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ABI® GeneMapper™ *ID* analysis software replaces and combines both GeneScan® and Genotyper ® data analysis programs. Fragment sizing and allele typing functions are performed in a single analysis, and the software includes data quality assessment features not available with previous software packages. The software was directly compared to the current laboratory STR data analysis software, GeneScan® and Genotyper®. All peaks evaluated with GeneMapper™ *ID* exhibited lower peak heights than their GeneScan®/ Genotyper® analysis counterparts. A mean percent decrease in peak height of 3.8% ± 0.9% was observed for all peaks greater than 500rfu. Observed stutter ratios were comparable to the default stutter filter settings of GeneMapper™ ID. Parallel analyses of 388 sample files resulted in absolute concordance for all reference samples and most evidentiary samples. The software performed better than GeneScan®/Genotyper® in labeling microvariants, baselining data, disregarding –A peaks, aligning below threshold data, and defining size standard peaks despite artifact interference. The Off Scale and Spectral Pull-up PQV did not fuction as expected. Overall, GeneMapper™ ID is an accurate and reliable method for sizing and genotyping STR fragments for forensic human identification analysis.

VALIDATION OF GENEMAPPER™ ID HUMAN IDENTIFICATION SOFTWARE

FOR FORENSIC STR DNA ANALYSIS

Christina L. Capt, B.S.

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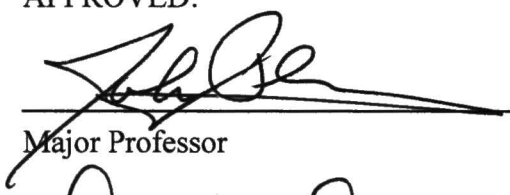

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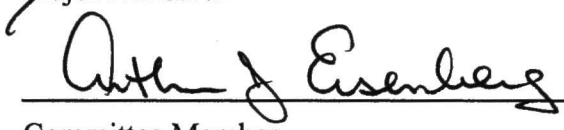

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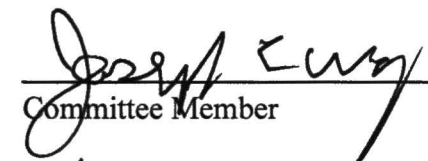

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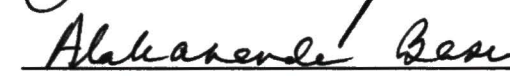

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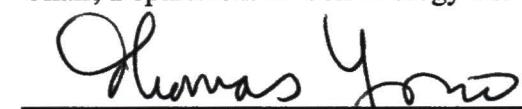

Dean, Graduate School of Biomedical Sciences

VALIDATION OF GENEMAPPER™ ID HUMAN IDENTIFICATION SOFTWARE FOR FORENSIC STR DNA ANALYSIS

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

By

Christina Lee Capt, B.S.

Fort Worth, TX

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CHAPTER 1

INTRODUCTION

Forensic short tandem repeat (STR) DNA analysis typically involves several distinct phases: extraction, quantitation, amplification, electrophoresis, detection, and analysis. DNA is extracted from biological material and in turn the concentration of DNA recovered is determined. Specific regions of the DNA are amplified. The amplified products are then separated and detected prior to analysis.

Capillary electrophoresis instrumentation is currently the platform of choice for STR fragment separation and detection. These instruments capture fluorescent data and convert it into a digital format. Electronic sample files must then be analyzed with specialty software programs.

Applied Biosystems (ABI) GeneScan® and Genotyper® analysis software is widely used to analyze fragment sample files generated by ABI PRISM® Genetic Analyzers. These software packages are hereafter referred to as GeneScan and Genotyper. GeneScan analysis software uses complex algorithms to assign a basepair size, peak height, and peak area to each peak identified within the sample file. The software calculates a sizing curve based on the migration of known fragments within an internal lane standard incorporated into each sample run (1). Genotyper uses the sizing data generated by GeneScan to assign allele designations to sample fragments. This is

accomplished by comparing the sizes of the sample fragments to the sizes obtained for the known alleles within the allelic ladder sample (2,3).

The ABI GeneMapper™ *ID* analysis software, hereafter referred to as GeneMapper or GeneMapper *ID*, replaces and combines both GeneScan and Genotyper data analysis programs. Fragment sizing and allele typing functions are performed in a single analysis. The software was designed specifically for AmpF*l*STR® data analysis and includes data quality assessment features not available with GeneScan and Genotyper analysis (4).

GeneMapper analysis is expected to improve efficiency of data analysis through simultaneous sizing and genotyping functions and by allowing data generated from different AmpF*l*STR® amplifications to be combined and analyzed within the same GeneMapper project. All marker panels and bin sets for all commercially available AmpF*l*STR® kits are available for each GeneMapper analysis project. Genotyper analysis requires separate templates and projects for a given AmpF*l*STR® amplification chemistry (4).

GeneMapper *ID* incorporates a quality value system for assessing data quality. This system was designed to reduce analysis time by allowing analysts to quickly sort problematic data for manual review. The software evaluates sizing quality (SQ) for each sample and a genotype quality (GQ) for each locus within a sample. SQ and GQ are both assigned a numerical value from zero to one. Default categorical range settings for SQ and GQ are: 0 to 0.25 for low quality and 0.75 to 1.00 for pass quality. All SQ and GQ values falling between the low quality and pass quality settings are assessed as check

quality by the software. Process Component-Based Quality Values (PQV) are component quality values used to assess both the size calling and genotyping processes (4).

PQV applicable for the entire sample file include:

Sample File Not Found (SFNF)	flagged when the sample file associated with a particular sample in a GeneMapper project cannot be located
Matrix Not Found (MNF)	flagged when a matrix file is not associated with a particular sample
Size Standard Not Found (SNF)	flagged when a size standard cannot be detected for a particular sample
Off Scale (OS)	flagged when off-scale data is detected within the size standard range of a particular sample
Sizing Quality (SQ)	evaluates overall sizing quality of a particular sample

All sample file PQV other than Sizing Quality are reported with pass or check flags by the software. SQ assessment is dependent upon the "similarity between the size standard fragment pattern and the actual size standard peak distribution pattern in the sample"(4).

PQV applicable for each locus within a sample file include:

Allele Number (AN)	flagged when the number of alleles detected exceeds the maximum number of expected alleles
Out of Bin Allele (BIN)	flagged when an off-ladder allele is detected
Peak Height Ratio (PHR)	flagged when peak height ratios of detected alleles fall below a specified setting
Low Peak Height (LPH)	flagged when a detected peak falls below a specified setting
Spectral Pull-Up (SPU)	flagged when the detected peak is less than a specified percent of a larger overlapping peak (± 1 data point)
Broad Peak (BD)	flagged when a detected peak is wider than a specified setting
Off Scale (OS)	flagged when an off-scale peak is detected within the marker range
Control Concordance (CC)	flagged when a designated positive or negative control sample is not concordant with the expected results
Overlap (OVL)	flagged when a peak in overlapping allelic size regions is labeled twice
Genotype Quality (GQ)	evaluates overall analysis quality for a particular locus

All locus or genotype PQV except GQ are reported with pass or check flags by GeneMapper *ID*. Genotype Quality is a function of the allele quality of each peak detected within a locus and the component based process quality values. Each PQV is assigned a particular weight by the user. If a particular PQV flag is triggered, the associated GQ will be reduced in proportion to flag weight. All PQV with weights of one will automatically reduce the Genotype Quality to zero. Allele number is the only PQV whose weight is preset to one by the software and cannot be adjusted by the user (4).

GeneMapper *ID* is also capable of performing concordance checks between overlapping loci of separate amplifications. This is useful for checking concordance between multiple amplifications of the same sample and for confirming concordance between overlapping loci of different amplification chemistries (4).

According to federal guidelines for forensic DNA analysis, the manufacturer or other responsible party must conduct developmental validation and verification before a novel methodology may be utilized for forensic DNA analysis (5,6). Applied Biosystems performed such a validation and reported their findings in the GeneMapper *ID* user guide. Their study evaluated: peak detection and genotyping functions; analysis algorithms; and workflow and data handling capabilities of GeneMapper *ID* analysis software using simulated forensic data (4).

The company compared peak height assessments between GeneMapper *ID* and GeneScan analysis software. For AmpF*l*STR® Profiler Plus™ and COfiler™ amplifications, a peak height difference range of 0-16 relative fluorescent units (rfu) was

reported. The 16 rfu peak height difference amounted to a 2.6% difference in peak height observed between GeneScan and GeneMapper analyses using advanced peak detection algorithms (4).

Peak detection concordance was examined by comparing 20,364 allele calls made by GeneScan/Genotyper and GeneMapper *ID*. 99.18% of those calls were deemed concordant between analyses. One hundred thirty-nine alleles, or 0.68% of the calls were labeled by GeneScan/Genotyper but were not detected by GeneMapper *ID* because they fell below threshold. Eighteen alleles, or 0.09% of allele calls, involved a single sample with a D18S51 microvariant. The microvariant was correctly labeled as a 12.2 allele by GeneMapper *ID* and as an off-ladder allele by Genotyper. Eight alleles, or 0.04% of the calls, were from mixed DNA samples and involved filtering of the minor contributor alleles in stutter positions by GeneMapper *ID* but not with Genotyper analysis. An additional two alleles, or 0.01% of all allele calls, were labeled by GeneScan/Genotyper but not by GeneMapper *ID*. Genotyper labeled a stutter peak and –A peak associated with a D21S11 allele; GeneMapper *ID* filtered both labels (4).

Algorithm testing encompassed the following parameters: global cut-off values, –A cut-off values, overall sizing quality, overall genotype quality, and process quality value flag verification. The overall sizing quality, global cut-off value and –A cut-off value algorithms performed reliably with all applied settings. The Out of Bin Allele (BIN), Allele Number (AN), Low Peak Height (LPH) and Control Concordance (CC) process quality value flags also functioned accurately for all analyses conducted. However, it was reported that overall Genotype Quality (GQ) and the Off Scale (OS),

Spectral Pull-Up (SPU), and Broad Peak (BD) flags did not function properly when processing off-scale data with advanced mode analysis parameters. Applied Biosystems acknowledges that markers containing pull-up peaks may be assessed incorrectly with GeneMapper *ID*'s PQV system. Instances of such data receiving Off Scale and Broad Peak check flags but passing Spectral Pull-up flags were reported. These loci did not contain off-scale peaks within their allelic marker range. During GeneMapper *ID* software training at the University of North Texas Health Science Center (UNTHSC), the Applied Biosystems training specialist also indicated that the Spectral Pull-Up flag does not function as designed. Instead of flagging loci exhibiting a percent pull-up greater than the user setting, it flags loci with pull-up percentages less than the customized setting. All other portions of the GeneMapper *ID* software verification performed accurately and reliably according to the developmental validation study findings (4).

Data analysis plays a critical part in the overall process of STR DNA analysis. It results in the generation of the final reported profiles for the identification of human biological material. These profiles may be used to link individuals to a crime, establish paternity and other kinships, identify human remains, and for entry into local, state, and national DNA databases. The significance and potential consequence of these analyses does not allow any room for error.

This study was designed to validate GeneMapper *ID* v3.2 for STR data analysis within the University of North Texas Health Science Center, DNA Identity Lab. Internal validation must be conducted prior to implementation of any new methodology or analysis platform within a forensic DNA laboratory. The technique or analysis method

must undergo rigorous testing to assess its accuracy, reliability, and reproducibility within the laboratory setting (5,6).

The internal validation study conducted at UNTHSC included several distinct evaluations. A peak height comparison study compared peak heights calculated by GeneMapper *ID* to parallel assessments made by GeneScan. A reproducibility evaluation tested both the reliability and precision of the software. A stutter study characterized stutter ratios for each locus within the AmpF*l*STR® Profiler Plus™ and COfiler™ amplification kits. These ratios were compared to the default stutter filter settings of GeneMapper *ID*. Evidentiary and reference sample concordance studies assessed the accuracy of GeneMapper *ID* for the analysis of forensic STR data. Profiles generated with GeneMapper *ID* were directly compared to counterpart analyses performed with the current laboratory STR data analysis software, GeneScan v3.7 NT and Genotyper v3.7 NT.

CHAPTER 2

MATERIALS AND METHODS

GeneMapper *ID*™ Analysis

All samples were analyzed with the factory provided size standard, "CE_F_HID_GS500" and respective "Profiler_Plus_v1" and "Cofiler_v1" marker panels using GeneMapper *ID* v3.2. The "HID_Reference Samples" analysis method was created expressly for reference sample STR analysis. This method utilizes an advanced peak detection algorithm, local southern method fragment sizing, light smoothing and a 100 rfu peak amplitude threshold. It applies a twenty percent cut-off value which filters all peaks within a category that are less than twenty percent of the highest peak within that category.

An additional analysis method, "HID_Evidentiary Samples" was created for evidentiary sample STR analysis. Except for filter and peak height ratio settings, this method is identical to the "HID_Reference Samples" analysis method. Instead of a comprehensive cut-off filter, it employs marker specific stutter filters using factory-provided stutter ratio settings. The peak height ratio setting was reduced from 70% for reference samples to 50% for evidentiary samples to accommodate commonly encountered stochastic effects associated with low copy number amplifications.

Except for low peak height (LPH), process quality value weights were set according to manufacturer suggestions for human identity analysis. LPH weight was set at one instead of the recommended 0.3 to ensure that apparent homozygous peaks less than 200 rfu were assessed as low quality. According to current laboratory STR interpretation guidelines, this data is not reportable. [Note: The LPH weight was adjusted to one after analysis of the peak height comparison samples was completed.]

Reference samples and evidentiary samples were analyzed with the "HID_Reference Samples" and "HID_Evidentiary Samples" analysis methods, respectively. Both analysis methods are included in Appendix A.

GeneScan® /Genotyper® Analysis

All samples were sized with GeneScan v3.7 employing the local southern method of sizing, light smoothing, and a 100 rfu threshold. The GeneScan analysis method is included in Appendix A. Genotyper v3.7 GS350 and GS500 macros were used to verify internal lane standard size assignment. Reference samples were genotyped with Genotyper v3.7 KAZAM 20% macro. This macro applies a twenty percent filter after genotypes are determined. All labeled peaks that are less than twenty percent of the highest peak within the category are filtered. Evidentiary samples were genotyped with Genotyper v3.7 KAZAM macro. This macro assigns genotypes and then filters stutter peaks according to marker specific stutter percentages provided by the software.

Peak Height Comparison Study

A stepwise dilution series was prepared with both 9947A and 9948 known control DNA samples (10ng/ul). Each series encompassed 2ng, 1ng, 500pg, 200pg, 150pg,

100pg, 75pg, 50pg, 25pg, 12.5pg and 6.25pg of DNA per 5ul. Dilutions were quantitated with ABI Quantiblot™ using 5ul assays. Standard 25ul, 28 cycle amplifications were performed with 5ul template addition using both AmpF*l*STR® Profiler Plus *ID*™ and COfiler™ human STR multiplex amplification kits (Applied Biosystems, Foster City, CA). Data was collected with an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and analyzed with both GeneMapper *ID* v3.2 and GeneScan v3.7/ Genotyper v3.7. GeneMapper *ID* analysis was conducted with the "HID_Reference Samples" analysis method, and standard reference sample parameters were used for GeneScan/Genotyper analysis.

Tabular peak height data was compiled for all analyses. The difference between the peak height value assigned by GeneMapper *ID* and the corresponding value derived by GeneScan/Genotyper was calculated for each peak. Relative peak height differences were calculated by dividing the peak height difference by the GeneScan/Genotyper peak height.

Reproducibility Evaluation

Twenty ABI Prism® 3100 sample files consisting of Profiler Plus *ID*™ and COfiler™ amplifications of ten different samples were analyzed by GeneMapper *ID* v3.2. The samples included five reference samples and five evidentiary samples. The reference samples consisted of both blood samples and buccal swabs. The evidentiary samples included: vaginal swab epithelial and sperm fractions; two bloodstains; and a tissue sample. The evidentiary profiles analyzed included two low-copy number (LCN) amplifications and two mixed DNA profiles. A low copy number amplification is an

attempt to capture STR data from low-level DNA samples by increasing PCR (polymerase chain reaction) cycle number and maximizing template input. These amplifications do not necessarily result in low signal (rfu) data. The two LCN amplifications included within the reproducibility study did exhibit low signal data.

Reference samples and evidentiary samples were analyzed with the "HID_Reference Samples" and "HID_Evidentiary Samples" analysis methods respectively using GeneMapper *ID* v3.2. Five replicates of this analysis were performed. For four replicates, sample files were organized into separate run folders with their corresponding ladders. For the fifth replicate, all sample files and ladders were compiled into a single folder.

GeneMapper *ID* recognizes folder organization as run divisions. All samples within a single run folder are assumed to be from the same CE run. Multiple ladders within a single folder are averaged by GeneMapper *ID* to calculate allelic bin offsets. Separate folders within the same project are treated as independent analyses by the software (4).

All recognized peaks within a sample file were evaluated for size (bp), peak height (rfu), genotype, and process quality value (PQV). These values were then compared for all replicate analyses of a sample file.

Stutter Study

Two hundred eighteen ABI Prism® 3100 sample files consisting of Profiler Plus *ID*™ and COfiler™ amplifications of 109 different reference samples were analyzed by GeneMapper *ID* v3.2. The reference samples consisted primarily of buccal swabs and a

few blood samples. Standard 25ul, 28 cycle amplifications were employed with 1-2ng of input DNA estimated from ABI Quantiblot™ DNA quantitation data. (Note: Some samples included in this study exhibited off-scale amelogenin data. All other markers exhibited data within scale.)

All samples were analyzed with an analysis method created expressly for validation purposes. It employs a 20 rfu peak amplitude threshold and no allelic filters or cut-off settings. All other settings are exactly as those intended for forensic STR analysis. This alteration allows for a more thorough detection of stutter peaks and a more comprehensive evaluation of stutter ratios.

Stutter ratios were calculated by dividing the peak height (rfu) of each stutter allele by the peak height (rfu) of its respective true allele. This data was collected independently for each of the thirteen markers represented within the Profiler Plus *ID*™ and COfiler™ amplification systems. Samples exhibiting heterozygous alleles one repeat unit apart were excluded on an independent basis for each marker evaluated.

Stutter ratios were plotted according to allelic repeat unit. Mean stutter percentage and standard deviation were calculated for each marker. The maximum and minimum observed stutter percentage values were also noted. These statistics were calculated from total data for a given marker and independently for homozygous and heterozygous data. Stutter percentage ranges for each marker were calculated with both 95% and 99% confidence intervals. The upper bound values of these intervals were compared to the GeneMapper *ID* v3.2 factory-provided marker specific stutter cut-offs.

Reference Sample Concordance Study

One hundred four ABI Prism® 3100 sample files consisting of Profiler Plus *ID*™ and COfiler™ amplifications of 52 different reference samples were analyzed by GeneMapper *ID* v3.2 and GeneScan v3.7/Genotyper v3.7. Samples consisted of buccal swabs and blood samples. Standard 28 cycle, 25ul amplifications were employed with 1-2ng of input DNA estimated from ABI Quantiblot™ DNA quantitation data.

GeneMapper™ *ID* v3.2 analysis was performed with the "HID_Reference Samples" analysis method, and GeneScan® v3.7/ Genotyper® v3.7 analysis used standard parameters for reference sample genotyping. Samples were manually edited for spikes, pull-up, and PCR-related artifacts.

A subset of these reference samples was used to evaluate the concordance check features of GeneMapper *ID*. The sample names of 22 counterpart Profiler Plus *ID*™ and COfiler™ amplifications were modified to comply with GeneMapper *ID* requirements for concordance verification. These files were reanalyzed as reference samples and prior to manual editing, an initial concordance verification of overlapping loci was performed. The data was reviewed and edited for pull-up peaks and amelogenin shoulders. The concordance verification was repeated. The D7S820 loci of all 11 Profiler Plus™ / COfiler™ pairs were then manipulated to purposely introduce artificial non-concordance. This was accomplished through deletion of an allele, renaming of an allele, labeling a –A peak, or labeling a stutter peak. A third concordance verification was then performed.

Within the reference sample subset, the sample types of six randomly chosen samples was changed to either "negative control" or "positive control." The data was reanalyzed and the control concordance (CC) PQV ratings were evaluated.

