Pentanucleotide Repeats:
Highly Polymorphic Genetic Markers Displaying Minimal Stutter Artifact

James W. Schumm, Jeffery W. Bacher, Laura F. Hennes, Trent Gu, Katherine A. Micka, Cynthia J. Sprecher, Ann M. Lins, Elizabeth A. Amiott, Dawn R. Rabbach, Jennifer A. Taylor, and Allan Tereba.
Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711 USA.

◇▷◁▷◁▷◁▷◁▷◁▷◁▷◁▷◁▷◁▷◁▷◁▷◁▷◁▷

INTRODUCTION

Short tandem repeat (STR) polymorphisms are becoming the standard genetic markers used throughout the world for development of forensic databases. The early work with polymorphisms based on dinucleotide repeats immediately revealed the presence of artifact bands, called stutter bands, which were associated with the PCR amplification of these loci. While the problem of stutter is much reduced with the implementation of current tetranucleotide repeat STRs, stutter bands are observed with current systems generally at levels of 2 – 15% of the total yield of amplification products. Additional reduction of the level of this artifact has been a goal of the forensic community since the first STR systems were identified. The motivation for this improvement is the simplification of interpreting results, especially in cases where mixtures of DNA, sometimes in unequal amounts, are present in sample material.

Our approach to achieving this goal has been the discovery and development of new genetic markers which offer a high degree of polymorphism, but do not reveal the typical level of stutter artifact displayed in existing systems. In reviewing the literature, we observed that the degree of stutter decreased considerably in tetranucleotide STR loci compared to dinucleotide repeat loci. We reasoned that genetic markers which contained longer repeats, e.g., pentanucleotide repeats, might display even less stutter. This work describes our search for pentanucleotide repeat markers with this characteristic, their isolation and initial characterization, and how they can be included into multiplex systems for efficient use in forensic science, paternity determination, and other forms of genetic analysis.

ISOLATION OF PENTANUCLEOTIDE REPEAT MARKERS

Two general approaches were employed to identify and isolate pentanucleotide markers. The first took advantage of the sequence information which has been generated and recorded by the Human Genome Project. There are 102 independent permutations of nucleotide sequences containing 5 nucleotides, once reverse complements (e.g., 5'-AAAAT-3' is the same as 5'-ATTTT-3' for searching purposes), similarities by shifting a single base in the sequence (e.g., AAAAT is the same as AAATA, AATAA, ATAAA, or TAAAA), and monomeric repeats (e.g., AAAAA) are removed. Each independent permutation containing at least four copies of the repeat sequence was used to screen the entire GenBank human database.

Once sequences containing pentanucleotide repeats were identified, this approach offered the efficiency of known flanking DNA sequence allowing us to design primers, amplify the corresponding candidate region from genomic DNA, and determine a crude value for heterozygosity at the locus. While dozens of candidate loci were identified, a more thorough search for loci was completed using a more directed method.

This second approach to isolate loci containing pentanucleotide repeats involved enrichment for these sequences using hybridization selection (Armor et al, 1994) and cloning them into bacterial hosts. This procedure is quickly summarized in Figures 1 and 2. In this approach, human genomic DNA samples were digested with the restriction endonuclease *MboI*. Resulting fragments ranging from 250 to 600 bp were size selected and ligated to linker DNAs which allowed us to amplify them, denature the amplified products, and hybridize them to small nylon filters each containing complementary pentanucleotide repeat sequences. Unhybridized material was removed in a washing step. The hybridized material was then released and collected during additional washing. This hybridized and released material, that was enriched for genomic sequences containing pentanucleotide repeats, was reamplified and cloned into a plasmid vector (i.e., pGEM-3Zf(+)) and transformed into competent *E. coli* cells (strain JM109).

The resulting transformants were identified by colony hybridization (Sambrook et al, 1989) using oligonucleotide probes containing pentanucleotide repeats. Bacterial cells which contained DNA leading to hybridization with the probes were visualized using an alkaline phosphatase label on the probe in conjunction with chemiluminescent detection (Bronstein and McGrath, 1989; Tizard et al,

1990). This approach produced many more pentanucleotide candidates than the search of GenBank, but required significantly more work to confirm each candidate including DNA purification and sequence determination for approximately 1500 candidate clones.

