500pg, 15 = 250pg], 9947A, or dH_2O to respective tubes. Added 4µl master mix to respective tubes and mixed. Centrifuged tubes 1 minute at 3000rpm. Placed on thermal cycler program SNP-PCR-Amp.
- **ExoI/SAP** – Made mix for a total of 210 reactions in 2 separate tubes. Vortexed first and added 220.5µl ExoI/SAP to both tubes and 640.5µl dH_2O to both tubes. Vortexed. Added 8.2µl mix to each PCR product and pipet mixed. Placed on thermal cycler program SNP-Exo/Sap.
- **SNP Extension** – Made mix for each panel for 70 reactions each. Vortexed reagents and added 175µl SNaPshot™ Ready Reaction mix (Lot #0206008), 35µl primer pool for panel 17 and 42µl primer pool for panels 12 and 15, 35µl 5X

sequencing buffer, and 245µl dH_2O for panel 17 and 238µl dH_2O for panels 12 and 15 to tubes. Made SNaPshot™ controls as before. To new labeled tubes, added 7µl SNP extension mix for each panel to respective tubes. Added 3µl ExoI/SAP product to tubes and pipet mixed. Placed on thermal cycler SNP-Ext program. Stored remaining ExoI/SAP products.

- **SAP** – Made mix for 210 reactions. Vortexed SAP and added 210µl to 1050µl dH_2O. Vortexed. Added 6µl to each extension product and pipet mixed. Placed on thermal cycler SNP-SAP program.

03/27/03

- **Reproducibility Assay for Panel 41** – As noted on 03/24/03, lack of panel 41 SNP primer pool, therefore will not be able to do 10 repeat reactions per sample. Instead, will try to do 5 repeat reactions per sample. Made PCR master mix for 40 reactions. Vortexed reagents and added 20µl 10X PCR buffer, 40µl $MgCl_2$, 6µl 25uM dNTPs, 4µl AmpliTaq® Gold (new lot #DO9313, exp. 09/30/04), 1.2µl primer pool, and 89.2µl dH_2O to tube. Vortexed. Labeled tubes for each sample, 5 each plus controls. Add 4µl PCR mix to each tube. Add 1µl DNA (250pg), 9947A, or dH_2O to respective tubes. Pipet mixed. Placed on thermal cycler SNP-PCR-Amp program. Aliquot 50µl Panel 41 SNP Extension primer mix made 03/24/03 into tube for Xavier to do casework.

- **ExoI/SAP** – Made mix for 35 reactions. Vortexed ExoI/SAP and added 73.5µl to 213.5µl dH_2O. Vortexed and added 8.2µl to each PCR product. Pipetted to mix. Placed on thermal cycler SNP-Exo-Sap program.
- **SNP Extension** – Labeled new tubes. Added 7µl SNP Extension primer mix into tubes. Only had enough for controls and 3 repeat reactions per sample. Will base SNaPshot™ control results on those from 03/24/03 run. Added 3µl ExoI/SAP product to respective tubes (used repeat PCR reactions 1-3 for each sample). Pipet mixed and placed on thermal cycler program SNP-Ext. Stored remaining ExoI/SAP samples.
- **SAP** – Made SAP mix for 24 samples. Vortexed SAP and added 24µl to 120µl dH_2O in tube. Vortexed. Added 6µl to each extension product and mixed. Placed on thermal cycler program SNP-Sap.

03/28/03

- **Reproducibility Assay for Panel 41 – Prepare Samples for ABI Prism® 3100 CE** – Made 120LIZ™/formamide mix for 24 samples. Vortexed 120LIZ™ (Lot #0302016) and added 12µl to 204µl formamide in tube. Vortexed. Added 1µl SAP product to designated wells of 96-well plate. Added 9µl 120LIZ™ mix to each well with sample plus one blank well. Placed septum cover on tray and centrifuged 1 minute at 3000rpm. Heat denatured 5 minutes at 95°C and snap cooled on ice 5 minutes. Prepare plate record, load tray, and run ABI® Prism 3100. Stored remaining SAP samples.