Evidentiary Sample Concordance Study

Two hundred eighty-four ABI Prism® 3100 sample files consisting of Profiler Plus *ID*™ and COfiler™ counterpart amplifications of different evidentiary samples were analyzed by GeneMapper™ *ID* v3.2 and GeneScan® v3.7/ Genotyper® v3.7. Samples were representative of typical materials submitted to this laboratory. They included bloodstains on various substrates, a beer can swab, a bite mark swab, cigarette butts, fingernail scrapings, a jacket scraping, bones, teeth, decomposed tissue, fetal material, urine, tissue blocks, vaginal swabs, vulvar swabs, perianal swabs, and a penile swab (Table 1). Amplification parameters were sample dictated. The data generated from these samples included 151 low copy number (LCN) amplifications and 59 mixed DNA profiles. [Note: LCN amplifications are indicative of increased cycle PCR and not necessarily low signal (rfu) data.]

The "HID_Evidentiary Samples" analysis method was employed for GeneMapper *ID* v3.2 analysis, and GeneScan v3.7/Genotyper v3.7 analysis was performed as previously described for evidentiary sample genotyping. Samples were manually edited for spikes, pull-up, and PCR-related artifacts only. All labeled stutter peaks remained unedited for software comparison.

Table 1. Types of samples analyzed for the evidentiary sample concordance study. AmpF*l*STR® Profiler Plus *ID*™ and Cofiler™ counterpart amplifications are counted independently.

Types of Samples Analyzed	
Bloodstains	62
Beer Can Swabs	2
Bitemark Swabs	2
Cigarette Butts	4
Fingernail Scrapings	4
Jacket Scrapings	2
Bones	106
Teeth	6
Decomp. Tissue	2
Fetal Material	28
Urine	2
Tissue Blocks	8
Vaginal Swabs	32
Vulvar Swabs	16
Perianal Swabs	4
Penile Swabs	2

CHAPTER 3

RESULTS

GeneMapper *ID* Analysis

Low quality Sizing Quality (SQ) assessments of internal lane standards incorporated with each sample file were observed throughout the validation study. Frequently this resulted from the loss of the 75bp and 400bp fragments within the specified analysis range. The range was repeatedly stretched to accommodate differences in sample migration. A final range of 3,700 to 13,000 data points was used. Additional low quality and check quality SQ results were observed for data containing CE artifacts such as pull-up peaks and spikes within the red, size standard fluor.

Profiler Plus™ and COfiler™ allelic ladders received check quality GQ ratings for some loci. When this occurred all other associated PQV indicators and SQ assessments were of passing quality. All ladders were visually inspected and checked for accurate allelic labeling. No inconsistencies or irregularities were noted. For all validation studies but the evidence concordance study, check quality GQ assessments were consistently indicated for the D3S1358, D21S11 and D18S51 loci of all Profiler Plus™ ladders and for the D3S1358 and D16S539 loci of all COfiler™ ladders. This trend held true for 74 out of 80 ladders used to analyze data in the evidentiary concordance study. Three Profiler Plus™ ladders also flagged D8S1179 in addition to the standard three check

quality loci, and one Profiler Plus™ ladder flagged additional FGA, D8S1179 and D7S820 loci. One Profiler Plus™ ladder received passing flags for all loci. A COfiler ladder received a low quality GQ for D3S1358 but passing flags for all other loci. The D3S1358 low quality assessment apparently resulted from shoulder peaks on all alleles. Otherwise the ladder was labeled correctly and did not appear to affect sample analysis within that run folder.

Peak Height Comparison Study

Step-wise dilutions were prepared using stock solutions of 10ng/ul per the manufacturer's specification. Quantitation data for the prepared dilutions of both 9947A and 9948 control DNAs indicated concentrations were **approximately 1.25X** more concentrated than expected. Concentrations were not adjusted because according to the quantitation data, the dilutions were proportional if not accurate, and imprecise concentration levels would not affect the validity of peak height comparisons between the software packages.

GeneMapper *ID* ranked Sizing Quality as passing for all associated sample files. Allele number flags and resultant low quality Genotype Quality assessments were designated for all loci with no results detected. Check quality GQ ratings were assigned to samples with either Low Peak Height flags or Peak Height Ratio flags. The only manual editing required involved the removal of an amelogenin shoulder peak from a 2ng amplification. GeneScan/Genotyper analysis also required the removal of a single amelogenin shoulder peak, but for a different 2ng amplification.

Allelic shifting of below threshold peaks was observed for the 25pg, 50pg, 75pg, and 100pg amplifications with GeneScan/Genotyper analysis. In these instances, below threshold peaks did not align properly with their corresponding allelic ladder fragments. Parallel GeneMapper *ID* analyses of the same sample files did not exhibit this phenomenon; below threshold peaks were correctly aligned with their corresponding allelic ladder fragments by GeneMapper *ID*.

All peaks evaluated with GeneMapper *ID* v3.2 exhibited lower peak heights (rfu) than their GeneScan/Genotyper v3.7 analysis counterparts. Differences in peak height ranged from 2 rfu at threshold to 296 rfu near detection limit. In general, the absolute difference in peak height increased with signal intensity. Relative peak height difference or the percent decrease in relative fluorescent units, however, was fairly constant when evaluating peaks 500 rfu and greater. A mean percent decrease of 3.8% ± 0.9% was observed for all peaks greater than 500 rfu. The spread was much greater as peaks approached threshold (100 rfu). A mean percent decrease of 4.8% ± 1.9% was observed for all peaks between 100 rfu and 500 rfu (Figure 1).

Both GeneMapper *ID* and GeneScan/Genotyper analyses of the Profiler Plus *ID*™ and COfiler™ amplifications of the 9947A dilution series produced complete profiles for the 200pg and greater amplifications. Similarly, both demonstrated near complete profiles for the 150pg amplifications, lacking only the D7S820 locus in both instances. No reportable results were observed for either software analysis at 50pg and lower.

Differences in software detection capabilities were observed for the 50pg, 75pg, and 100pg 9947A amplifications. Three differences had no net effect on data capture.

These three occurrences were markers, that according to current laboratory STR interpretation guidelines, would be reported as "inconclusive" when analyzed with GeneScan v3.7/Genotyper v3.7 and as "no result" when analyzed with GeneMapper *ID* v3.2. Inconclusive designations are currently reported for loci with apparent homozygous peaks less than 200 rfu and for heterozygous loci possessing a below-threshold allele. Three other observed differences between software analyses exposed the possibility of data loss with GeneMapper *ID* v3.2 analysis. In these three instances, conclusive or reportable data was derived with GeneScan/Genotyper analysis, while GeneMapper *ID* analysis produced inconclusive results.

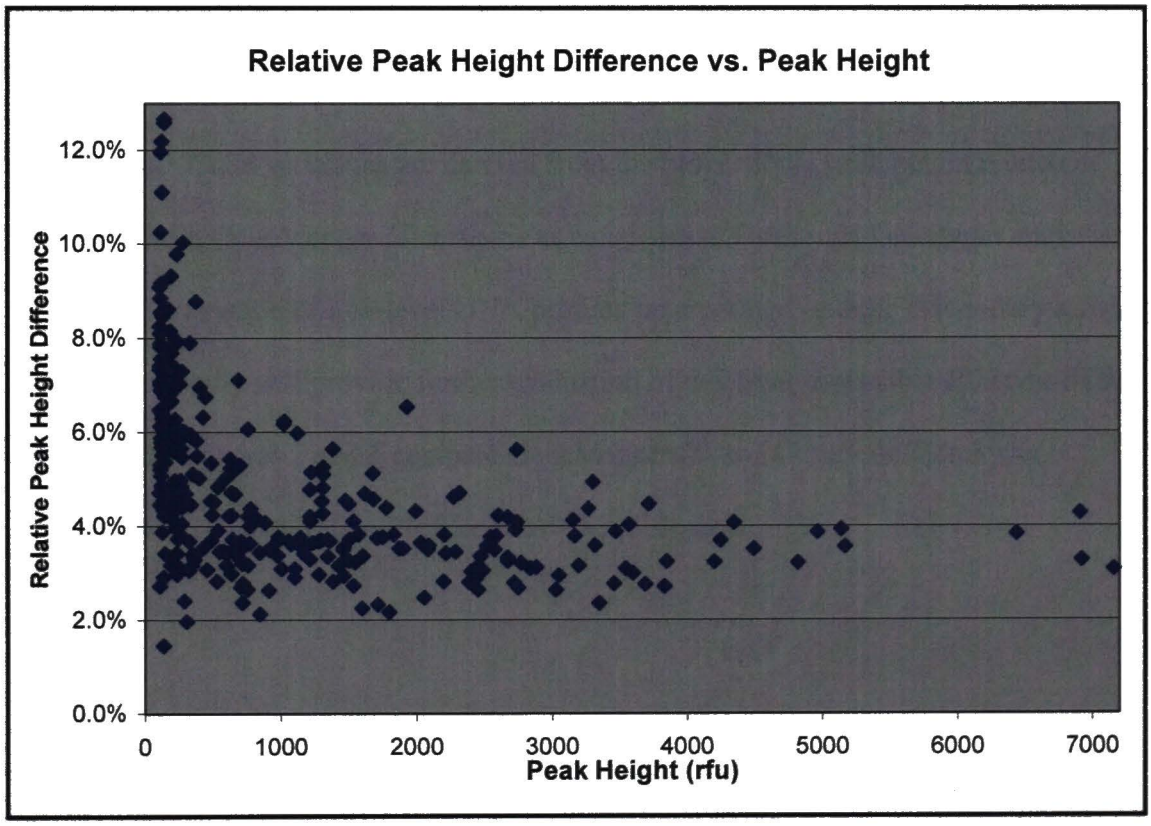


Figure 1. Relative peak height difference versus peak height. For peaks greater than 500 rfu, the relative peak height difference ranges from approximately 2% to 6%. For peaks less than 500 rfu, the range is approximately 2% to 12%.

Evaluation of the 9948 dilution series amplifications produced comparable findings. Both GeneMapper *ID* and GeneScan/Genotyper analyses of the Profiler Plus *ID*™ and COfiler™ amplifications of the 9948 dilution series generated complete profiles for the 150pg and greater amplifications. Several peaks within the 150pg profiles hovered above threshold for both analyses. No reportable results were observed for either software analysis at 50pg and lower.

Differential detection capability was observed for the 50pg, 75pg, and 100pg 9948 amplifications. Six differences did not affect data yield, whereas three other differences represented a reduced capability of GeneMapper *ID* to capture the existing data in these low-level DNA samples.

Table 2 cites all observed genotyping disparities of the software peak height comparison. These variations are derived from the proportional peak height reduction associated with GeneMapper *ID* analysis as compared to GeneScan/Genotyper analysis. Additional evaluation of low-level DNA profiles incorporated into the evidentiary sample concordance study will provide further evaluation of this trend's possible effect on STR data capture. Figures 2 and 3 compare GeneMapper *ID* and GeneScan/Genotyper analyses of the 9947A 100pg Profiler Plus™ amplification.

Table 2. Software Data Detection Discrepancies.

Amplification	Marker	GeneScan® Genotyper® Result	GeneMapper™ *ID* Result
9947A_100pg_Profiler	D3S1358	14 (143rfu), 15 (101 rfu)	Inconclusive: 14 (135rfu), 15 (bt)
	VWA	17 (115rfu), 18 (100 rfu)	Inconclusive: 17 (109rfu), 18 (bt)
	FGA	Inconclusive	No Result
	D21S11	30 (202 rfu)	Inconclusive: 30, (187rfu)
9947A_75pg_Cofiler	D16S539	Inconclusive	No Result
9947A_50pg_Profiler	D8S1179	Inconclusive	No Result
9948_150pg_Profiler	D7S820	11 (210rfu)	Inconclusive: 11 (197rfu)
9948_100pg_Profiler	FGA	Inconclusive	No Result
9948_100pg_Cofiler	TPOX	Inconclusive	No Result
	D7S820	Inconclusive	No Result
9948_75pg_Profiler	D3S1358	15 (123rfu), 17 (113rfu)	Inconclusive: 15 (116rfu), 17 (bt)
9948_75pg_Cofiler	D3S1358	Inconclusive	No Result
	CSF1PO	10 (114rfu), 11 (105rfu)	Inconclusive: 10 (105rfu), 11 (bt)
9948_50pg_Profiler	D3S1358	Inconclusive	No Result
9948_50pg_Cofiler	D5S818	Inconclusive	No Result

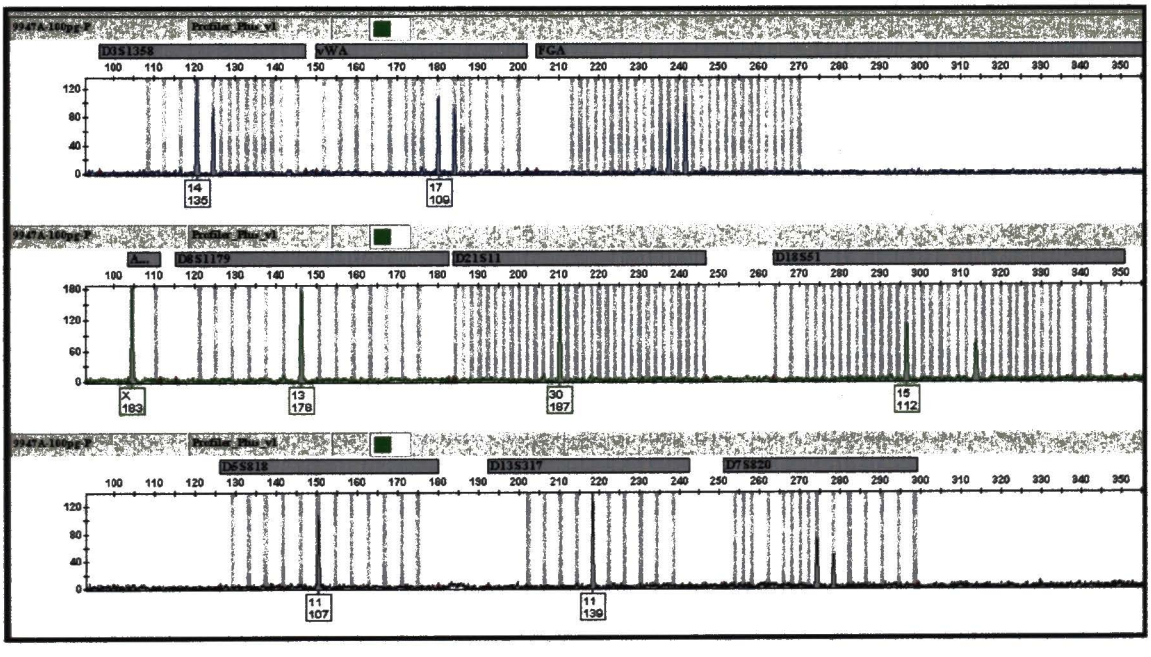


Figure 2. GeneMapper™ *ID* electropherogram of 9947A 100pg amplification. D3S1358 and vWA are inconclusive with GeneMapper™ *ID* analysis, but are reportable with GeneScan®/Genotyper® analysis. No results were obtained for FGA with GeneMapper™ *ID* analysis, whereas inconclusive results were obtained with GeneScan®/Gentoyper®.

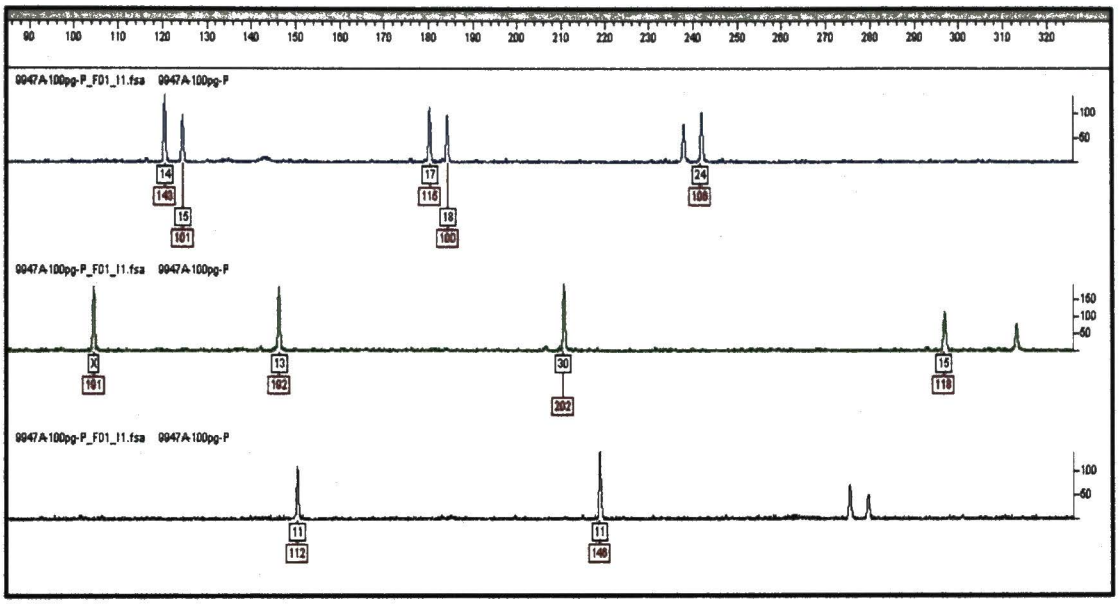


Figure 3. Genotyper® electropherogram of 9947A 100pg amplification. D3S1358 and vWA are conclusive with GeneScan®/Genotyper® analysis, but are inconclusive with GeneMapper™ *ID* analysis. Inconclusive results were obtained for FGA, whereas no results were obtained with GeneMapper™ *ID* analysis.

Reproducibility Evaluation

All replicate analyses of each sample file produced identical sizes, peak heights, and genotypes for each peak identified and assessed by the software. The four replicates containing individual run folders were equivalent to the replicate containing a single compiled run folder.

Slight differences in manual editing requirements were observed for two samples between the four individual-run-folder replicates, and the one compiled-run folder replicate. These two samples both required the removal of an off-ladder amelogenin shoulder peak for all four of the individual-run-folder analyses. These data revisions were not required by the compiled-run folder replicate. The amelogenin shoulder peaks affected BIN and PHR process quality values resulting in an overall low quality GQ. This effect was not observed in the compiled-run folder replicate. No other apparent

differences in Process Quality Values or software performance were noted between replicate analyses. Mixed DNA and low-copy number samples performed as reliably as single source reference samples.

Stutter Study

Observed stutter ratios differed between markers. Mean stutter percentages ranged from 2.2% for THO1 to 7.7% for D18S51. Stutter ratios also differed between alleles within a marker. In general, larger molecular-weight alleles exhibited higher percentages of stutter than smaller molecular-weight alleles of the same marker. Microvariants also showed markedly lower stutter percentages than their bordering whole-repeat constituents. Ratios did not differ significantly between heterozygous and homozygous data for a given marker (Figure 4). All stutter plots for the AmpF*l*STR® Profiler Plus *ID*™ and COfiler™ loci are available in Appendix B.

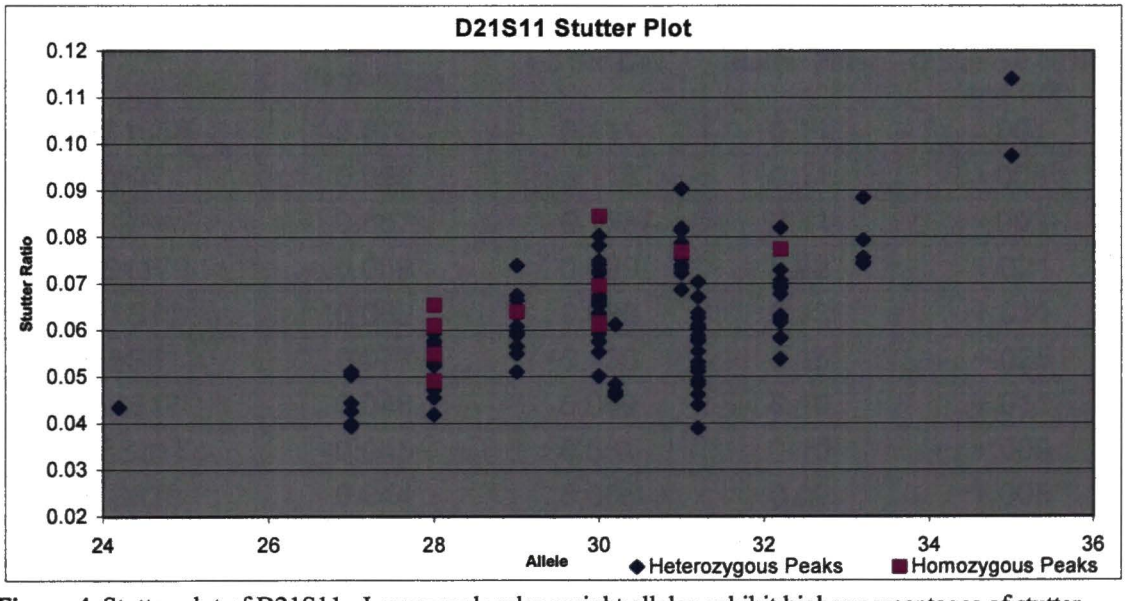


Figure 4. Stutter plot of D21S11. Larger molecular-weight alleles exhibit higher percentages of stutter than lower molecular-weight alleles. Microvariants show markedly lower stutter percentages than their bordering whole-repeat constituents. Ratios do not differ significantly between heterozygous and homozygous data.