HETEROZYGOSITY AND MICROVARIANT ANALYSIS WITH PENTANUCLEOTIDE REPEAT MARKERS

Once DNA sequences of loci containing pentanucleotide repeats were determined, either through a Gen-Bank search or direct isolation and sequencing, primers were designed and employed to amplify each candidate locus. Following development of successful amplification parameters, pooled DNA, i.e., DNA isolated from 15 separate individuals and mixed together, was used to provide a preliminary indication of the number of alleles present at each locus. In most cases, only one or two amplification fragments were observed among the 15 individuals indicating a lack of significant polymorphism at the locus. However, some samples displayed three or more alleles at the tested locus (Figure 3).

Those pentanucleotide loci displaying four or more alleles were re-tested using individual DNA samples to provide a more accurate indication of the polymorphic content of the locus. For example, Figure 4 displays the DNA profiles of 24 African-American individuals following amplification, separation, and detection of alleles at the Penta D locus.

For those loci displaying a significant amount of polymorphism in the individual DNA screen, a more thorough population screen was performed in Caucasian-American, African-American, Hispanic-American, and Asian-American DNA samples. The inclusion of Asian-American samples in this early stage is crucial because some previously developed markers which show a high degree of heterozygosity in other groups have sometimes revealed less polymorphism in this group.

A collaboration between our group and the Bode Technology Group to evaluate at least 400 alleles in each of the four population groups is now nearly complete. The preliminary heterozygosity determinations for four specific pentanucleotide loci are shown in Table 1. At least 200 individuals were evaluated in each population. The pentanucleotide repeat loci being characterized are quite polymorphic. This high degree of polymorphism is illustrated by comparison with the heterozygosity of the least polymorphic (TPOX) and most polymorphic (FGA) loci selected as core STR loci for the CODIS database, and the most polymorphic locus (vWA) contained in the previously developed multiplex STR system, the *Gene-*

Print[TM] PowerPlex[TM] 1 System. The levels of heterozygosity for these three loci are also displayed in Table 1.

Previously, it has been observed that STR loci which are highly polymorphic often have correspondingly high numbers of undesirable microvariant alleles (i.e., alleles differing from one another by lengths other than the repeat length, Moeller et al, 1994, Rolf et al., 1997). The presence of microvariant alleles complicates separation, interpretation, and assignment of alleles. However, in genotype determination of the pentanucleotide loci, few or no microvariant alleles were revealed despite their highly polymorphic nature. The size range of alleles identified and any microvariants observed with a frequency of greater than one per thousand are listed in Table 2 for the Penta B, Penta C, Penta D, and Penta E loci.

CHROMOSOME LOCALIZATION OF PENTANUCLEOTIDE REPEAT LOCI

While the particular location of genetic markers does not have a significant impact on their use in general forensic analyses, any situation which requires paternity determination benefits from having independent inheritance of the markers by retaining the full discrimination power of each marker. Chromosome localization provides a preliminary test of this characteristic as markers which are genetically unlinked (or on different chromosomes) usually satisfy this criteria. Both physical and genetic means have been employed to localize many of the pentanucleotide loci to their positions on human chromosomes.

The physical approach we used to assign new loci to appropriate chromosome arms is called radiation hybrid mapping (Boehnke et al, 1991; Walter et al, 1994). A summary of this procedure is displayed in Figures 5 and 6. In this method human cells are X-irradiated. This breaks the chromosomes into fragments and kills the cells. As the human cells die, they are fused with rodent cells which contain their own complement of DNA. Different human DNA fragments, a genomic subset, are recovered in different rodent-human fusions. Independent fusions are purified and their DNA isolated.

Primers for candidate loci are used to amplify the DNA isolated from independently derived rodent-human fusions (Figure 6). Loci which are physically close to one another in the original human cells will be co-amplified from the same rodent-human fusion DNA template more often than those loci which are farther apart or randomly associated. A standard set of rodent-human fusions has been developed to allow different laboratories to compare results (Hudson et al, 1995; Gyapay et al, 1996). Using this set, we have been able

to correlate the physical locations not only with other new pentanucleotide markers, but also with many previously assigned STR markers.