03/29/03

- **Reproducibility Assays for Panels 12, 15, 17 – Prepare Samples for ABI Prism® 3100 CE** – Retrieved samples from storage and 2 Micro-Amp® Optical 96-well plates. Added 1µl SAP product to designated wells for all panels (plate A – panel 12 and panel 17 KD, XA, JW; plate B – panel 15 and panel 17 DH, SS, MM). Made 120LIZ™/formamide mix for 104 samples for plate B. Vortexed 120LIZ™ and added 52µl to 884µl formamide. Vortexed and added 9µl to each well. For plate A, made 2 sets of 120LIZ™/formamide mix. For panel 12 added 32µl 120LIZ™ (lot #0301013) to 544µl formamide and for panel 17 samples, added 18µl 120LIZ™ (lot#0207011) to 306µl formamide. Vortexed both tubes. Added 9µl mix to respective wells. Centrifuged plates 1 minute at 3000rpm. Heat denatured 5 minutes at 95°C and snap cooled on ice 5 minutes. Change buffer and set up plate records in ABI Prism® 3100. Load trays and run.

03/30/03

- **Analyzed reproducibility Genescan results for all panels**. No results were obtained for Panel 17. I realized I may not have added enough AmpliTaq® Gold DNA Polymerase to the PCR master mix for this panel because the tube was almost empty and I may have gotten more air in the pipet tip than fluid. I will redo the reproducibility assays for Panel 17 using a new tube of AmpliTaq® Gold DNA Polymerase, Lot #DO9313.

- **Reproducibility Panel 17 - PCR Amplification** – I labeled 62 PCR tubes for the 6 samples, 10 reactions each plus 2 control tubes. Made PCR master mix for the samples by first vortexing reagents and adding to a 1.5ml tube: 35µl 10X PCR buffer, 70µl $MgCl_2$, 10.5µl 2.5mM dNTPs, 7µl AmpliTaq® Gold DNA Polymerase (Lot # DO9313, exp. date 09/30/04), 2.1µl Panel 17 primer pool, and 156.1µl dH_2O. Vortexed and added 4µl to all tubes. Added 1µl 100pg DNA to respective sample tubes and 9947A or dH_2O to control tubes. Pipetted to mix. Placed on thermal cycler SNP-PCR-Amp program. Made more SNP Pool for Panel 17 extension mix. Made 50µl by adding 4.15µl each of the 120uM primers (6) and 5µl each of the 100uM primers (3) to 10.1µl dH_2O for a total volume of 50µl. Vortexed all reagents and added to a 1.5ml tube: 175µl SNaPshot™ Ready Reaction Mix (Lot #0206008), 35µl Panel 17 primer pool, 35µl 5X Sequencing buffer, and 245µl dH_2O. Vortex. Made SNaPshot™ controls as before. Store mix and controls until needed for extension step.

03/31/03

- **Reproducibility Panel 17 – ExoI/SAP** – Made ExoI/SAP mix for 70 reactions by vortexing ExoI/SAP and adding 147µl to 427µl dH_2O. Vortexed and added 8.2µl to each PCR product, pipet to mix. Placed on thermal cycler SNP-Exo-Sap program.
- **SNP Extension** – Labeled new tubes. Vortexed extension mix made 03/30/03 and added 7µl to each new tube. Add 3µl ExoI/SAP product to respective tubes and

pipet to mix. Retrieve SNaPshot™ controls and place all tubes on thermal cycler for SNP-Ext program.

- **SAP** – Made SAP mix for 70 reactions. Vortex SAP and add 70µl to 350µl dH_2O. Vortex and add 6µl mix to each SNP extension product. Pipet mix and place on thermal cycler SNP-SAP program.
- **Prepare samples for ABI Prism® 3100 CE** – Added 1µl SAP product to designated wells of a Micro-Amp® Optical 96-well plate. Made 120LIZ™/formamide mix for 68 reactions by first vortexing 120LIZ™ (Lot #0207011) and adding 34µl to 578µl formamide. Vortexed and added 9µl to all wells with sample plus 2 additional blank wells. Added 10µl plain formamide to remaining empty wells to total 80 full wells. Covered plate with septum and centrifuged 1 minute at 3000rpm. Heat denatured 5 minutes at 95°C and snap cooled on ice 5 minutes. Leave plate at room temperature until ready to load on ABI Prism® 3100 on 04/01/03. Stored remaining SAP products at 4°C.

04/01/03

- Load tray from 03/31/03 into ABI Prism® 3100. Set up plate record and ran samples. Analyzed Panel 17 Reproducibility samples. Stored samples at 4°C.

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