One outlier was observed for the CSF1PO data set. When removed, standard deviation for mean stutter percentage of CSF1PO decreased from 1.8% to 1.3%. This sample will be reamplified and its stutter ratios recalculated. The observed elevated stutter percentage may be due to some immeasurable intrinsic aspect of this sample or an atypical amplification event.

Table 3 compares intra-laboratory stutter ratio data with the default stutter filters incorporated into GeneMapper *ID* v3.2 software. The upper bound 99% CI is consistent with the default filter cut-offs within GeneMapper *ID* v3.2 for most markers. The upper bound 99% CI for D8S1179, D21S11, D18S51, and D16S539 are notably lower than the GeneMapper *ID* default values. The default values for these markers are at least 0.02 percent higher than their respective experimental values.

Table 3 Comparison of observed and factory-provided AmpF*l*STR® marker stutter ratios.

Marker	Observed Mean Stutter Percentage	Observed Mean + 3 Std Dev.	GM *ID* v3.2 Stutter Filter	Difference Between Observed 99%CI and GM
D3S1358	0.070	0.111	0.11	-.001
VWA	0.066	0.118	0.11	-.008
FGA	0.067	0.113	0.11	-.003
D8S1179	0.059	0.099	0.12	+.021
D21S11	0.063	0.099	0.13	+.031
D18S51	0.077	0.135	0.16	+.025
D5S818	0.048	0.089	0.10	+.011
D13S317	0.045	0.091	0.10	+.009
D7S820	0.044	0.085	0.09	+.005
D16S539	0.051	0.098	0.13	+.032
TH01	0.022	0.049	0.06	+.011
TPOX	0.024	0.050	0.06	+.01
CSF1PO	0.052	0.091	0.09	-.001

Reference Sample Concordance Study

Twenty-eight out of 104 sample files were reported to contain off-scale data by GeneMapper *ID*. Two sample files were given a low quality score for Sizing Quality. These files were Profiler Plus *ID*™ and COfiler™ counterpart amplifications of the same extract. Upon review, this sample was extremely overblown and contained ROX pull-up peaks in excess of 4000 rfu. The software correctly labeled all pertinent ROX peaks despite the low quality assessment. All other sample files received a passing SQ.

The GS500 and GS350 Genotyper macros also had difficulty with the same overblown sample. The macros assigned size labels to the ROX pull-up peaks of these sample files while ignoring many of the true size standard peaks. Six other samples for which GeneMapper *ID* accorded a passing SQ had ROX pull-up peaks labeled by Genotyper.

Excluding allelic ladder assessments, GeneMapper *ID* accorded a passing GQ to 787 loci, a check GQ to 3 loci, and a low quality GQ to 94 loci. All three of the check GQ loci were due to PHR flags. Review of the data confirmed that these markers exhibited peak height ratios less than the seventy percent PHR trigger setting (Figure 5). Sixty-five low quality GQ markers were due to off-scale data and associated artifacts. OS flags carry a weight of 0.8 for overall GQ assessment. If triggered, GQ is automatically reduced to low quality. Additional flags observed for off-scale data included BIN, PHR, and AN. These flags were attributed to extraneous –A and pull-up peak labels.

Of the remaining twenty-nine low quality GQ loci, 25 were due to ameloginin shoulder peak labels, three were due to –A off-ladder allele calls, and one was due to a true allele labeled as off-ladder by GeneMapper *ID*. These loci exhibited various combinations of the BIN, PHR, and AN flags depending on their respective allelic constitutions. No flags were observed for the LPH, SPU, BD, or OVL process component-based quality values.

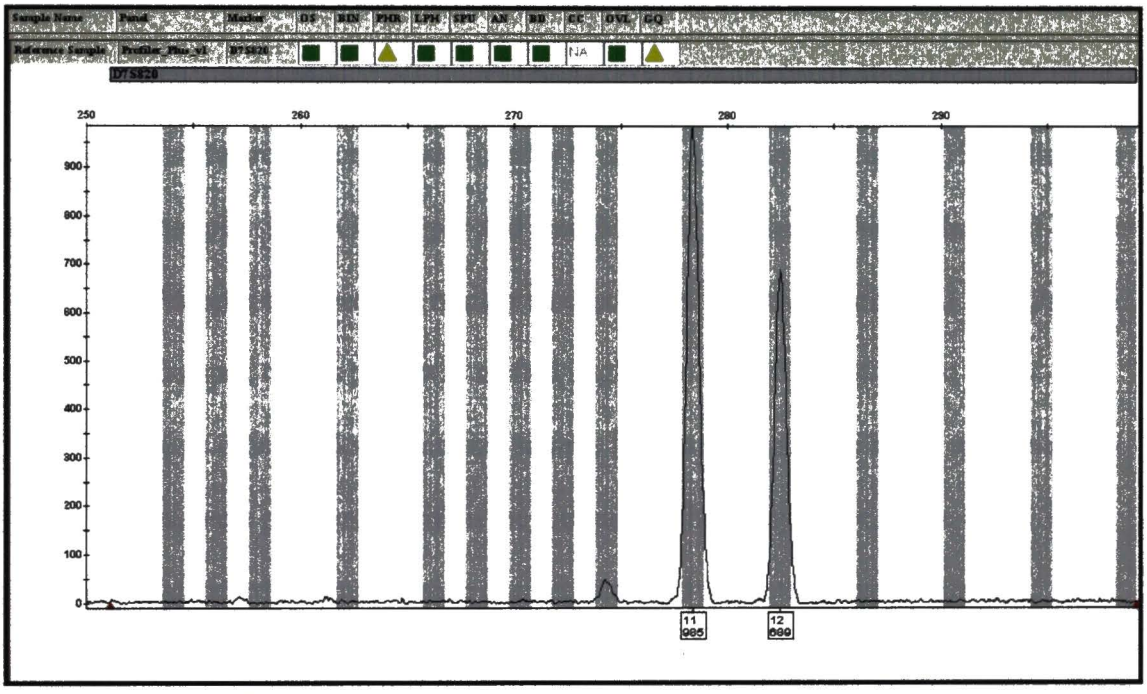


Figure 5. Reference sample with PHR flag and check quality GQ for D7S820. The peak height ratio for this locus is 0.699 which is slightly less than the PHR flag setting of 0.7.

After manual data review and editing, the number of markers exhibiting low quality GQ was reduced to 36. OS flags were observed for all 36. These markers were reviewed again and all GQ were subsequently overridden. All were either markers with off-scale data or overlapping off-scale data that had not required previous editing.

The Off Scale PQV did not function as expected. According to the user manual, OS flags are triggered for all loci containing off-scale peaks (4). All loci with observed off-scale peaks in this study received Off Scale PQV flags by the software. However, OS flags were also triggered for many loci that did not contain off-scale data. These loci exhibited above threshold pull-up peaks resulting from off-scale data of an overlapping locus. Markers with overlapping off-scale data that did not produce corresponding pull-up peaks above 100 rfu did not receive OS flags. All samples received passing Spectral Pull-Up (SPU) assessments.

Both software packages were unable to assign a genotype to a D3S1358 allele for a particular sample. Both Genotyper and GeneMapper *ID* labeled this allele as off-ladder (OL). GeneMapper assessed this marker as check BIN and low quality GQ. On closer inspection, the apex of this peak was shifted slightly to the left of the allelic bin boundary. Most other peaks within this sample file, particularly the larger molecular weight alleles, were positioned within the left half of their respective allelic bins. The internal size standard fragments were correctly labeled for both software analyses. This marker required manual genotyping with both analyses.

Two counterpart amplifications of the same sample contained D3S1358 microvariants. GeneMapper *ID* correctly auto-typed the 16.2 allele in both instances. This allele required manual typing with Genotyper analysis.

Manual editing requirements significantly differed between the two software analysis packages. On the whole, GeneMapper *ID* required a greater amount of manual editing than counterpart GeneScan/Genotyper analyses of the reference sample files.

Additional edits required by GeneMapper *ID* but not Genotyper included: 36 amelogenin off-ladder shoulder calls, 3 amelogenin split peak calls, 2 D8S1179 split peak calls, and a pull-up peak off-ladder call. The two D8S1179 split peak calls belonged to the same off-scale amplification. GeneMapper *ID* labeled the splits individually, whereas Genotyper treated the alleles as single peaks. The off-ladder pull-up peak labeled by GeneMapper *ID* was outside of the D3S1358 allelic marker range in Genotyper and not labeled accordingly. Absolute concordance was observed between all manually edited reference profiles generated with GeneMapper *ID* v3.2 and those generated by GeneScan/ Genotyper v3.7.

Total analysis time for the 104 reference profiles amounted to 46 minutes for GeneMapper *ID* and 67 minutes for Genotyper. These times include full data review and all manual editing required for both analyses. It also includes time spent documenting all data editing, which was noted for validation purposes only and would not be required for casework analyses.

The initial concordance check for all unedited sample files within the data subset did not function as expected. GeneMapper *ID* correctly identified six non-concordant D3S1358 pairs resulting from off-ladder pull-up peak labels and three non-concordant amelogenin pairs resulting from amelogenin off-ladder shoulder labels. However, two non-concordant amelogenin pairs and one non-concordant D3S1358 pair were not recognized by the software. An explanation for this discrepancy could not be determined. The post manual editing concordance check did not identify any non-concordant loci.

All 11artificially generated, non-concordant D7S820 pairs were detected by GeneMapper *ID*. Control Concordance (CC) flags and resultant low quality GQ assessments were correctly accorded to all falsely designated "positive control" and "negative control" sample files.

Evidentiary Concordance Study

GeneMapper *ID* assessed SQ as pass for 320 sample files, check for 7 sample files, and low quality for 37 sample files. The 7 SQ check sample files and 18 of the 37 SQ low quality sample files contained amelogenin pull-up peaks in ROX. Three of these sample files also contained pull-up from additional loci. GeneMapper *ID* correctly labeled all requisite internal lane standard fragments despite pull-up peak interference for all but one sample file. GS350 and GS500 Genotyper macros often labeled the pull-up peaks of these samples and depending on the severity of pull-up would ignore all or some of the true size standard peaks. One sample file received a low quality SQ due to the presence of a 3500 rfu spike within the analysis range. Both GeneMapper *ID* and Genotyper correctly labeled all size standard fragments for this sample.

Sixteen sample files from three separate 3100 CE runs were assigned low quality SQ values due to missing 400bp peaks in the analysis range. Upon review of the raw data, the 400bp ROX peak was not collected for 11 of the files and was greater than the upper limit of the analysis range (13,000 data points) for the remaining five files. SQ was overridden for all 17 sample files because all were low-copy number samples and did not possess any fragments greater than 340bp. Both GeneMapper *ID* and Genotyper correctly assigned labels to the detected 75bp to 350bp ROX peaks of these samples.

The internal lane standards for three sample files were incorrectly labeled by GeneMapper *ID*. All three received low quality SQ assessments of 0.0 by the software. Two of these sample files exhibited drop-out of internal lane standard peaks. One sample lost the 340bp ROX peak; the other lost both the 300bp and 340bp ROX peaks. For the sample file missing a 340bp fragment, GeneMapper *ID* correctly labeled the 75bp to 300bp fragments, but the 340bp to 400bp labels were incorrectly assigned. GeneMapper *ID* did not assign any sizes for any detected ROX fragments, for the sample file missing both the 300bp and 340bp fragments. Size standard fragment labels were manually edited and the SQ values were overridden for both samples to assess GeneMapper *ID*'s ability to correctly genotype samples with sub-par size standards. The Genotyper size standard macros correctly labeled all detected ROX fragments for both samples. One sample was completely mislabeled by GeneMapper *ID* despite detection of all size standard fragments. This sample possessed an amelogenin pull-up peak in excess of 4000 rfu (Figure 6).

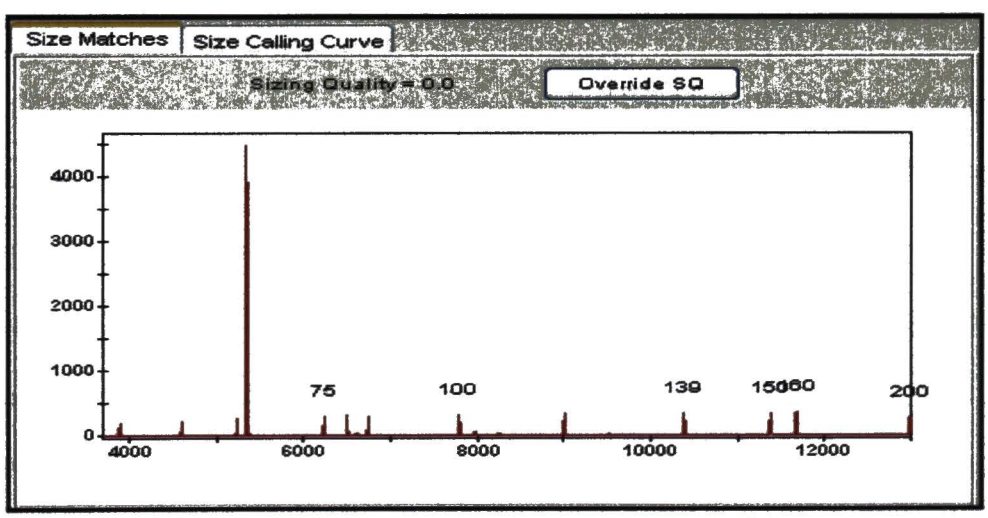


Figure 6. Sample file with internal size standard incorrectly labeled by GeneMapper™ *ID*. An amelogenin pull-up peak in excess of 4000 rfu may have affected sizing. The ROX fragments were manually labeled, and sizing quality was overridden.

The ROX fragments were manually labeled and the SQ value was overridden for this sample. Only the ROX pull-up peak of this sample was labeled by the Genotyper GS500 macro.

Prior to data review and editing, GeneMapper *ID* accorded a passing GQ to 1,252 loci, a check GQ to 110 loci, and a low quality GQ to 1,052 loci. These numbers do not reflect ladder quality assessments. The majority of data receiving passing GQ was reportable. Although, it should be noted that some of these loci were actually associated with mixed DNA samples. These loci received passing GQ because all additional alleles (>2) were below threshold and not counted by the software. Two loci with passing GQ required additional editing. Both were amelogenin loci exhibiting split X peaks with two X labels. Thirteen passing GQ loci would be reported as inconclusive according to current STR interpretation guidelines. Three of these loci were associated with LCN amelogenin data exhibiting allelic drop-out of the X allele and detection of only a single Y peak. Ten were LCN heterozygous loci exhibiting one allele greater than 200 rfu and one allele below threshold.

The 110 loci with check quality GQ were associated with PHR flags. Review of the data confirmed that these loci exhibited peak height ratios less than the 50% PHR trigger setting. These loci largely belonged to mixed DNA samples and low-copy number amplifications. The 1,052 low quality GQ assessments were due to: off-scale data and associated artifacts (assessed with various combinations of OS, BIN, PHR, AN, and SPU flags); mixtures (assessed with PHR and/or AN flags); no result loci (assessed with AN flags); low peak height data (assessed with LPH flags); and loci requiring off-

ladder label editing (assessed with various combinations of BIN, PHR, AN, and SPU flags). Off-ladder labels were associated with amelogenin shoulder peaks, –A peaks, and pull-up peaks. No BD or OVL flags were triggered for the evidentiary sample data.

Eighty of the 284 evidentiary sample files were reported to contain off-scale data by GeneMapper *ID*. One hundred sixty-two low quality GQ loci were due to off-scale data and associated artifacts. BIN, PHR, and AN flags were often observed in association with loci receiving OS flags. These flags were attributed to the extraneous –A and pull-up peak labels associated with off-scale data.

OS genotype flags were triggered for markers containing off-scale data and for some markers with allelic ranges that overlapped off-scale peaks. Not all markers with overlapping off-scale data received OS flags. This difference seems to correlate with the presence or absence of detected pull-up peaks (>100 rfu). Nine of the 80 sample files containing off-scale data according to GeneMapper *ID* analysis did not receive additional OS genotype flags in this manner. Six were triplicate counterpart Profiler Plus *ID*™ and COfiler™ amplifications of the same bone sample. Two were counterpart amplifications of the same epithelial fraction and one was a COfiler™ LCN bone extract amplification. These nine sample files contained a total of 27 loci with off-scale peaks or overlapping off-scale peaks resulting in detectable pull-up peaks that were not given OS genotype flags by the software. Although lacking OS flags, all 27 loci were still graded low quality GQ by the software (Figure 7).

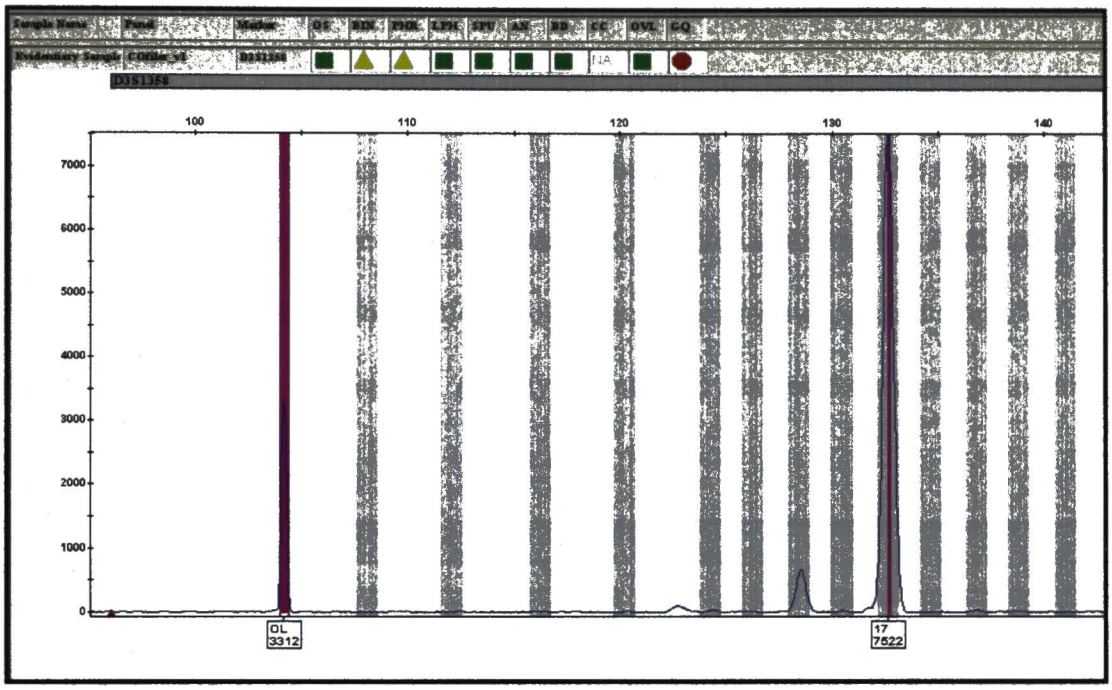


Figure 7. D3S1358 locus with off-scale data that was not assigned an Off Scale (OS) flag by GeneMapper™ *ID*. The BIN and PHR flags are attributed to the off-ladder amelogenin pull-up peak at 104bp, and the peak height difference between the pull-up peak and the true allele.

Nine loci received Spectral Pull-up (SPU) flags. BIN, PHR, AN, and OS flags of various combinations were also triggered for these samples. All nine exhibited a labeled pull-up peak with a signal less than five percent of that attributed to the overlapping peak.