The genetic approach to chromosome localization used in this study is linkage analysis. Basically, DNA samples from family members of large human pedigrees are generally available for this type of work. The inheritance of particular alleles is studied for each locus. If two loci are "closely linked", then alleles of each locus which are present together in a parent are inherited together more often in children. If loci are far apart or randomly associated (e.g., on different chromosomes), then regardless of the allele content of the particular two loci in the parent, the alleles are randomly associated in the children.

Work defining both physical and genetic inheritance of pentanucleotide markers has been performed in collaboration with Cindy Helms and Helen Donis-Keller at the University of Washington (St. Louis, MO). The preliminary assignments of several pentanucleotide loci to regions of particular chromosomes based on these experiments are consistent using both methods and are displayed in Table 2.

CHARACTERIZATION OF STUTTER IN PENTANUCLEOTIDE REPEAT LOCI

Five pentanucleotide loci (Penta A, Penta B, Penta C, Penta D, and Penta E) were selected for a controlled evaluation of stutter fragments associated with these loci. In each case, 1 ng of twenty separate DNA samples was amplified at the individual locus and the resulting products were separated and analyzed for peak heights of the main product and the stutter fragment product for each heterozygote allele using an ABI Prism[TM] 377 DNA Sequencer and GenoTyper[TM] software (Perkin Elmer Biosystems, Foster City, CA). The same procedure was employed in evaluation of several tetranucleotide repeat loci commonly used in forensic and paternity analyses. An example of the typical results observed for pentanucleotide and tetranucleotide loci is illustrated in Figure 7.

A summary of the average amount of stutter observed in a variety of loci is displayed in Figure 8. This characteristic varies according to the locus being evaluated[1]. Furthermore, there is a pronounced trend to lower amounts of stutter observed in pentanucleotide loci versus the previously developed systems. For selected tetranucleotide repeat loci, averages of 4 to 6% stutter are typical and in particular instances alleles may reveal more than 12% stutter. All of the evaluated pentanucleotide repeat loci displayed average stutter of less than 2%, with several loci less than 1%. The clean nature of

the amplification product is easily visualized for the Penta D locus in Figure 4 using the Hitachi FMBIO® II fluorescent scanner. Despite intense signal of the amplified authentic alleles, no stutter at any allele is visible in this image.

INTRODUCTION OF PENTANUCLEOTIDE REPEAT MARKERS INTO MULTIPLEX STR SYSTEMS

The combined properties of high power of discrimination, few microvariants and very low levels of stutter make pentanucleotide STR loci ideal markers for forensic DNA analyses. Efforts are currently in progress to incorporate the best pentanucleotide repeat loci into STR multiplex systems. In particular, the *GenePrint*[TM] Power-Plex[TM] 2 System and the *GenePrint*[TM] PowerPlex[TM] 16 System will contain pentanucleotide repeat loci.

A prototype of the PowerPlex[TM] 2 System has been developed. It allows co-amplification of 9 STR loci (including all the INTERPOL loci selected for pan-European use and all the SGM loci developed by the Forensic Science Service, United Kingdom) plus Amelogenin, a gender identification locus. One of these loci is Penta E. The multiplex system design is displayed in Figure 9. Five STR loci are labeled with fluorescein and four STR loci plus Amelogenin are labeled with carboxy-tetramethylrhodamine (TMR). The amplified product may be detected in combination with an internal lane standard with evenly space fragments labeled in a third dye, carboxy-X-rhodamine (CXR).

A prototype of the PowerPlex[TM] 16 System allows co-amplification of 15 STR loci (including all of the CODIS 13 core STR loci) plus Amelogenin, the gender identification locus. This multiplex system design is displayed in Figure 10. In addition to all the loci present in the same configuration and the same dye content as the PowerPlex[TM] 2 System, six additional loci are present in a new dye[2]. Two pentanucleotide loci, Penta E and Penta D, are included in this system.

An example of the output of a single amplification reaction containing primers for the 16 loci of the Power-Plex[TM] 16 System prototype is displayed in Figure 12. This prototype of the system is displayed in three fluorescent colors for the polymorphic loci and a fourth color for the internal lane standard (i.e, size marker). Performance of both the PowerPlex[TM] 2 and PowerPlex[TM] 16 Systems is more fully described in work presented at this symposium by Lins et al, (1998). These multiplex systems have been designed for optimum performance in demanding situations of forensic analysis and paternity determination. Amplification of so many loci simulta-

neously allows use of minimal amounts of sample material as well as efficient sample throughput. In addition, with a single amplification reaction, there is less chance of sample mix-up from multiple separate amplification reactions.

Compatibility with use of degraded sample material has also been incorporated into the designs. For example, in the PowerPlex[TM] 16 System, twelve CODIS STR loci generate amplification products completely below 372 bases (Figure 11). Only the rarer alleles of the FGA locus (less than 2% of the observed FGA alleles) are larger and these are all separated from one another by four bases allowing their easy assignment. Eight of the CODIS STR loci and five of the SGM loci produce amplification products completely contained below 261 bases, even for all rare alleles.

Additional power of discrimination is achieved by incorporation of the Penta E and Penta D loci. With the paucity of microvariants observed with these systems, the Penta E and Penta D alleles are all separated from one another by 5 bases except in extremely rare circumstances (less than 1 in 500 allele calls). This allows easy and confident allele separation and determination. The matching probability and power of exclusion for both multiplex systems are summarized in Table 3. The corresponding values for the Powerplex[TM] 1 and FFFL Systems (Lins et al, 1998) are included for comparison. We are continuing to collaborate with the Bode Technology Group to evaluate additional population characteristics such as final genotype frequencies and Hardy-Weinberg Equilibrium calculations (Weir, 1996) of independence of all 19 STR loci represented in the FFFL and PowerPlex[TM] 16 Systems.

Footnotes:

[1]Repeat size is not the only factor affecting the amount of stutter found at a particular locus. Other major factors include the total number of repeats present within the allele (i.e., larger alleles tend to display more stutter) and whether the repeat is interrupted (i.e., STR loci containing one or more repeat units having a different DNA sequence than core repeat unit generally display less stutter) (Walsh et al, 1996). The two complementary strands of an amplification product may also reveal different amounts of stutter (data not shown). However, these factors do not change the significance of the effects of repeat length described in this work.

[2]The final selection of the dye to be used for this purpose has not been determined (December, 1998) despite progress with the existing prototype.

REFERENCES

1. Armor, J., Neumann, R., Gobert, S., and Jeffreys, A.J. (1994). Isolation of human simple repeat loci by hybridization selection. Hum Mol Genet 3(4): 599-605.

2. Boehnke, M., Lange, K., and Cox, D.R. (1991). Statistical methods for multipoint radiation hybrid mapping. Am J Hum Genet 49: 1174-1188

3. Bronstein, I., and McGrath, P. (1989). Chemiluminescence lights up. Nature 338: 599-600.

4. Gyapay G., Schmitt, K., Fizames, C., Jones, H., Vega-Czarny, N., Spillett, D., Muselet, D., Prud'homme, J., Dib, Cl, Auffray, C., Morissette, J., Wiessenbach, J., and Goodfellow, P.N. 1996. A radiation hybrid map of the human genome. Hum Mol Genet 5(3): 339-346.

5. Hudson T.J., et al. (1995). An STS-based map of the human genome. 59 (4 SUPPL.): pA26

6. Lins, A.M., Micka, K.A., Sprecher, C.J., Taylor, J.A., Bacher, J.W., Rabbach, D.R. Bever, R.A., Creacy, S.D., and Schumm, J.W. (1998) Development and Population Study of an Eight Locus Short Tandem Repeat (STR) Multiplex System. Journal of Forensic Sciences 43: 1178-1190.

7. Lins, A.M., Sprecher, C.J., Micka, K.A., Rabbach, D.R., Taylor, J.A., Bacher, J.W., Hennes, L.F., Tereba, A., Gu, T., Amiott, E.A., Nassif, N.A., and Schumm, J.W. (In Press). The evolution of short tandem repeat (STR) multiplex systems. In: Proceedings from the Second European Symposium on Human Identification 1998. Innsbruck, Austria: Promega Corporation.