Any allele edits made by the user are logged under allele edit history and result in an automatic override of the associated GQ. Excluding allelic ladder evaluations, post-editing GQ marker data included 1,468 pass, 107 check, and 727 low-quality assignments.

Eleven post-editing low-quality GQ values were due to BIN and PHR flags. All 11 possessed off-ladder allele calls within their respective allelic marker range. When off-ladder calls resulted in more than two labeled peaks per category, AN flags were also triggered. Off-ladder calls included: one spurious off-ladder call associated with an

increased cycle number amplification and near off-scale data; three authentic alleles labeled as off-ladder because their –A peaks were higher than their respective +A peaks; one D21S11 microvariant (Figure 8); three reproducible 98.5bp off-ladder calls present in triplicate amplifications of the same bone sample; and three 138.59bp to 138.82bp off-ladder D5S818 calls present in identical profiles generated by independent amplifications of three different items of evidence. Genotyper data also possessed the same post-editing off-ladder allele calls.

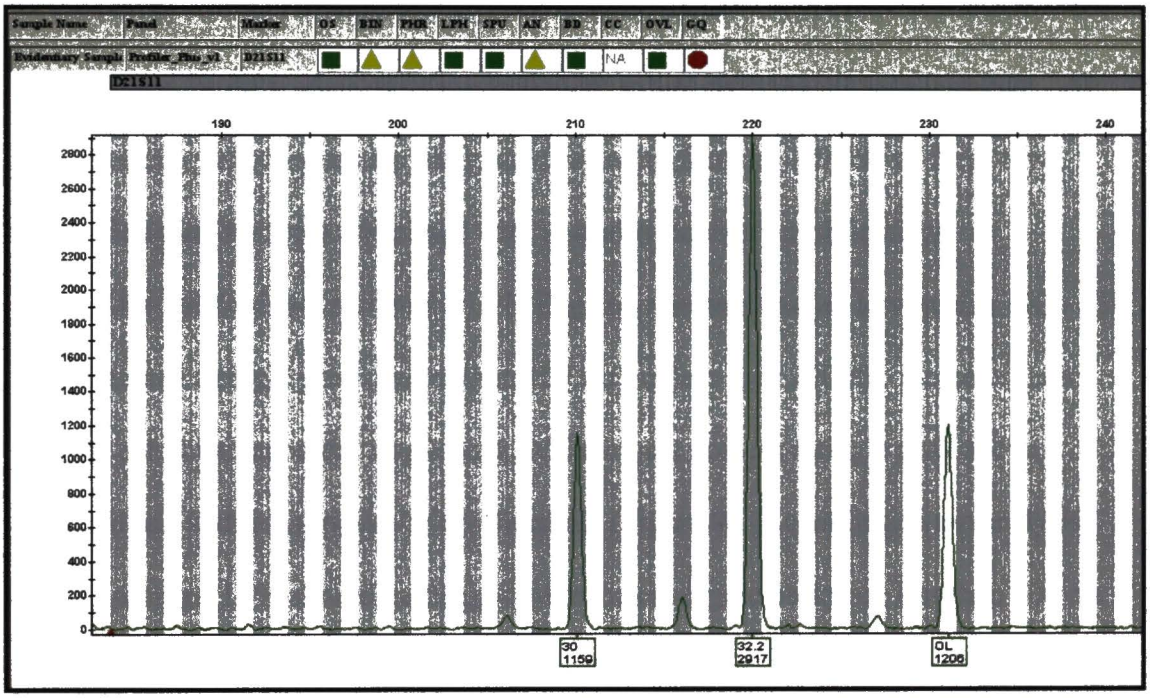


Figure 8. D21S11 locus of mixed DNA sample exhibiting microvariant allele. GeneMapper™ *ID* labeled as off-ladder allele. BIN, PHR, and AN flags were triggered.

The off-ladder peaks due to disproportionate –A were all associated with separate amplifications and extractions of the same tissue block sample. This sample was severely inhibited and required dilution and increased cycle amplification to generate only a partial profile. All peaks associated with this sample were very broad and exhibited the

same –A greater than +A phenomenon. It appears that the software was able to correctly type these –A/+A peaks, if it was able to distinguish the peaks and assess them independently. When the –A/+A peaks were smoothed into a single peak, they were labeled as off-ladder. Genotyper appeared to treat these peaks in a similar manner.

Twenty-five low-quality GQ values can be attributed to OS flags triggered by off-scale data within the marker range. Three hundred ninety-one low-quality GQ assessments resulted from markers for which no results were obtained. In these instances, the AN flag is triggered and GQ is automatically reduced to low-quality. OS and OVL are the only other process-component values reported in these instances. All other process quality values are designated as not applicable. All 391 of these low-quality designations were associated with low copy number amplifications.

One hundred loci received a LPH flag and resultant overall low-quality GQ. All 100 contained a single peak greater than 100 rfu but less than 200 rfu. Some of these samples exhibit an apparent single peak, while others possess a single peak above threshold and additional peaks below threshold (Figure 9). According to the analysis method settings, LPH should be flagged when heterozygote peaks are less than 100 rfu and apparent homozygote peaks are less than 200 rfu. Because the peak amplitude threshold setting is also set at 100 rfu, this flag should not trigger for heterozygote peaks less than 100 rfu. All but one of these flags were associated with low-copy number amplifications. One standard amplification received a flag for CSF1PO which is a higher molecular weight allele and more susceptible to allelic drop-out. All but two of these loci would be non-reportable according to current laboratory STR interpretation guidelines.

The remaining two are associated with low-copy number mixtures, and as such all peaks breaking the 100 rfu threshold are reportable.

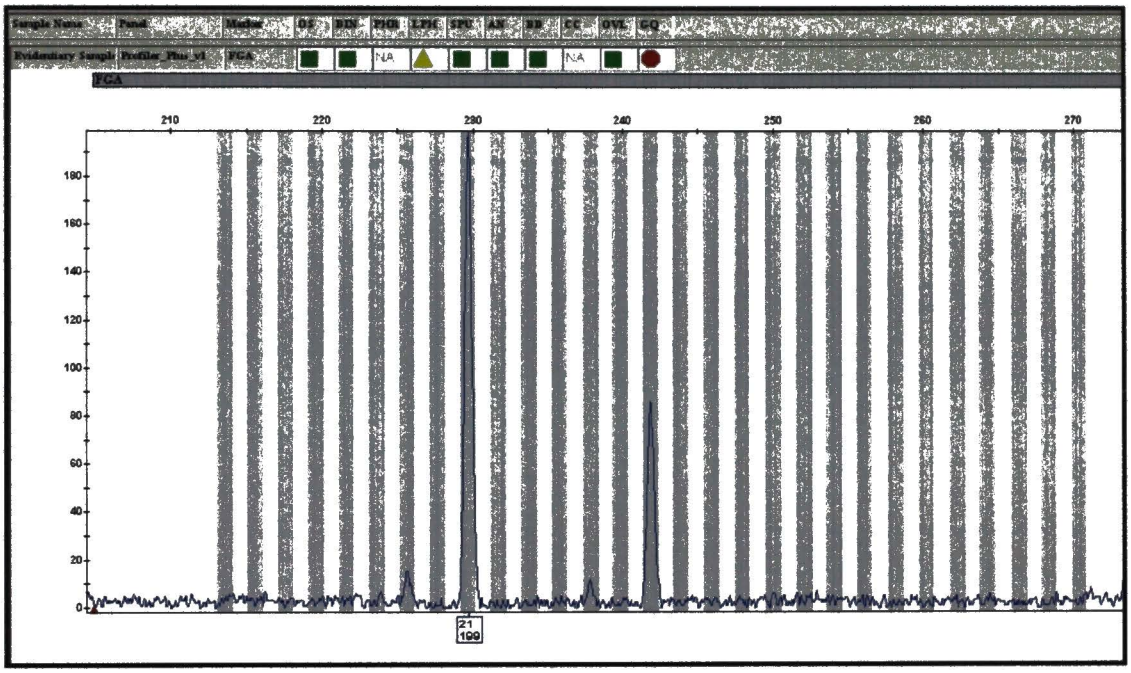


Figure 9. FGA locus exhibiting allelic drop-out of larger allele. Locus received LPH flag and low quality GQ by GeneMapper™ *ID*.

Data from 185 markers associated with mixed DNA profiles also maintained their low-quality GQ values post manual editing. One hundred fifty-five loci presented with PHR and AN flags, and 30 loci presented with AN flags only (Figure 10).

Fifteen loci associated with low copy number amplifications also displayed PHR and AN flags. Seven of these flagged loci resulted from stutter peaks not filtered by the software and eight were associated with primarily single-source amplifications in which one to two markers exhibited a low-level peak in addition to the major contributor peaks. These sample files may have been derived from true mixed DNA samples; or they may

be associated with artifacts and spot-contamination resulting from increased cycle number amplifications.

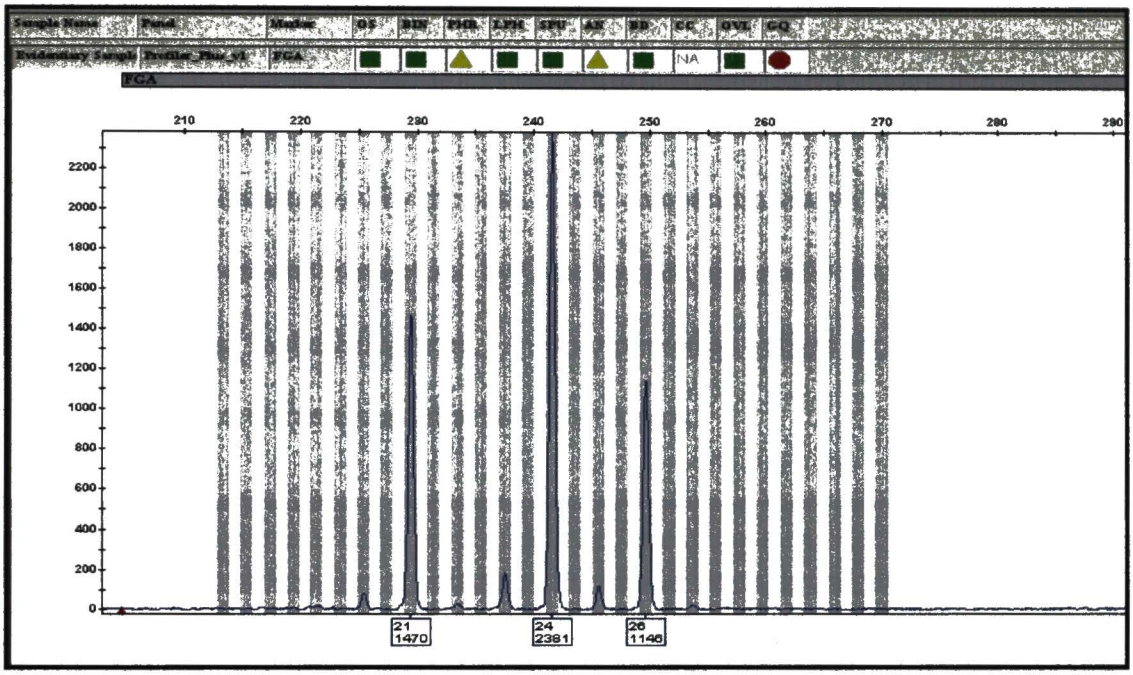


Figure 10. FGA locus of mixed DNA sample analyzed with GeneMapper™ *ID*. PHR and AN flags were triggered.

Manual editing requirements differed significantly between the GeneMapper *ID* and GeneScan/Genotyper analyses. In direct contrast to the reference sample concordance study findings, GeneScan/Genotyper analyses required more overall editing than counterpart GeneMapper *ID* analyses. Data revisions performed during Genotyper analysis but not GeneMapper analysis of the evidentiary sample set included: 149 markers requiring –A edits; 23 markers requiring pull-up peak deletions; 3 amelogenin split-peak corrections; 26 amelogenin shoulder removal edits; and one spike requiring two peak deletions in Genotyper and no deletions in GeneMapper. The differential –A

edits were largely due to differential detection of –A between the two analysis programs. The majority of the –A detected and labeled in Genotyper was present at below threshold levels in GeneMapper. Some –A peaks were barely below threshold, but the majority of –A peaks exhibiting differential recognition were in the 40 rfu to 80 rfu range. This represents a peak height reduction of 20% to 60%, which far exceeds the previously noted reduction characterized by the peak height comparison study (Figures 11 and 12). Some of the –A peaks not recognized by GeneMapper, apparently resulted from peak smoothing effects. In these instances, –A was observed as a slight shoulder on a single +A peak, and not a discrete peak attached to a +A peak.

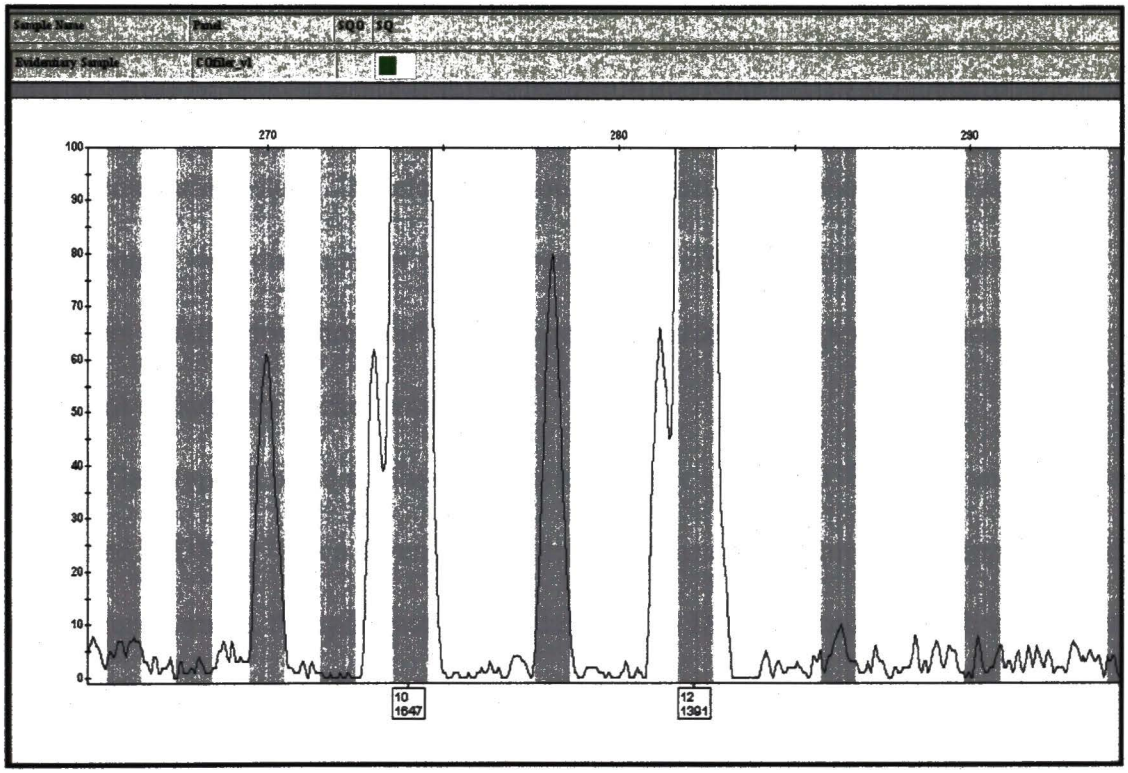


Figure 11. GeneMapper *ID* analysis of D7S820 locus. –A peaks are approximately 62 rfu and 67 rfu. With GeneScan/Genotyper analysis these peaks are 113 rfu and 103 rfu.

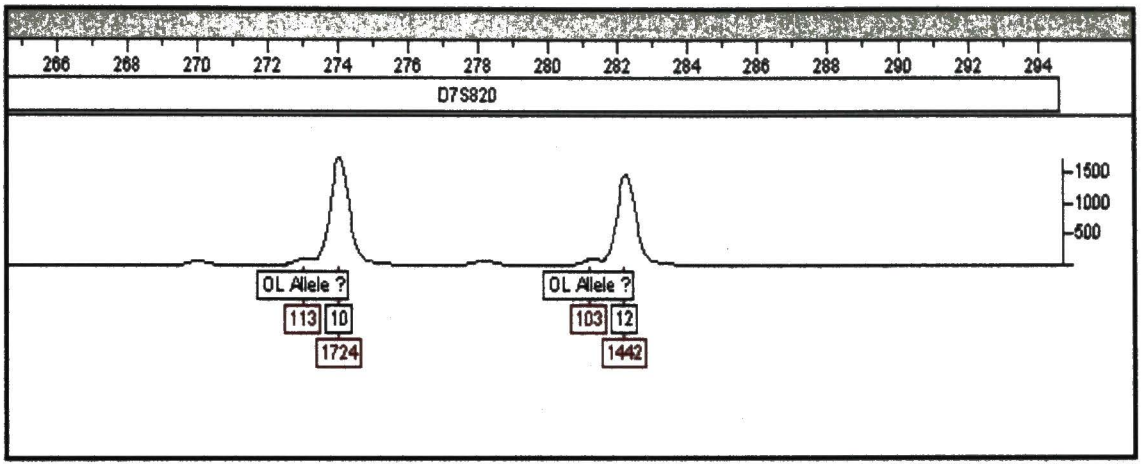


Figure 12. GeneScan/Genotyper analysis of D7S820 locus. –A peaks were labeled by the software and required manual editing. GeneMapper analysis of this sample file exhibited –A peaks below threshold.

Differential pull-up peak recognition can also be largely attributed to differences in peak height detection between the two software programs. These peaks exhibited relative peak height reductions similar to the proportions discussed previously in the peak height comparison study. Amelogenin shoulders detected by Genotyper but not by GeneMapper were due to either reduced peak height effects causing amelogenin shoulders to fall below threshold or amelogenin shoulder peak apexes positioned either on or just outside the marker boundary.

Genotyper labeled an observed electrophoretic spike in D3S1358 as an off-ladder allele and in D8S1179 as a seven repeat allele. For the counterpart GeneMapper analysis of the same sample, the spike was below threshold in D8S1179 and above threshold but not labeled in D3S1358. Several samples exhibited areas of elevated baseline after analysis with GeneScan/Genotyper and necessitated the removal of multiple off-ladder labels. Corresponding areas of baseline were much smoother with GeneMapper *ID* analysis. Below threshold data shifting was observed with Genotyper analysis. Some

spike and pull-up peaks also did not properely align with Genotyper analysis, especially if these peaks were below threshold. This did not occur for counterpart GeneMapper *ID* analyses.

All allele calls made by GeneMapper *ID* v3.2 were concordant with corresponding allele calls made by GeneScan/Genotyper v3.7. However, the profiles generated did demonstrate some degree of non-concordance. Out of the 284 sample files analyzed, 154 exhibited absolute concordance, 101 demonstrated non-concordance at stutter positions, 35 produced examples of non-concordance due to decreased peak height, and one exhibited non-concordance at amelogenenin.

All stutter position non-concordance can be attributed to stutter peaks labeled by Genotyper but not GeneMapper *ID*. Software variation in stutter filter settings and the observed peak height reduction of GeneMapper *ID* analysis were both considered as possible explanations for the non-concordance. Table 4 offers a direct comparison between default filter settings utilized by the two software programs. Stutter filter settings for all thirteen markers are higher for Genotyper KAZAM macros. Therefore any stutter position labels assigned by Genotyper but not by GeneMapper should not be related to differences in stutter filter settings. Peak height reduction also did not appear to play a significant role in the observed discrepancy; corresponding stutter peaks were above threshold with GeneMapper analysis.

The ability of Genotyper to effectively filter labeled stutter peaks seems to be correlated with the presence or absence of a labeled –A peak on the true allele. The KAZAM macro filters stutter peaks by removing labels from peaks followed by a higher,

labeled peak within 3.25bp to 4.75bp. When a stutter peak is followed by a labeled –A peak, the software does not filter the stutter peak even if it falls below the stutter filter setting. This may be due to the stutter ratio calculation being based on the height of the –A peak instead of the height of the true allele.

Table 4. Profiler Plus ID™ & COfiler™ stutter filter setting comparison. Genotyper KAZAM macro filter settings are from the respective Amp*Fl*STR® Profiler Plus™ v4.1 NT and Amp*Fl*STR® COfiler™ v4.1 NT Genotyper templates.