8. Moeller A, Meyer E, Brinkmann B. (1994) Different types of structural variation in STRs: HumFES/FPS, HumVWA and HumD21S11. Int J Leg Med 106: 319-323.

9. Rolf, B., Schuerenkamp, M., Junge, A., and Brinkmann, B. (1997). Sequence polymorphism at the tetranucleotide repeat of the human beta-actin related pseudogene H-beta-psi-2 (ACTBP2) locus. Int J Leg Med 110: 69-72.

10. Sambrook J, Fritsch EF, Maniatis T (eds.) (1989) Molecular cloning - A laboratory manual. 2nd edition, Cold Spring Harbor Laboratory Press. pp. 1.90-1.104.

11. Tizard, R. Cate., R.L., Ramachandran, L.K., Wysk, M., Voyta, J.C., Murphy, O.J., and Bronstein, I. (1990). Imaging of DNA sequences with chemiluminescence. Proc Natl Acad Sci, U.S.A. 87: 4514-4518.

12. Walsh, P.S., Fildes, N.J., and Teynolds, R. (1996). Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. Nucl. Acids Res. 24 (14): 2807-2812.

13. Walter, M.A., Spillett, D.J., Thomas, P., Weissenbach, and Goodfellow, P. (1994). A method for constructing radiation hybrid maps of whole genomes. Nature Genet 7: 22-28.

14. Weir, B.S. Genetic data analysis II. Sunderland: Sinauer Associates, Inc., 1996.

Table 1

Locus	Fraction of Heterozygotes			
	African-American	Asian-American	Caucasian-American	Hispanic-American
Penta B	.74	.84	.85	.79
Penta C	.73	.77	.75	.80
Penta D	.88	.75	.86	.84
Penta E	.83	.90	.88	.83
TPOX	.75	--	.65	.67
vWA	.83	--	.82	.75
FGA	.85	.92	.87	.89

Table 2

Locus	Allele Range (Repeat Numbers)	Microvariants (Frequency >0.001)	Chromosome Location
Penta B	5-31	NONE	7q
Penta C	3-15	NONE	9p
Penta D	2.2-17	2.2*,3.2*,13.3	21q
Penta E	5-24	NONE	15q

*Sequence analysis indicates that the Penta D allele 2.2 and Penta D allele 3.2 contain an identical five base deletion outside the repeat region. The next larger known allele is allele 5. Therefore, Penta D allele 13.3 is the only allele which differs from other alleles by less than 5 bases.

Table 3

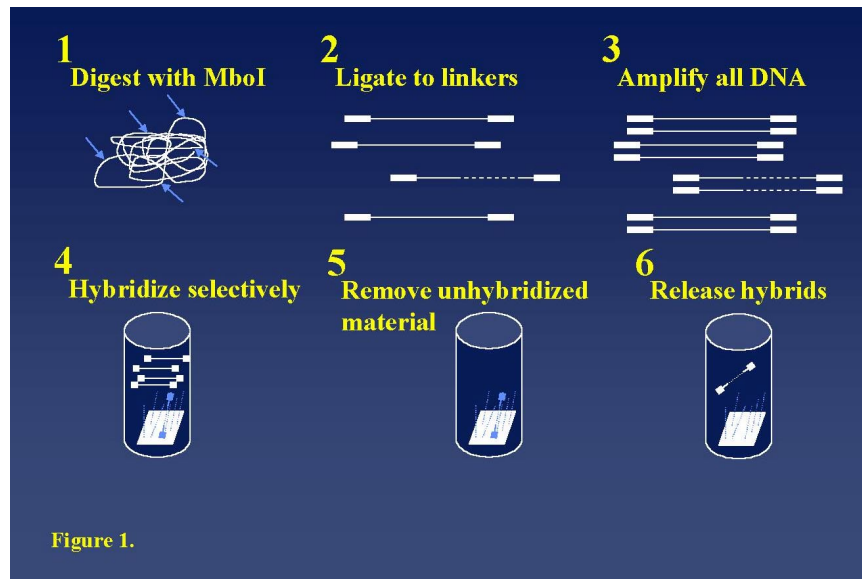|	Matching Probabilities		
	African-American	Caucasian-American	Hispanic-American
FFFL System	1.68×10^4	2.66×10^3	3.28×10^3
PowerPlex^{TM} 1 System	2.74×10^8	1.14×10^8	1.45×10^8
PowerPlex^{TM} 2 System	3.00×10^{11}	8.46×10^{10}	1.02×10^{11}
PowerPlex^{TM} 16 System	1.40×10^{18}	1.83×10^{17}	2.94×10^{17}
	Power of Exclusion		
	African-American	Caucasian-American	Hispanic-American
FFFL System	0.9459	0.9406	0.9019
PowerPlex^{TM} 1 System	0.9982125	0.9968853	0.9973337
PowerPlex^{TM} 2 System	0.9999219	0.9999242	0.9997134
PowerPlex^{TM} 16 System	0.9999996	0.9999994	0.9999983

Figure 1. Hybridization Selection. Genomic sequences containing pentanucleotide repeats were enriched using hybridization selection which employed the following steps.

1) Human genomic DNA was digested with restriction endonuclease *Mbo* I.
2) DNA linkers were ligated to the ends of the digested DNA.
3) Primers were used to amplified the materials generated in step (2)
4) Amplified materials were hybridized to filters with immobilized pentanucleotide repeat sequences.
5) Unhybridized materials were washed away.
6) Enriched hybridized materials were released at increased temperature.

Figure 2. Cloning of materials enriched by hybridization selection.

7) Hybridization selected materials were reamplified and cloned into the plasmid vector, pGEM-3Zf(+)
8) Cloned fragments were transformed into competent *E. coli* JM109 cells
9) Colonies containing pentanucleotide sequences were identified by hybridization with an alkaline phosphatase-labeled probe containing pentanucleotide sequences.

Using Pooled DNA to Estimate Number of Alleles

**Pooled DNA
54 CEPH Individuals**

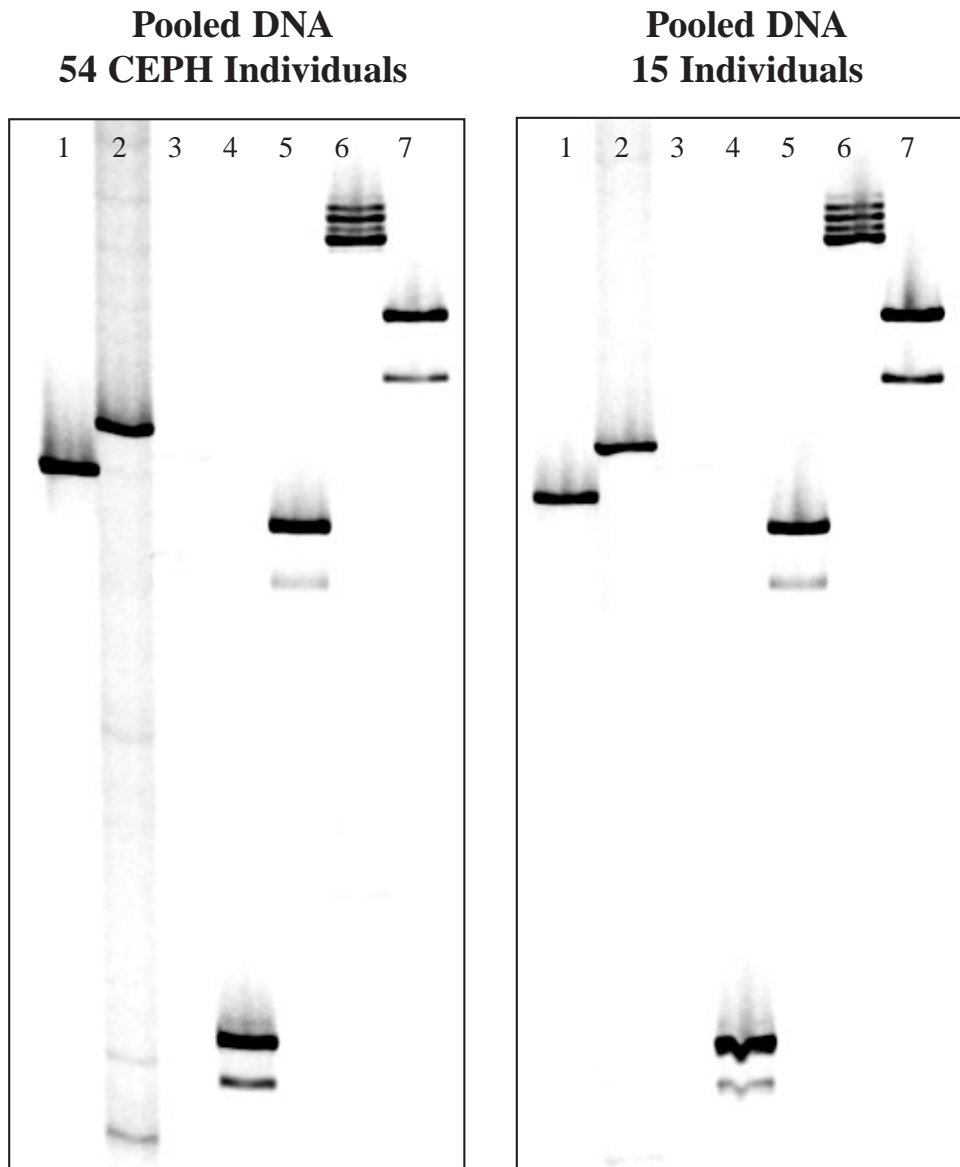**Pooled DNA
15 Individuals**