Marker	GM *ID* v3.2 Stutter Filters	GT_{NT} v3.7 Stutter Filters	Difference Between GM and GT Filters
D3S1358	0.11	0.124	-0.014
VWA	0.11	0.124	-0.014
FGA	0.11	0.124	-0.014
D8S1179	0.12	0.136	-0.016
D21S11	0.13	0.150	-0.020
D18S51	0.16	0.191	-0.031
D5S818	0.10	0.111	-0.011
D13S317	0.10	0.111	-0.011
D7S820	0.09	0.099	-0.009
D16S539	0.13	0.150	-0.020
TH01	0.06	0.064	-0.004
TPOX	0.06	0.094	-0.034
CSF1PO	0.09	0.099	-0.009

An interesting additional discrepancy between GeneMapper *ID* and Genotyper analyses is the labeling of THO1 stutter peaks. Genotyper labels these peaks as off-ladder (OL), whereas GeneMapper labels in an X.3 format (X=whole repeat unit).

Out of the 35 samples exhibiting degrees of non-concordance due to peak height reduction with GeneMapper *ID* analysis, seventeen resulted in a loss of reportable data. Some of these samples were low-copy number mixtures where low-level alleles were lost with GeneMapper analysis. Others were single-source with one or more alleles hovering above threshold in Genotyper analysis that were subsequently lost with GeneMapper analysis. The remaining eighteen samples did not result in a net loss of reportable data. Most were examples of inconclusive loci obtained with Genotyper analysis that transformed into no result loci with GeneMapper analysis. Table 5 lists all observed non-concordance due to decreased GeneMapper *ID* peak heights. Data loss occurred for both low-copy and standard cycle amplifications. Data loss within standard cycle amplifications was limited to one single source sample. All other instances involve a loss of low-level alleles for mixed DNA samples.

A non-concordant genotype at amelogenin was observed for a LCN mixed DNA amplification. GeneMapper *ID* typed this sample as X,Y; Genotyper typed as X. The Y peak was detectable within the Genotyper analysis and could be manually labeled. Peak height ratios of Y to X were 0.028 for both analyses.

Table 5. Observed evidentiary sample non-concordance between GeneMapper™ *ID* and GeneScan®/Genotyper® anlaysis due to peak height reduction of GeneMapper™ *ID* analysis

Sample Number	Sample Type	Data Type	Loci	Data Loss?
1	BONE	LCN/SS	THO1	YES
2	BONE	LCN/SS	AMEL,D8	YES
3	BONE	LCN/MIXTURE	D18	YES
4	BONE	LCN/SS	D7	YES
5	BLOODSTAIN ON CLOTH	LCN/MIXTURE	D21	YES
6	BONE	LCN/SS	D5	YES
7	BONE	LCN/SS	AMEL	YES
8	BONE	LCN/SS	D21	YES
9	BLOODSTAIN ON CLOTH	STD/MIXTURE	D16,THO1,TPOX	YES
10	BLOODSTAIN ON DENIM	LCN/MIXTURE	CSF1PO	YES
11	FINGERNAIL SCRAPINGS	LCN/MIXTURE	FGA	YES
12	VAGINAL SWAB	STD/MIXTURE	TPOX	YES
13	VAGINAL SWAB	STD/MIXTURE	D7	YES
14	VULVAR SWAB	STD/MIXTURE	D13	YES
15	BLOODSTAIN SWAB	STD/MIXTURE	D5	YES
16	JACKET SCRAPING	STD/MIXTURE	D18	YES
17	CIGARETTE BUTT	STD/SS	CSF1PO	YES
18	BONE	LCN/SS	AMEL,D8,D5	NO
19	TISSUE BLOCKS	LCN/SS	D5	NO
20	BONE	LCN/SS	FGA	NO
21	BONE	LCN/SS	FGA	NO
22	BONE	LCN/SS	THO1	NO
23	BONE	LCN/SS	D18,D7	NO
24	BONE	LCN/SS	CSF1PO	NO
25	BONE	LCN/SS	CSF1PO	NO
26	BONE	LCN/SS	D7	NO
27	BONE	LCN/SS	THO1	NO
28	BONE	LCN/SS	VWA	NO
29	BONE	LCN/SS	D8	NO
30	BLOODSTAIN ON CLOTH	STD/SS	D8	NO
31	BONE	LCN/SS	D7	NO
32	BONE	LCN/SS	TPOX	NO
33	BONE	LCN/SS	CSF1PO	NO
34	VAGINAL SWAB	LCN/SS	D3	NO
35	VULVAR SWAB	STD/SS	D8 (minor)	NO

LCN = Low Copy Number Amplifications; STD = Standard Cycle Amplifications; SS = Single-Source

Operational Errors Observed with GeneMapper *ID* Analysis

During the course of this validation study, several operational errors were observed with GeneMapper *ID* analysis. On several different occasions, GeneMapper *ID* locked-up or froze. Any data modifications that had not been previously saved were lost. This malfunction may have been partially related to the abnormally large projects used for validation purposes.

When samples from different run date and times or from the same run date but different times were combined into a single analysis folder and imported into GeneMapper *ID*, run date and times were uniformly converted to match the correct information from one sample within the folder. This error occurred for several of the above studies. Whether this error occured during all such instances has yet to be confirmed. This error does not affect data analysis.

Errors also occurred when trying to maintain selections while toggling between samples and genotypes tabs and plots. Samples selected in the genotypes tab should remain selected when switching to the samples tab and should be displayed in the samples plot. This feature malfunctioned on several occasions. The malfunction may have been associated with differential sorting mechanisms applied to the samples and genotypes tabs.

During preliminary analysis of project data, it was occasionally necessary to delete a sample file or an entire folder from a project. Often data deletions were followed by the importation of replacement data. Inconsistencies in the software's response to this sort of data manipulation were observed.

The plots per pane printing feature does not work as described in the GeneMapper user manual. This setting should allow the user to determine the number of plots printed per page (with a range of one to six) when multiple samples are selected and printed within the samples and genotypes plot windows. Plots per pane was set to three to accommodate all three data fluors (5-FAM, JOE, and NED) of each sample on a single page. On all printing attempts, four plots per page were printed.

CHAPTER 4

DISCUSSION

Peak Height Comparison Study

As compared to corresponding GeneScan/Genotyper analysis, a distinct decrease in peak-height was observed for all fragments analyzed with GeneMapper *ID*. For peaks greater than 500 rfu, the average observed relative decrease is between three and six percent. The percent decrease for peaks less than 500 rfu can be significantly higher.

The decreased peak detection of GeneMapper *ID* analysis can reduce the software's data capture capability as compared to GeneScan/Genotyper analysis. Peaks detected with capillary electrophoresis instrumentation may not be reportable with GeneMapper *ID* analysis using current laboratory peak threshold settings. This potential reduction in data capture was realized for six sample files within the peak height comparison evaluation and seventeen sample files within the evidentiary concordance study. All affected samples involve the loss of low-level peaks associated with either low-copy number amplifications or mixed DNA profiles with low-level contributors. Lowering peak amplitude thresholds within the bounds of instrument sensitivity would increase the software's data capture ability as compared to the current analysis software.

The observed relative decrease in peak height is greater than that reported for the developmental validation conducted by Applied Biosystems (4). In part, this appears to

be due to differences in analysis parameter settings for GeneScan and GeneMapper. The software's respective settings differ for minimum peak half-width, peak window size, and baseline window size. The difference in baseline window size is the most significant and most plausible candidate for resultant peak height disparities.

To examine this possibility, the 9947A dilution series was reanalyzed in GeneMapper using the current GeneScan analysis settings and conversely reanalyzed in GeneScan/Genotyper using the recommended GeneMapper HID analysis settings. The GeneMapper simulated GeneScan analysis resulted in higher overall peak heights as compared to the original GeneMapper 9947A dilutions series analysis. The new peak heights were still lower than the original GeneScan/Genotyper analysis results. The GeneScan/Genotyper analysis using GeneMapper *ID* analysis parameters resulted in lower overall peak heights as compared to its original analysis of the same dilution series. The new peak heights were still higher than the original GeneMapper *ID* analysis results. The analysis parameter simulation exercise demonstrates that the differences in analysis parameter settings were partially responsible for the observed peak height reduction of GeneMapper *ID* analysis. GeneMapper *ID* sacrifices a small measure of peak height for smoother baselines.

Reproducibility Evaluation

The reproducibility study demonstrated the ability of GeneMapper *ID* to analyze Profiler Plus *ID*™ and COfiler™ STR data in a reliable and consistent manner. This knowledge provides confidence that repeated analyses involving the same sample files

will generate identical results and therefore provides a valid foundation for peer review of electronic STR data.

The compiled-run folder replicate pushed the limits of GeneMapper precision. Compiled samples were run over the course of several months. Therefore, files with different rates of migration arising from fluctuations in various electrophoretic parameters were potentially combined into a single analysis. Assignments of peak size, peak height, and genotype were identical to those derived for the individual-run folder analyses. This replicate also demonstrated the validity of combining relevant ladders from several runs and allowing GeneMapper *ID* to average the ladder offsets and bin calculations to genotype associated sample files.

Stutter Study

Stutter data characterized by this study is comparable to Applied Biosystems stutter reports on AmpF*l*STR® loci (3). Observed stutter ratios differed within and between markers. In general, larger molecular-weight alleles exhibited higher percentages of stutter than smaller molecular-weight alleles of the same marker. Microvariants also expressed markedly lower stutter percentages than their bordering whole-repeat unit counterparts. Characteristic stutter ratio ranges and average stutter ratios were determined for each marker. These calculations validate the utilization of GeneMapper *ID* v3.2 stutter filter pre-sets for the analysis of AmpF*l*STR® Profiler Plus *ID*™ and AmpF*l*STR® COfiler™ data. For highly imbalanced mixtures, it may still be necessary to manually label stutter peaks filtered by the software after GeneMapper

analysis. All marker summaries and stutter plots will be made available to aid analysts under such circumstances.

Reference Sample Concordance Study

Absolute concordance observed between all manually edited reference profiles generated with GeneMapper *ID* and GeneScan/Genotyper counterpart analyses validates the use of GeneMapper *ID* for reference sample STR analysis. The greater amount of manual editing required by GeneMapper *ID* analysis of reference samples is largely due to shoulder labels on amelogenin peaks. The 20% cut-off filter is not applied to amelogenin. Amelogenin has an independent, adjustable cut-off filter. If set to 20% for future reference sample analysis, manual editing requirements should approximate GeneScan/Genotyper reference sample editing. The Genotyper KAZAM(20%) macro applies a 20% filter to all STR markers plus amelogenin.

The Control Concordance (CC) flag for assigned positive control and negative control sample types functioned properly during the GeneMapper *ID* concordance feature evaluation. All post-editing sample concordance checks accurately identified all instances of non-concordance between counterpart Profiler Plus™ and COfiler™ amplifications. However, pre-manual editing concordance checks did not fully identify all non-concordant loci.

Evidentiary Sample Concordance Study

Demonstrated concordance of all allele calls made by GeneMapper *ID* with corresponding allele calls made by GeneScan/Genotyper, validates the use of GeneMapper *ID* for evidentiary sample STR analysis. Significant non-concordance of

stutter position allele calls observed between the analyses is a notable improvement in STR data analysis. GeneMapper *ID* makes far fewer –A calls than GeneScan/Genotyper analysis, and therefore significantly reduces manual data editing requirements. Whether this is due to decreased peak detection, improved peak detection algorithms, or a combination of both is undetermined.

As stated previously, GeneMapper and GeneScan/Genotyper analysis of low-copy number and mixed DNA evidentiary sample files may not result in equivalent data capture. Thirty-five out of 284 evidentiary sample files resulted in a loss of reportable data with GeneMapper *ID* analysis.

Recommended Software Improvements for GeneMapper *ID*

The following is a list of suggestions for future improvement of GeneMapper *ID* human identification analysis software.

1. A right click option for changing labels to include peak height or peak size on an individual basis. Currently, this is an all or none option. Excessive labeling can be confusing and can cause page spill-over during printing.
2. A fully-functioning plots per pane setting feature. The current feature does not function as intended. This would allow for simultaneous printing of a large volume of samples with all data for a single sample printed on a single page.
3. A raw data multiple selection feature. Currently, if wanting to review raw data from multiple samples in different run folders, the analyst must select the respective samples in either the Samples or Genotypes tab and choose view

raw data. Only the first sample selection is displayed. The other selected samples must be located and selected in the navigation pane to view their raw data. Highlighting of the selected samples in the navigation pane currently expedites the search, but this process is still very difficult for large projects with multiple run folders.

4. A Genotype Quality and PQV history or memory feature. Any modification of a locus results in an automatic GQ override and erasure of all associated PQV flags. This occurs whether or not the problems that originally triggered the PQV flags and resultant low quality GQ were appropriately addressed. Theoretically, an analyst could remove a nominal –A label for a particular locus and the GQ would turn to green even if the sample still exhibits LPH, BIN, AN or other issues that require further attention. All of the PQV information is lost once editing occurs. Analysts must be certain all problems associated with a given locus are thoroughly evaluated and corrected with any initial editing. These samples will not be sorted or identified with subsequent GQ low quality to the top sorting mechanisms. Anyone reviewing project data will not have access to the original GeneMapper quality value assessments.

Conclusions

Overall, GeneMapper *ID* provided for a more efficient and more informative data analysis as compared to current laboratory fragment analysis software. Its ability to combine both Profiler Plus™ *ID* and COfiler™ amplifications into a single project

decreased analysis time and provided a convenient approach for comparing counterpart and multiple amplifications of the same sample. The software's ability to perform independent analyses for separate run folders within the same project also expedited data analysis and any required data comparisons. This capability allows for the simultaneous analysis of all data associated with a particular case despite any differences in CE run dates.

GeneMapper *ID* required a greater degree of manual editing than GeneScan/Genotyper for reference sample analysis and less manual editing than GeneScan/Genotyper for evidentiary sample analysis. The software performed better than GeneScan/Genotyper in labeling microvariant peaks, baselining data, identifying fewer –A peaks, aligning below threshold data, and correctly defining size standard peaks despite pull-up and spike interference. Compared to GeneMapper *ID*, GeneScan/Genotyper exhibited increased peak height detection, increased data capture capability and better amelogenin shoulder filtering during reference sample analysis. However, these differences may be eliminated with further optimization of GeneMapper *ID* analysis settings.

The concordance check features incorporated into GeneMapper *ID* analysis quickly alert the analyst to problems with controls and non-concordance between duplicate and counterpart amplifications of an individual sample. These checks are critical quality assurance procedures routinely performed by analysts. When extraction and amplification controls do not produce the expected results, all associated samples may require re-extraction or re-amplification depending on the specific circumstances.

Any discrepancies noted between multiple amplifications of an individual sample are of significant concern and require further investigation. The software's ability to confirm D3S1358, amelogenin, and D7S820 concordance between Profiler Plus™ and COfiler™ counterpart amplifications should facilitate data review of reference database samples. As previously indicated, the sample concordance check may not function appropriately prior to manual data editing.

GeneMapper's quality value system provides analysts with an immediate quality assessment of sample data prior to manual review. The analyst may quickly sort and immediately address problematic data. Low quality Sizing Quality (SQ) assessments were observed for non-detection of a size standard peak(s), off-scale data pull-up into Rox, and significant electrophoretic spikes. The analyst may choose to override low quality SQ and reanalyze the associated samples. GeneMapper *ID* incorrectly labeled size standards for three sample files associated with this study. Two were due to a loss of size standard fragments, and one was due to extreme amelogenin pull-up. All three were rated low quality SQ by the software. The size standards were manually labeled which automatically results in an SQ override. Upon reanalysis, the software did not exhibit any genotyping difficulty for the affected samples.

Genotype Quality (GQ) assessments are reported for each locus of an individual sample. Post-manual editing, low quality GQ were most often observed for loci with off-scale peaks or overlapping off-scale peaks, apparent homozygous alleles less than the 200 rfu setting, loci with more than two detected peaks, and no result loci. Observed, check quality GQ were always associated with loci exhibiting peak height ratios less than the

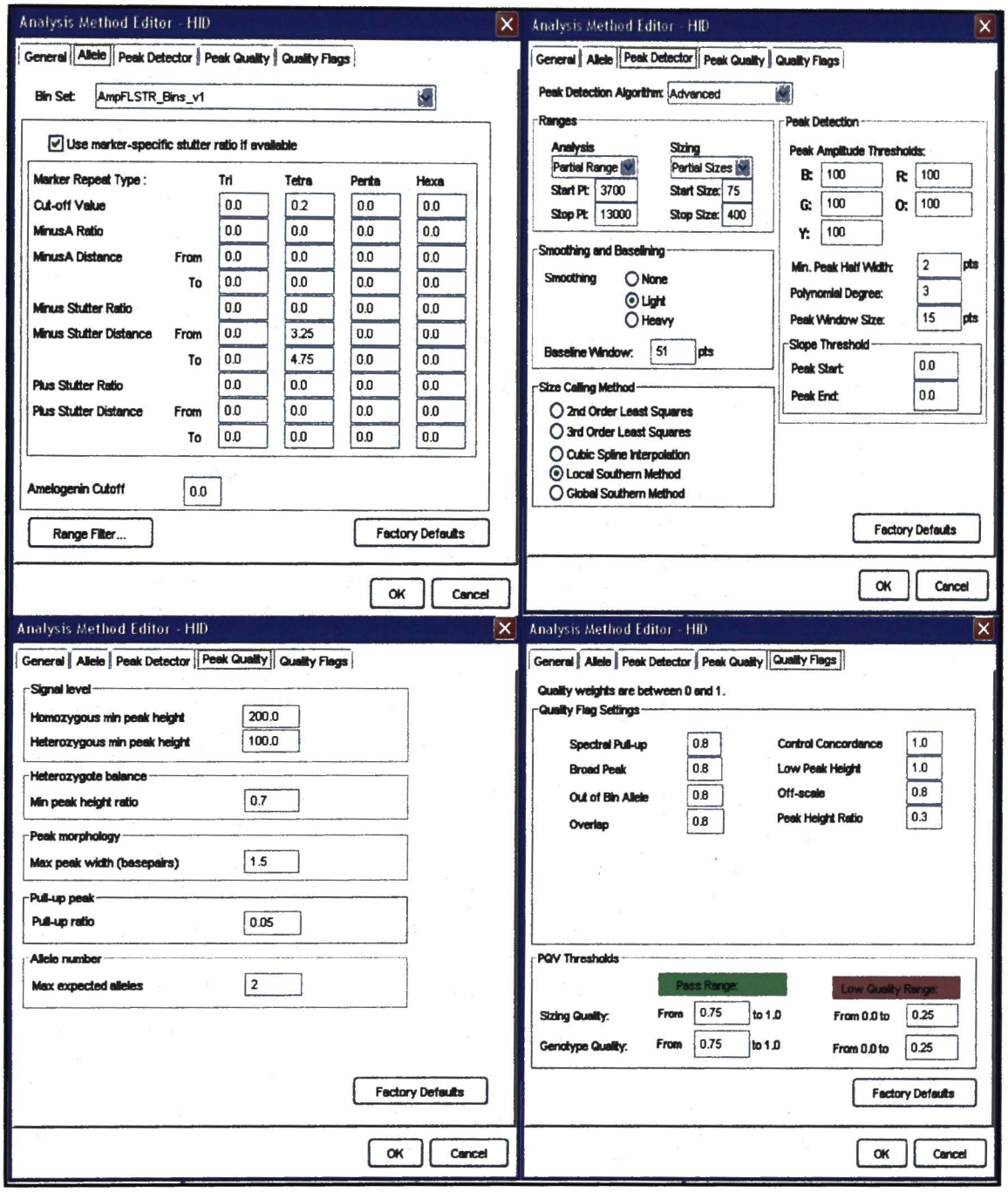
seventy percent and fifty percent settings. Analysts should bear in mind that the genotype Off Scale process quality value may not be uniformly applied and the Spectral Pull-up PQV does not function as intended.