Figure 3. Amplification analysis of pooled DNA samples. Individual primer pairs were used to amplify a mixture of 15 DNA samples at one locus in a single reaction. Amplification products were separated using a 4% denaturing polyacrylamide gel and detected using the Hitachi FMBIO® II fluorescent scanner.
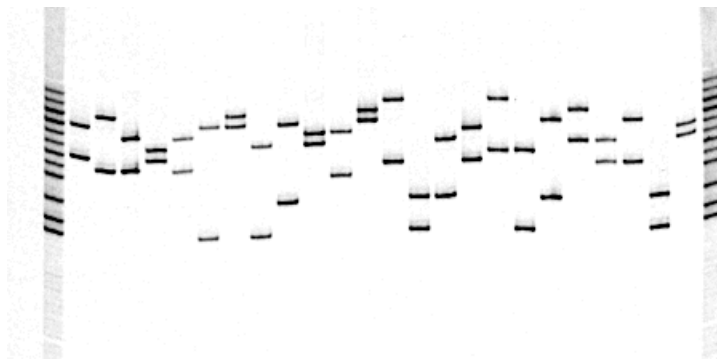
Figure 4. Amplification of Penta D using individual DNA samples. Twenty-four separate DNA samples were amplified using primer pairs for the Penta D locus. Amplification products were separated using a 4% denaturing polyacrylamide gel and detected using the Hitachi FMBIO® II fluorescent scanner. The first and last lanes include pooled (P) DNA samples.

Figure 5. Radiation hybrid analysis. Human cells are X-irradiated which break chromosomes into fragments containing different human loci (indicated by numbers). As the cells die, they are fused with healthy rodent cells containing their own complement of DNA. Resulting purified rodent-human hybrid cells contain different fragments of human DNA. The hybrid products are isolated and grown in culture.
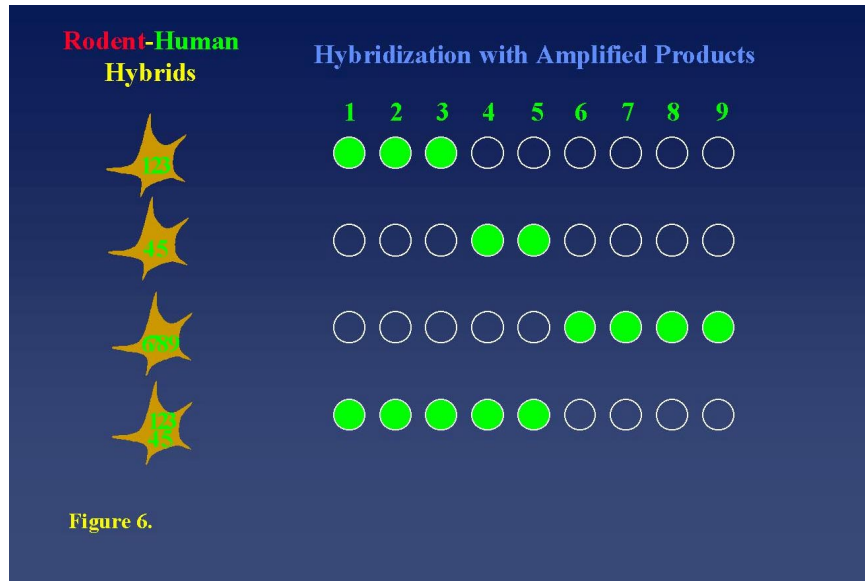
Figure 6.