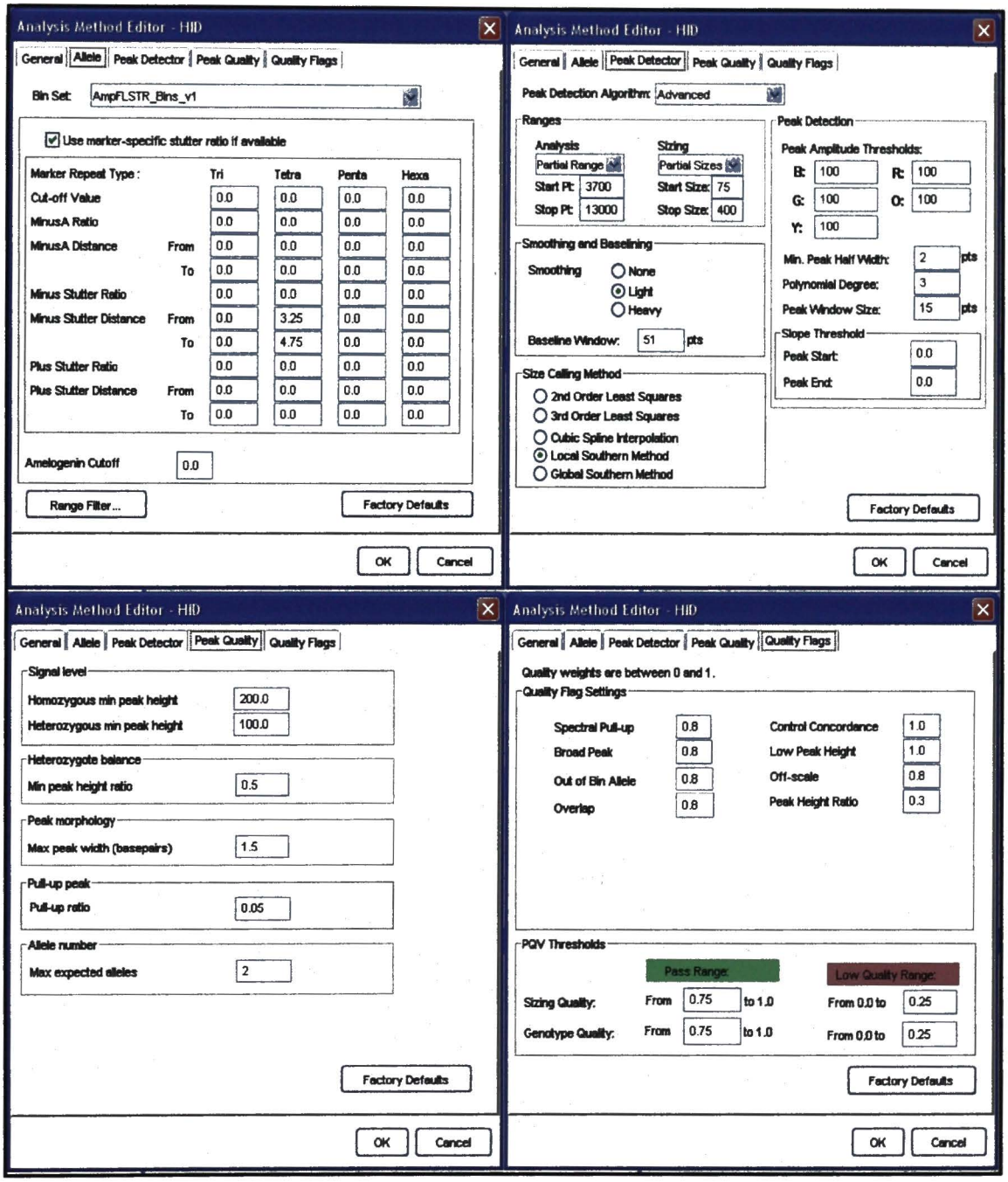
The peak height reduction of GeneMapper *ID* analysis proposes somewhat of an obstacle for low copy number DNA analysis. Unless interpretation thresholds are adjusted accordingly, analysts may be required to work harder to obtain data from difficult samples. All other attributes of GeneMapper *ID* analysis will be a noted improvement over current analysis software. GeneMapper *ID* is an accurate and reliable method for sizing and genotyping STR fragments for forensic human identification analysis.

APPENDIX A

ANALYSIS METHODS

GeneMapper™ *ID* HID Reference Samples Analysis Method

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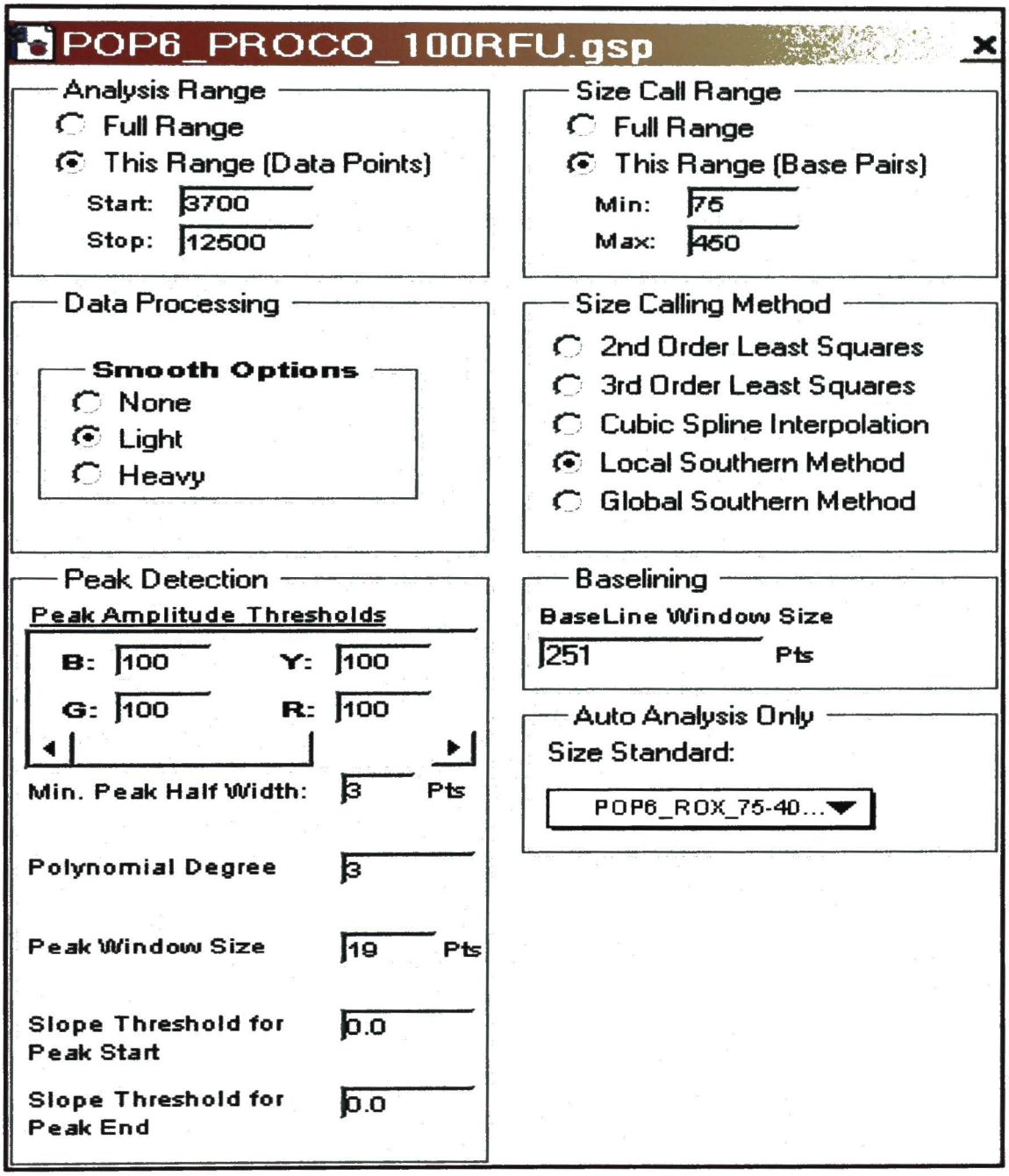
Peak Detector
Peak Quality
Quality Flags
Bin Set:
AmpFLSTR_Bins_v1
Use marker-specific stutter ratio if available
Marker Repeat Type :
Tri
Tetra
Penta
Hexa
Cut-off Value
MinusA Ratio
MinusA Distance
From
To
Minus Stutter Ratio
Minus Stutter Distance
Plus Stutter Ratio
Plus Stutter Distance
Amelogenin Cutoff
Range Filter...
Factory Defaults
OK
Cancel
Peak Detection Algorithm:
Advanced
Ranges
Analysis
Sizing
Partial Range
Partial Sizes
Start Pt:
Stop Pt:
Start Size:
Stop Size:
Smoothing and Baselining
Smoothing
None
Light
Heavy
Baseline Window:
pts
Size Calling Method
2nd Order Least Squares
3rd Order Least Squares
Cubic Spline Interpolation
Local Southern Method
Global Southern Method
Peak Detection
Peak Amplitude Thresholds:
B:
R:
G:
O:
Y:
Min. Peak Half Width:
Polynomial Degree:
Peak Window Size:
Slope Threshold
Peak Start:
Peak End:
Signal level
Homozygous min peak height
Heterozygous min peak height
Heterozygote balance
Min peak height ratio
Peak morphology
Max peak width (basepairs)
Pull-up peak
Pull-up ratio
Allele number
Max expected alleles
Quality weights are between 0 and 1.
Quality Flag Settings
Spectral Pull-up
Broad Peak
Out of Bin Allele
Overlap
Control Concordance
Low Peak Height
Off-scale
Peak Height Ratio
PQV Thresholds
Pass Range:
Low Quality Range:
Sizing Quality:
Genotype Quality:
From
to 1.0
From 0.0 to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Stop: 12500
Size Call Range
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This Range (Base Pairs)
Min: 75
Max: 460
Data Processing
Smooth Options
None
Light
Heavy
Size Calling Method
2nd Order Least Squares
3rd Order Least Squares
Cubic Spline Interpolation
Local Southern Method
Global Southern Method
Peak Detection
Peak Amplitude Thresholds
B: 100
Y: 100
G: 100
R: 100
Min. Peak Half Width: 3 Pts
Polynomial Degree 3
Peak Window Size 19 Pts
Slope Threshold for Peak Start 0.0
Slope Threshold for Peak End 0.0
Baselining
BaseLine Window Size
251 Pts
Auto Analysis Only
Size Standard:
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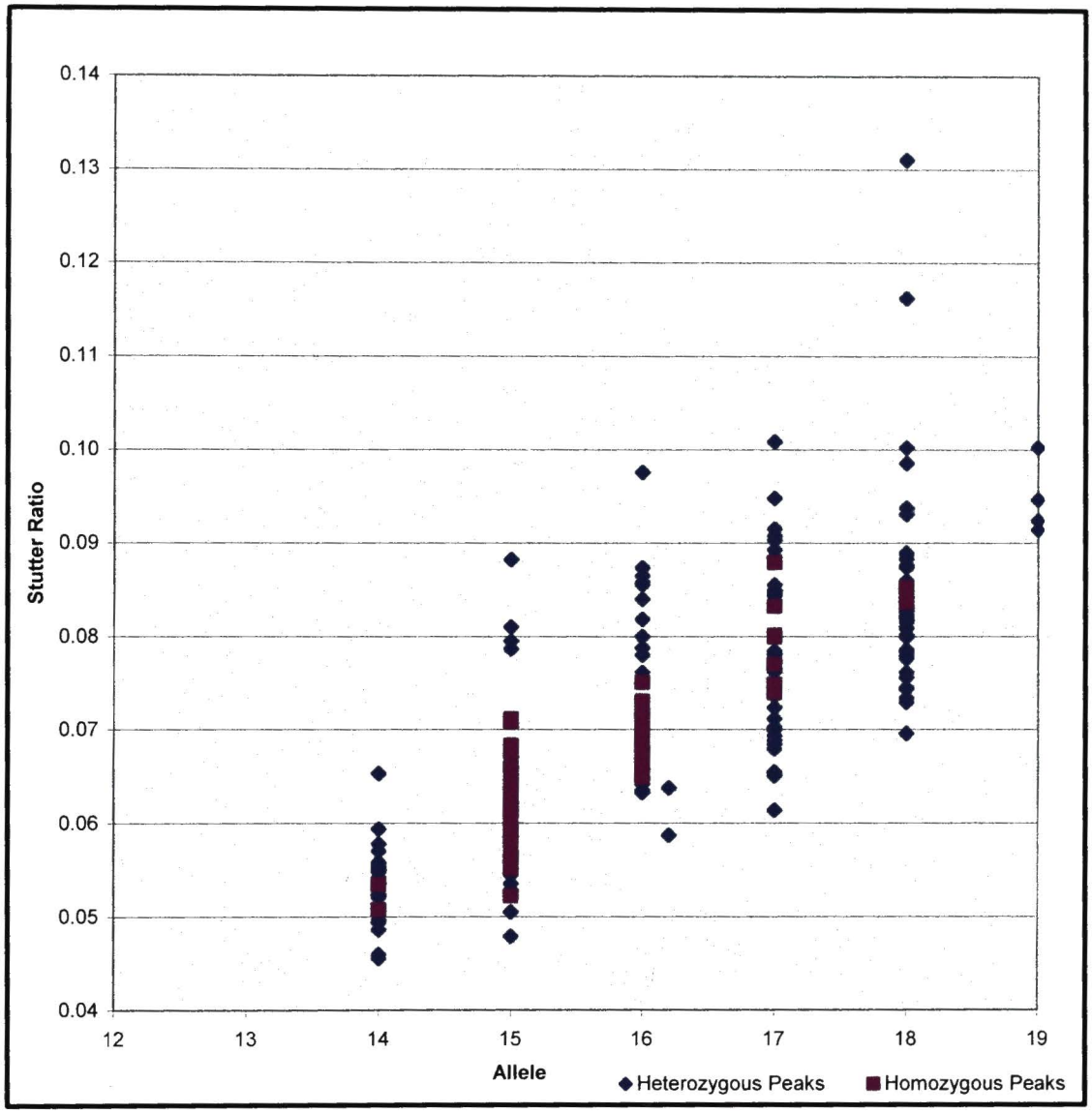
APPENDIX B

AMPF*l*STR® PROFILER PLUS *ID* AND COFILER STUTTER PLOTS

D3S1358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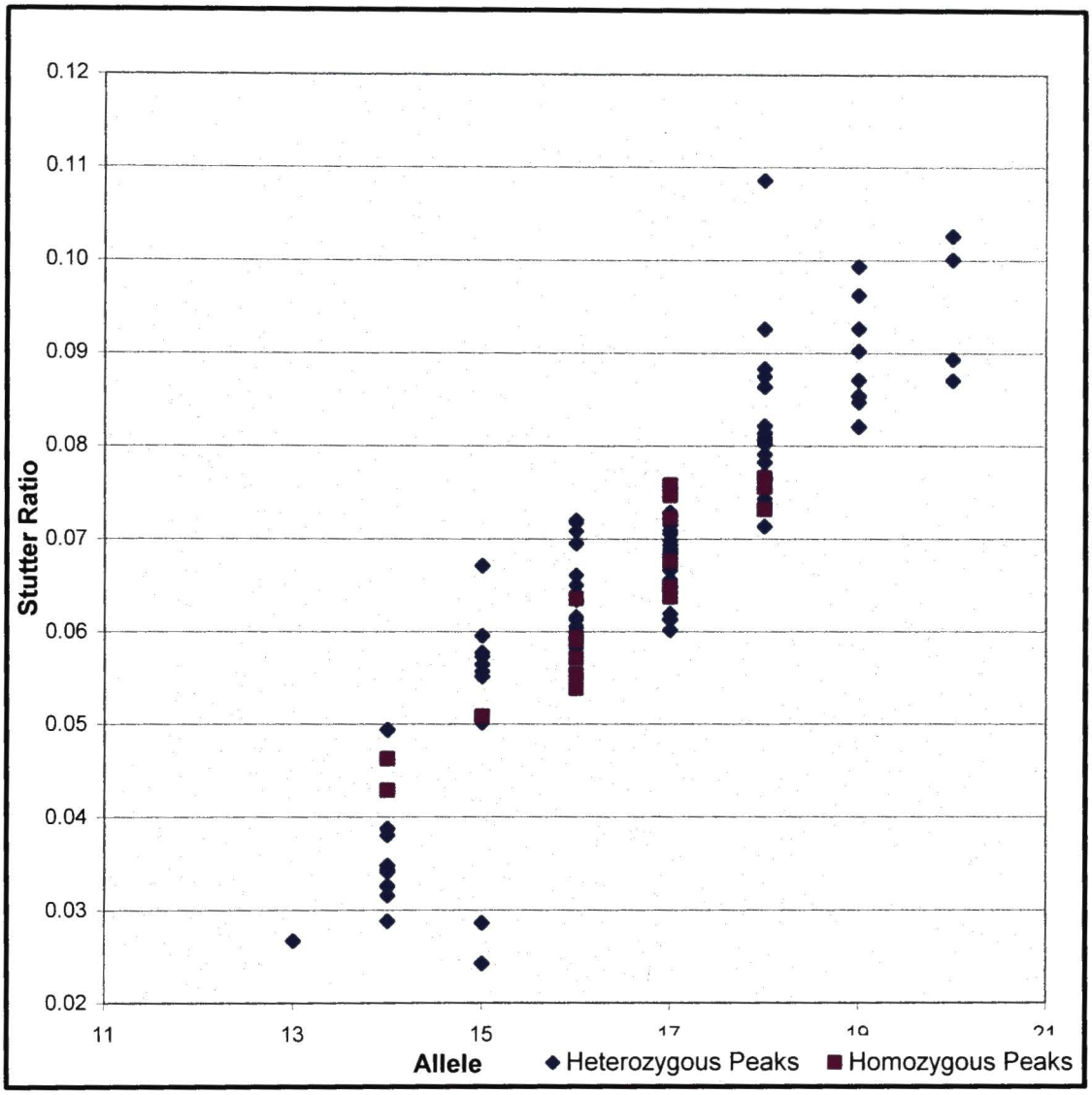
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
Stutter Ratio
12
13
14
15
16
17
18
19
Allele
Heterozygous Peaks
Homozygous Peaks

vWA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0.11
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0.05
0.04
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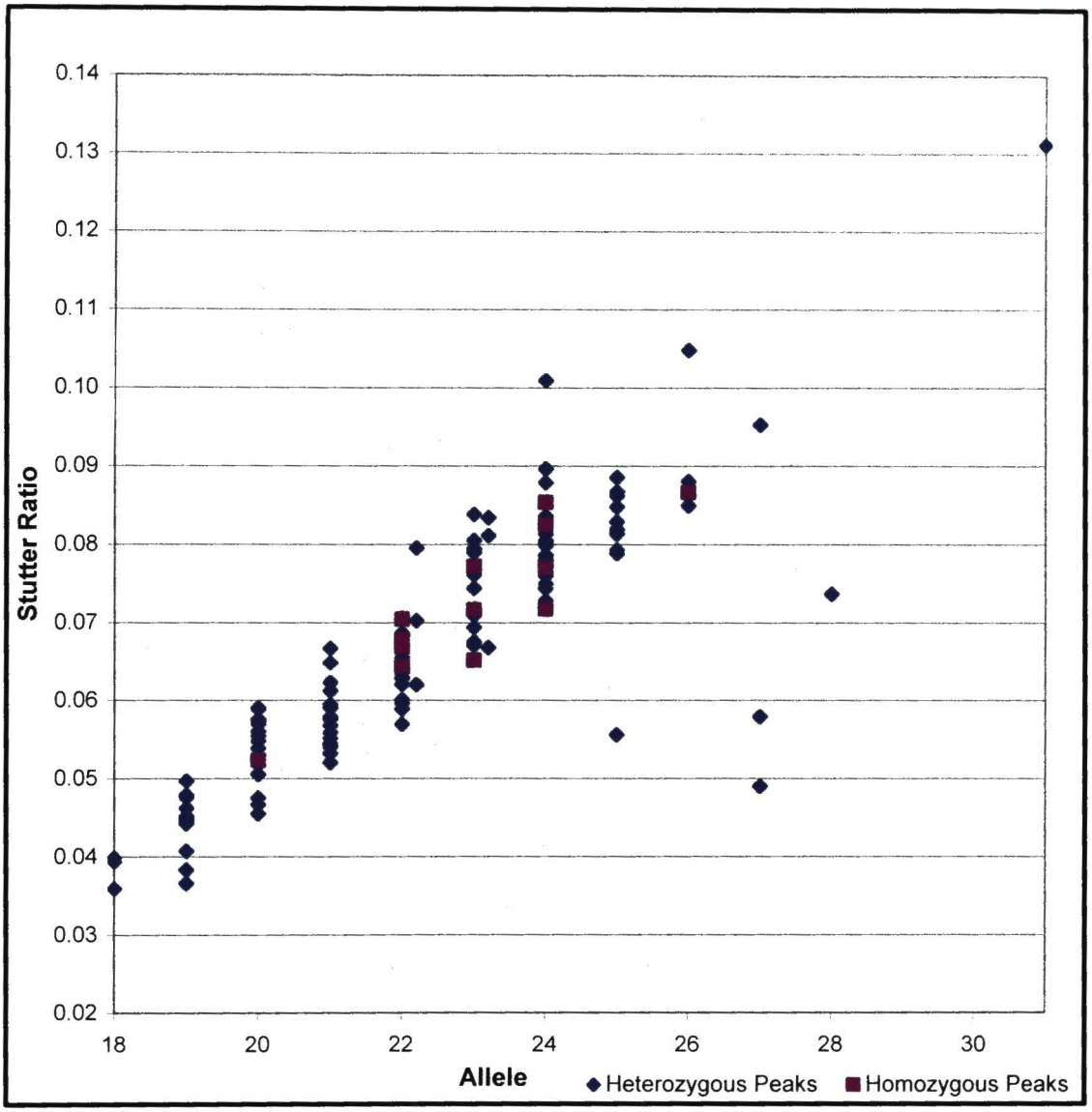
Stutter Ratio
11
13
15
17
19
21
Allele
Heterozygous Peaks
Homozygous Peaks

FGA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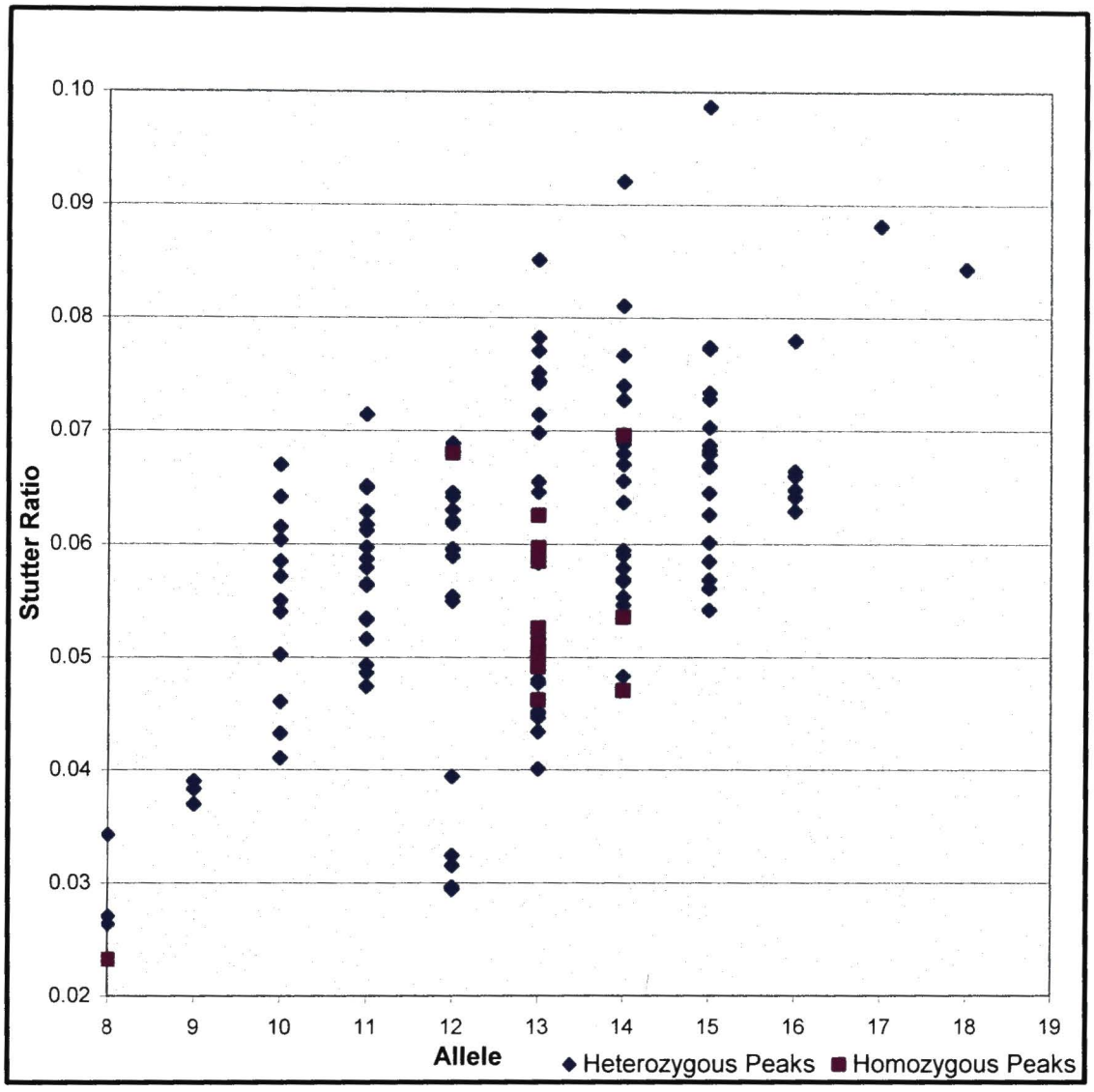
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
Stutter Ratio
18
20
22
24
26
28
30
Allele
Heterozygous Peaks
Homozygous Peaks

D8S1179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0.04
0.03
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Stutter Ratio
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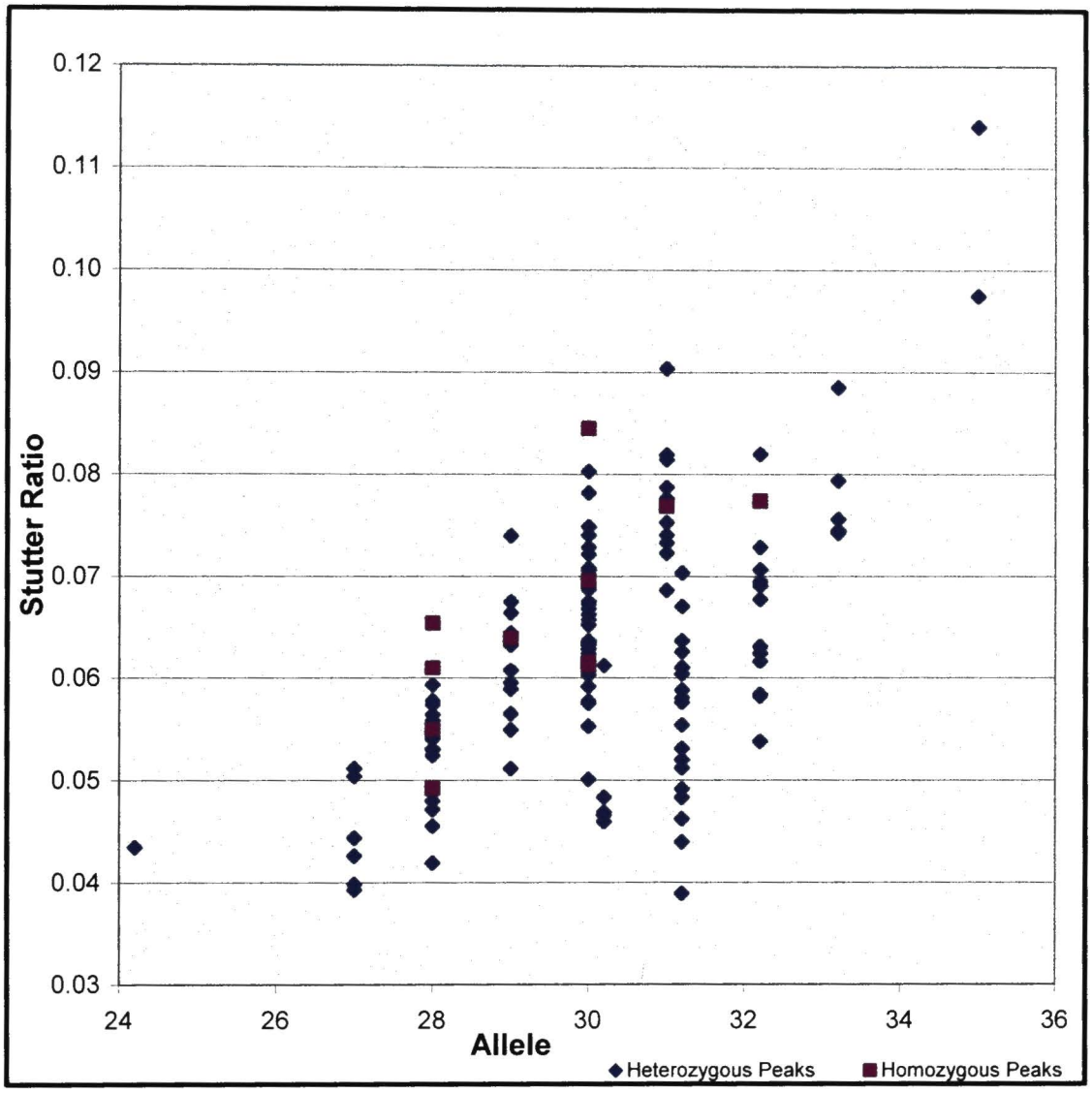
9
10
11
12
13
14
15
16
17
18
19
Allele
Heterozygous Peaks
Homozygous Peaks

D21S11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0.07
0.06
0.05
0.04
0.03
Stutter Ratio
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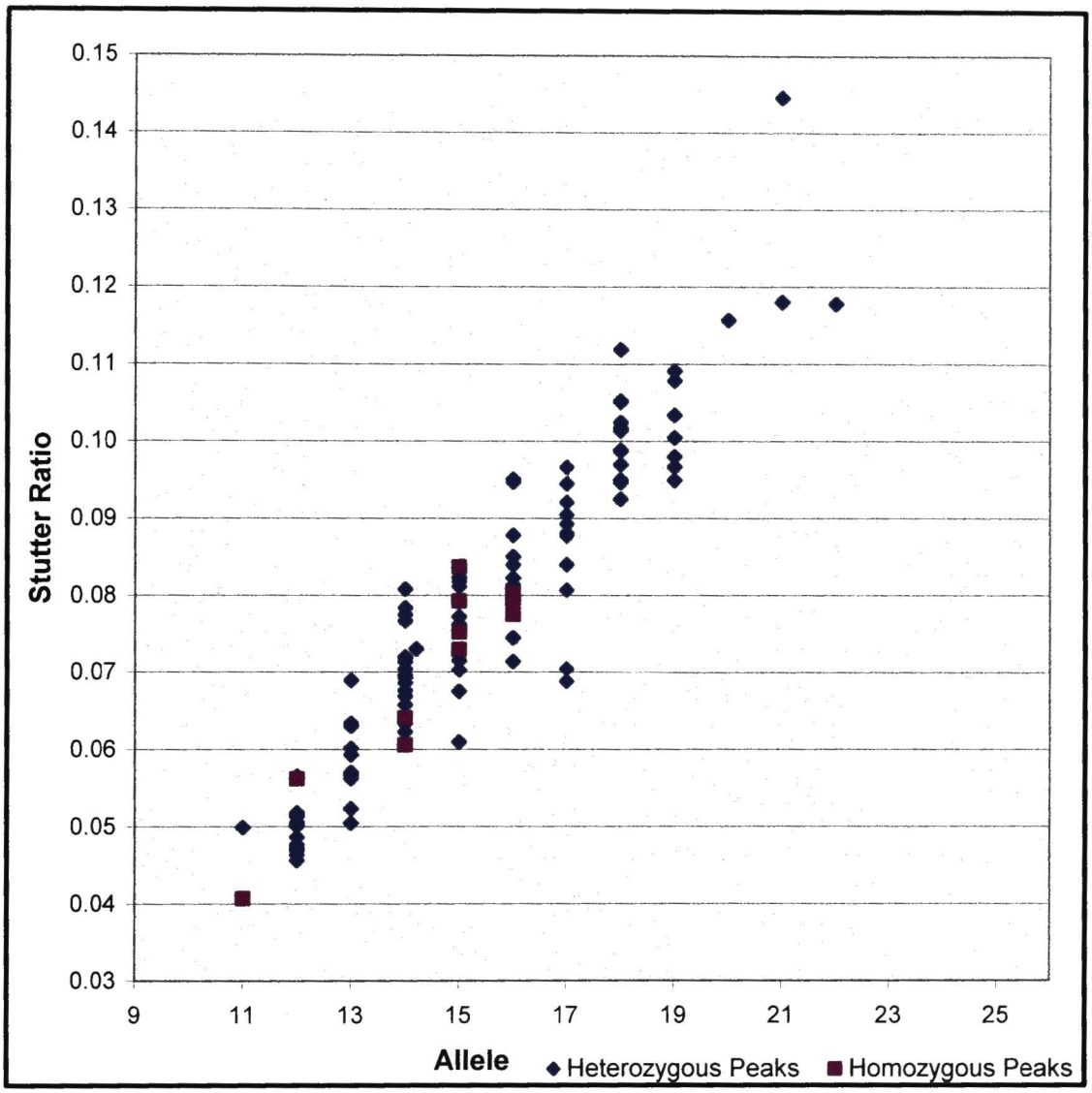
26
28
30
32
34
36
Allele
Heterozygous Peaks
Homozygous Peaks

D18S51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Stutter Ratio
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15
17
19
21
23
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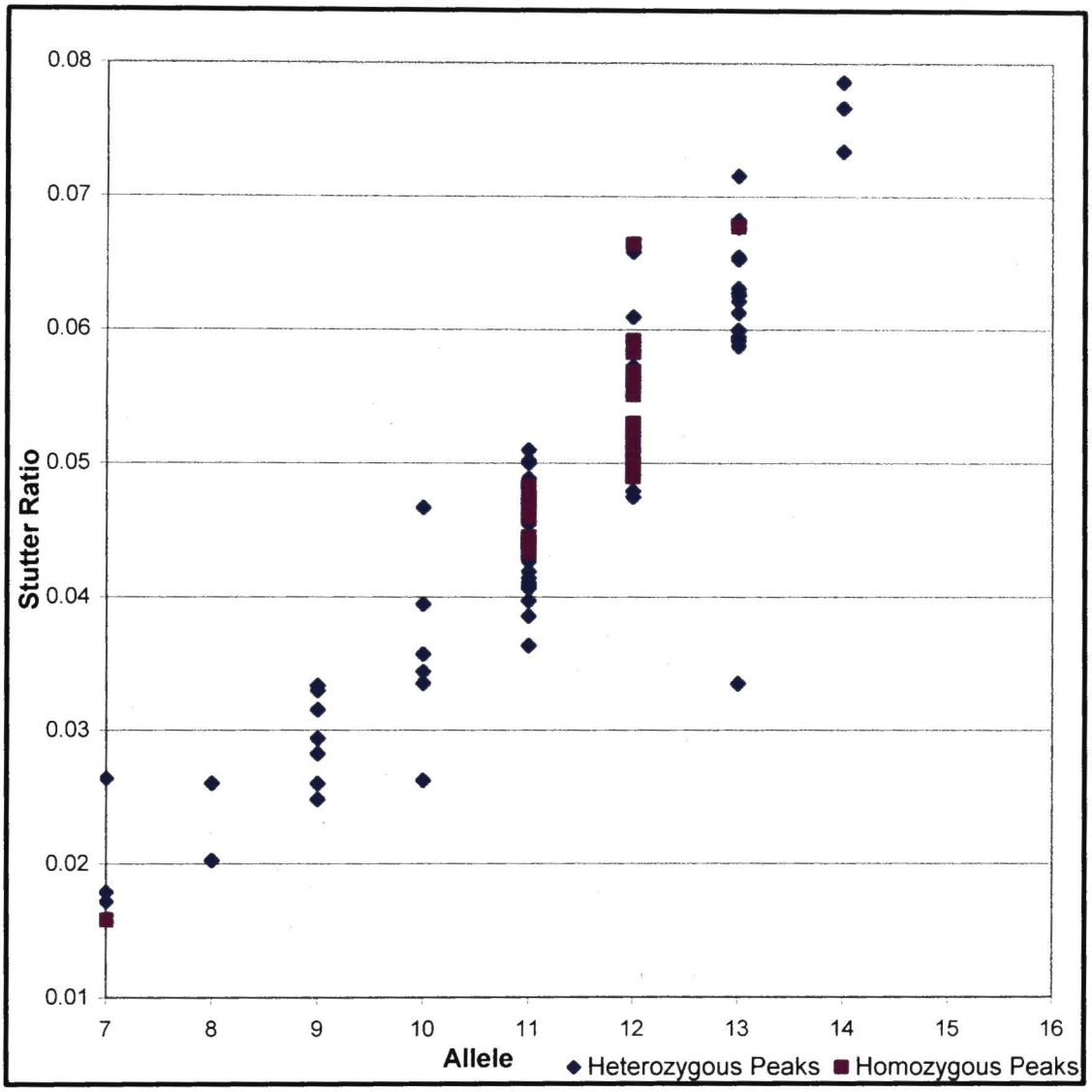
Allele
Heterozygous Peaks
Homozygous Peaks

D5S818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Stutter Ratio
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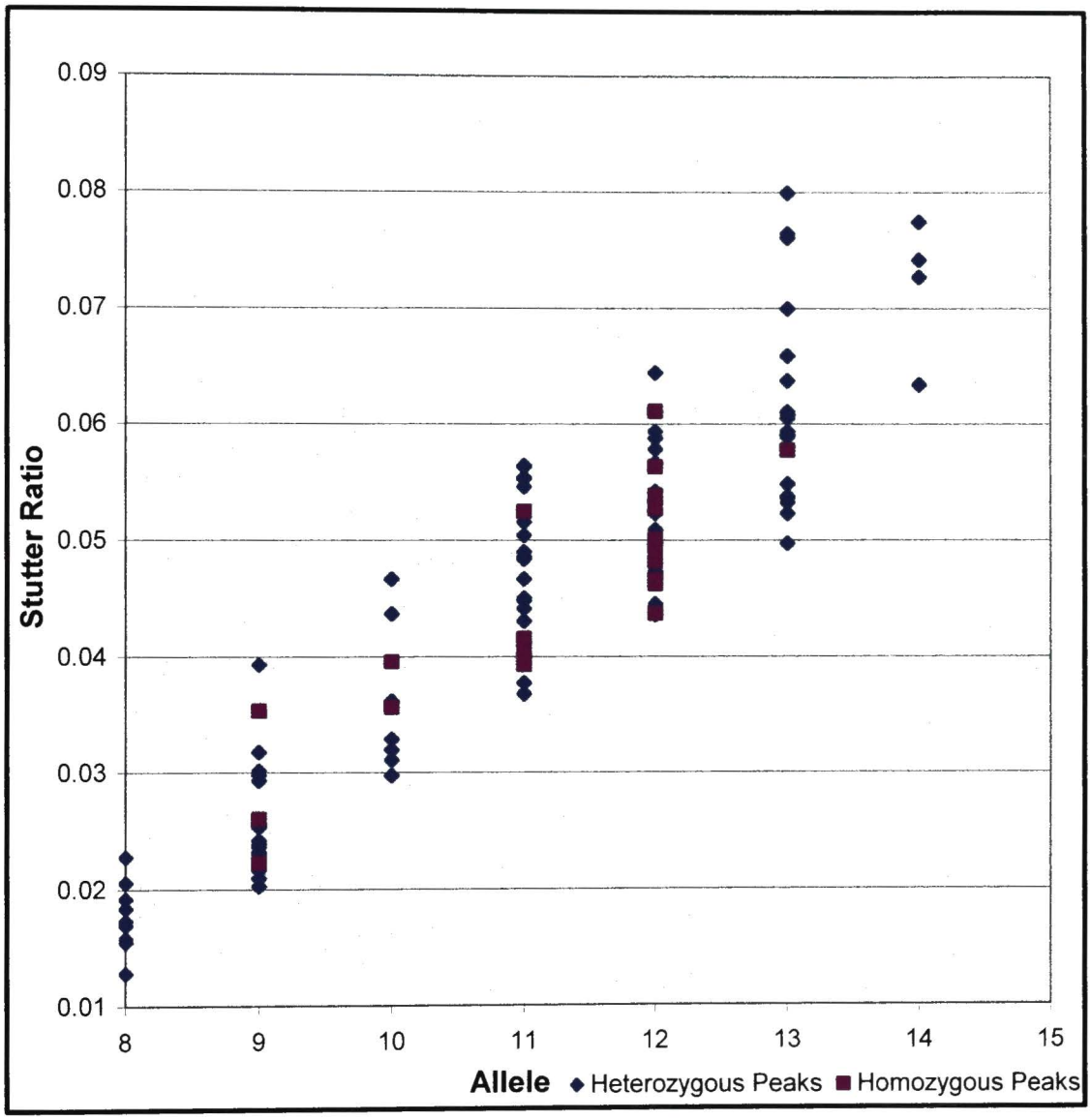
14
15
16
Allele
Heterozygous Peaks
Homozygous Peaks

D13S317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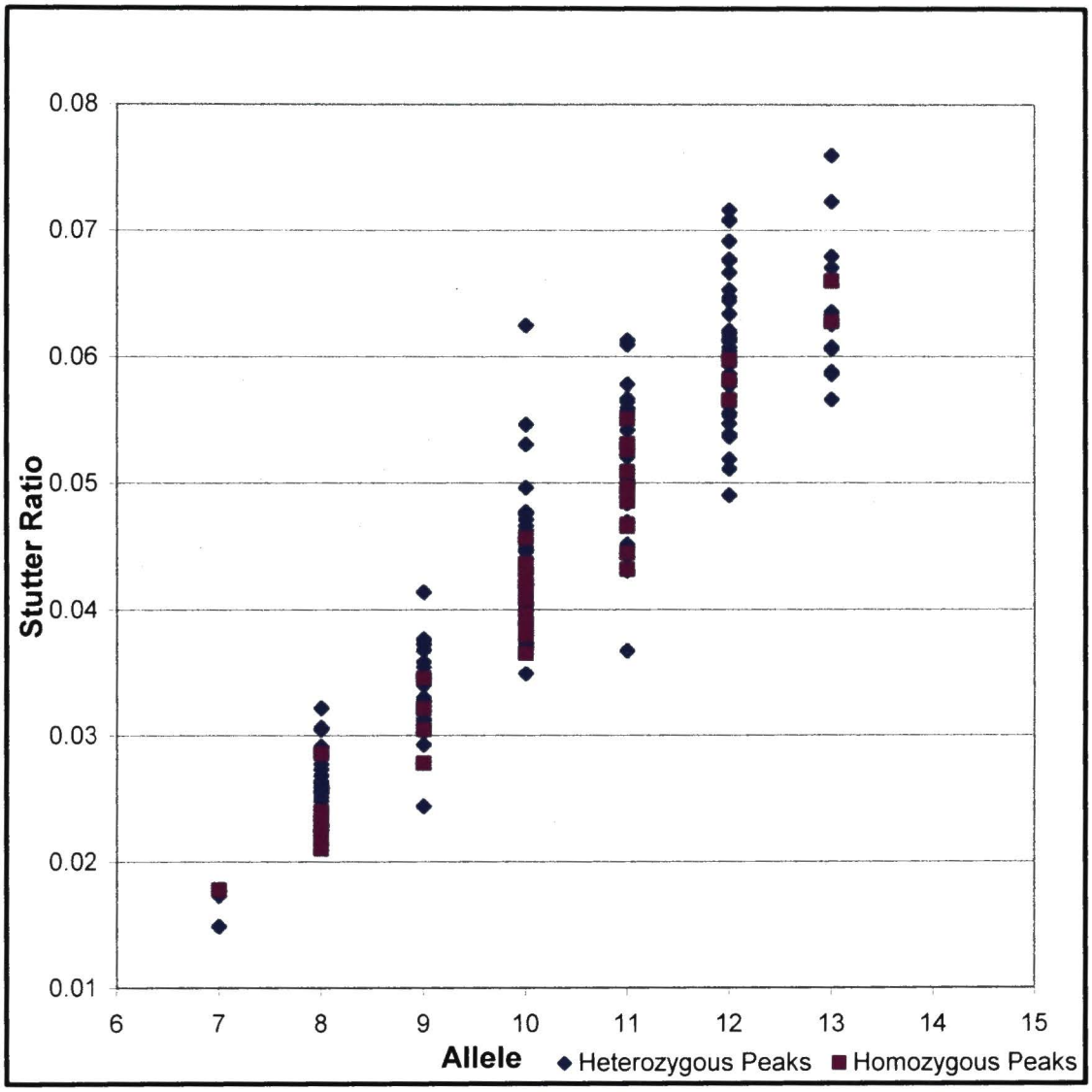
15
Allele
Heterozygous Peaks
Homozygous Peaks

D7S820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0.01
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12
13
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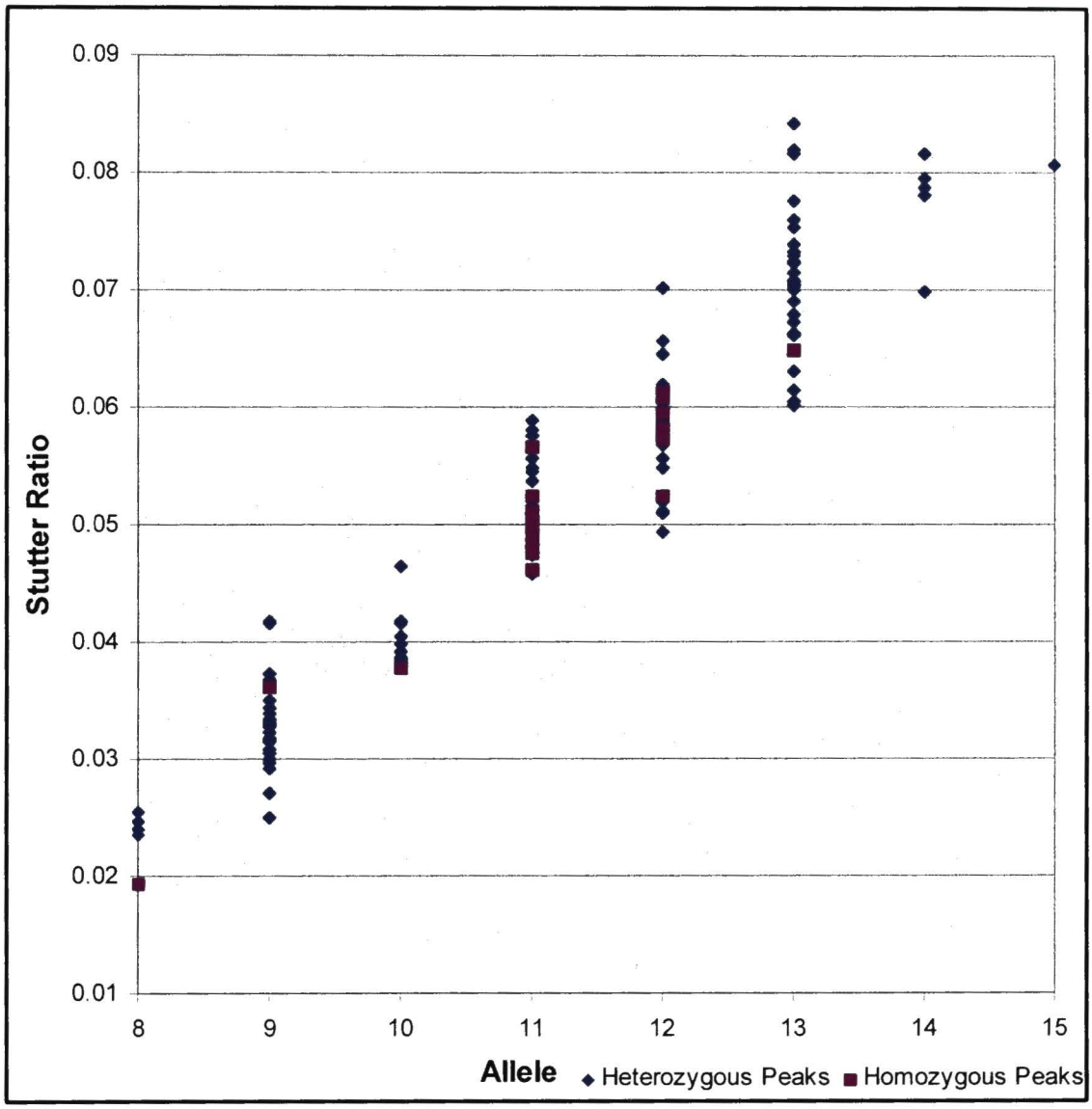
15
Allele
Heterozygous Peaks
Homozygous Peaks

D16S538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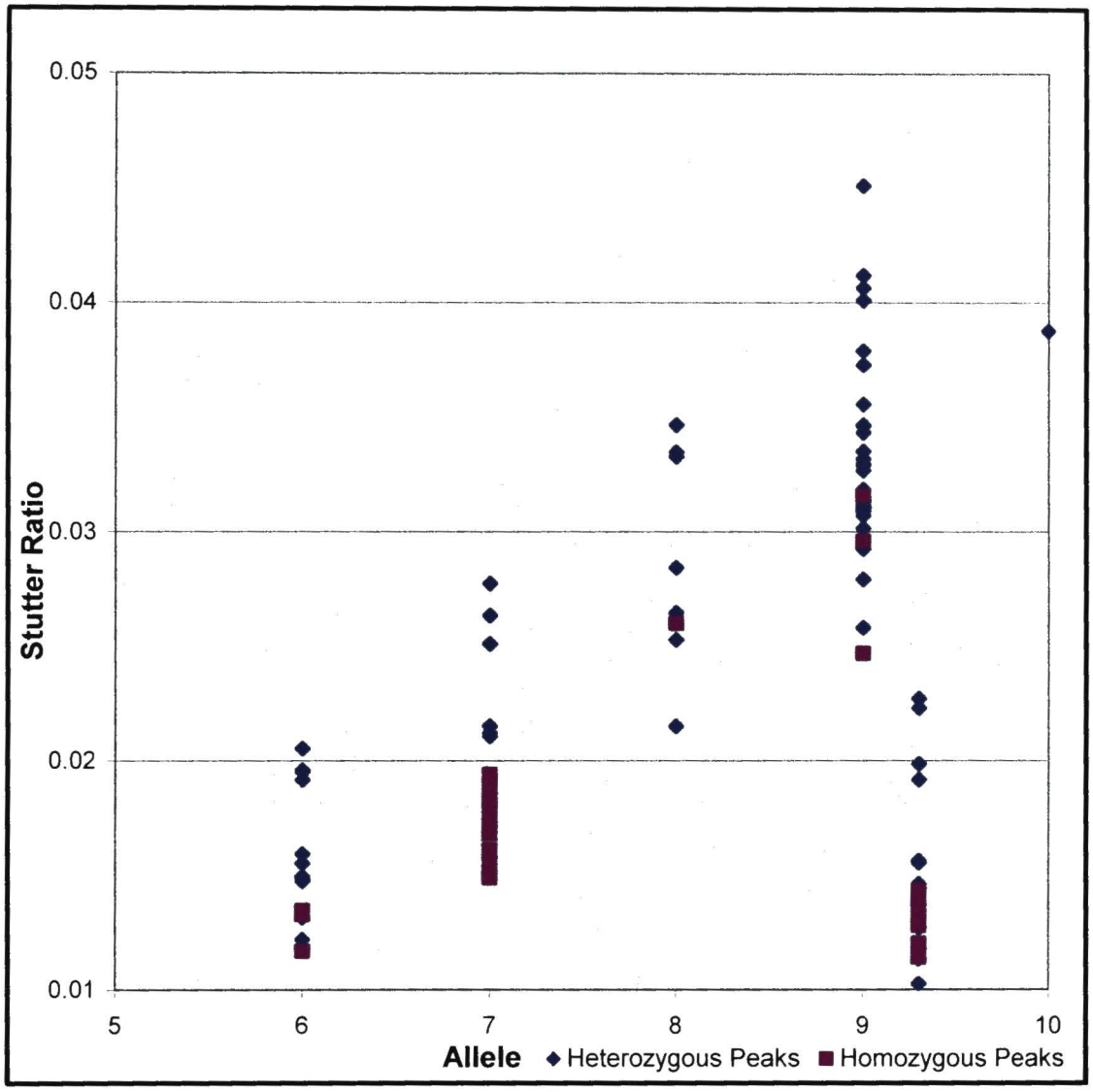
Allele
Heterozygous Peaks
Homozygous Peaks

TH01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Stutter Ratio
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7
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Allele
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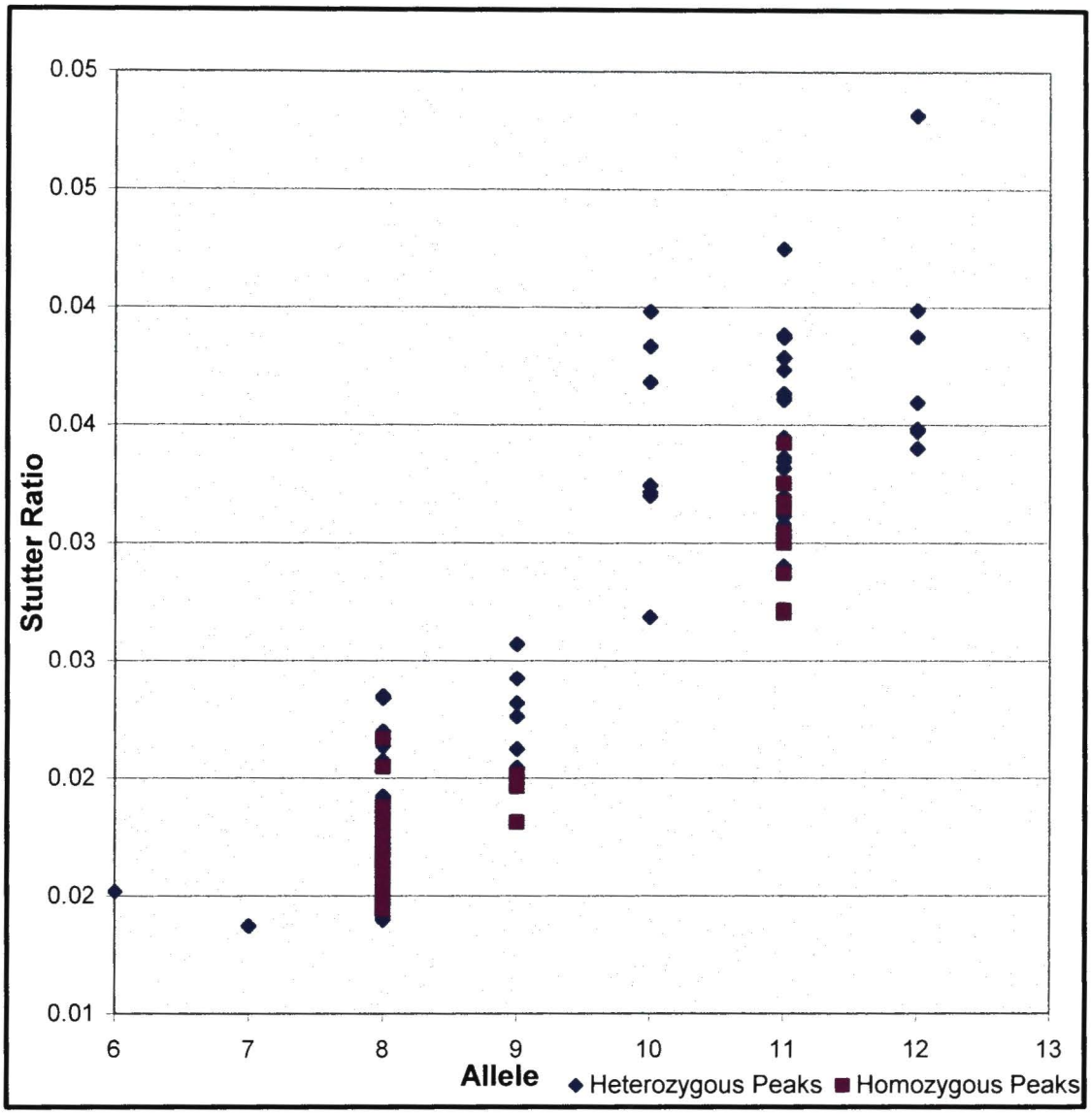
Homozygous Peaks

TPOX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0.02
0.01
Stutter Ratio
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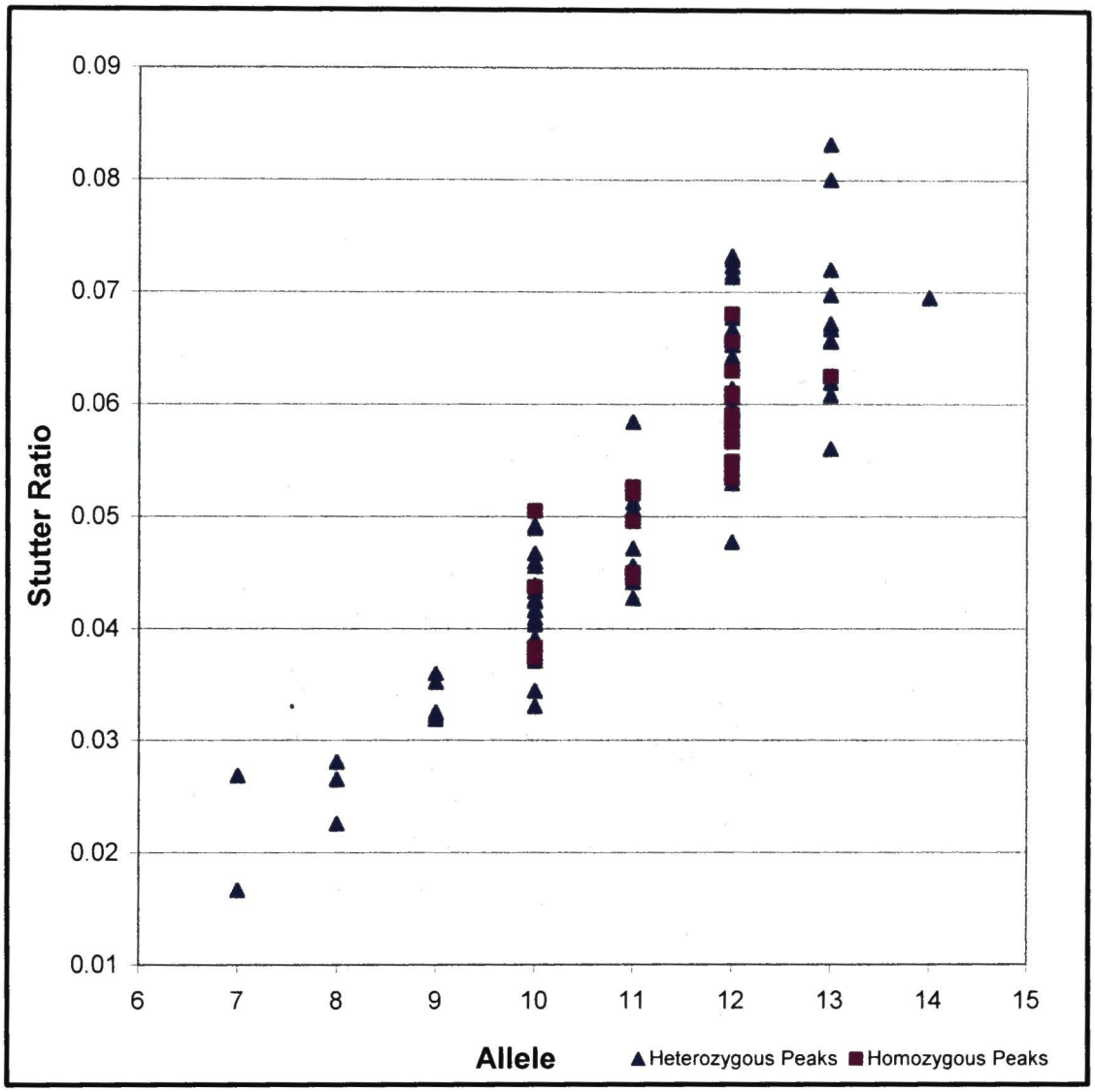
Allele
Heterozygous Peaks
Homozygous Peaks

CSF1PO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Stutter Ratio
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Allele
Heterozygous Peaks
Homozygous Peaks

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