Figure 6. Radiation hybrid analysis (continued from Figure 5). Purified DNA samples from a collection of rodent-human hybrid cells are used as amplification templates for primers to specific loci (indicated by the numbers 1 through 9). Examples of successful amplification are indicated by filled circles. Loci which are physically close to one another on the original human chromosome fragments (such as 1, 2, and 3 in this example) are more likely to amplify using the same rodent-human hybrid DNA templates (first and fourth row of filled circles for loci 1, 2, and 3 in this example). Those loci which come from different chromosomes or are distantly located on the same chromosome will amplify with same template less frequently (e.g., co-association of 1 and 4 is rare and of 1 and 6 does not occur in this example).
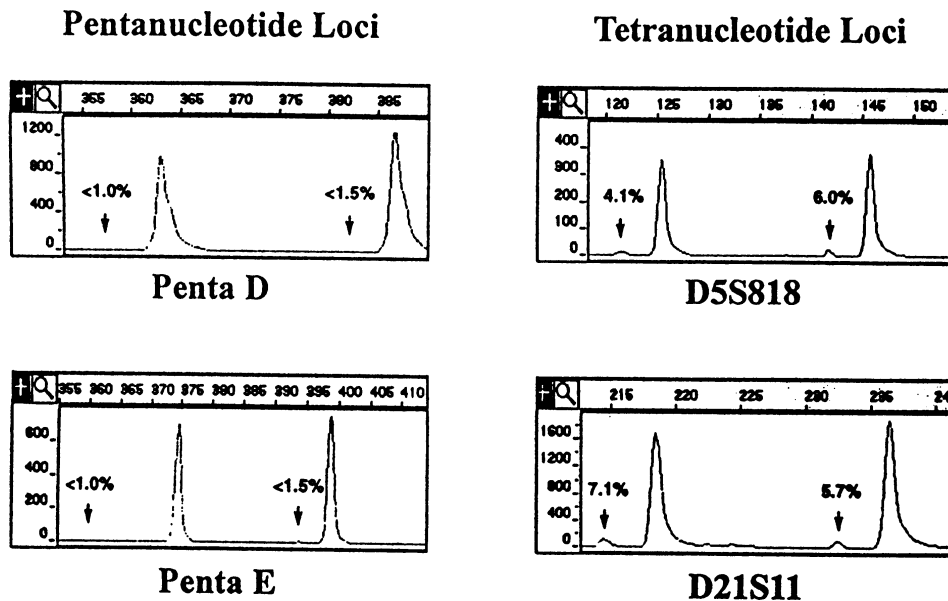
Pentanucleotide Loci   ## Tetranucleotide Loci

Penta D

D5S818

Penta E

D21S11

Figure 7. Stutter determination. Amplification products from individual pentanucleotide loci were separated and detected with the ABI Prism^{TM} 377 DNA Sequencer. Peaks representing amplification products of four different loci are displayed. Arrows indicate the amount of stuter observed in each case. The peak height of each allele for twenty heterozygous DNA samples was determined for each predominant fragment and the associated stutter peak (one repeat length smaller). Percentage stutter was determined by dividing the peak height of the stutter peak by the peak height of the predominant peak and multiplying by 100.
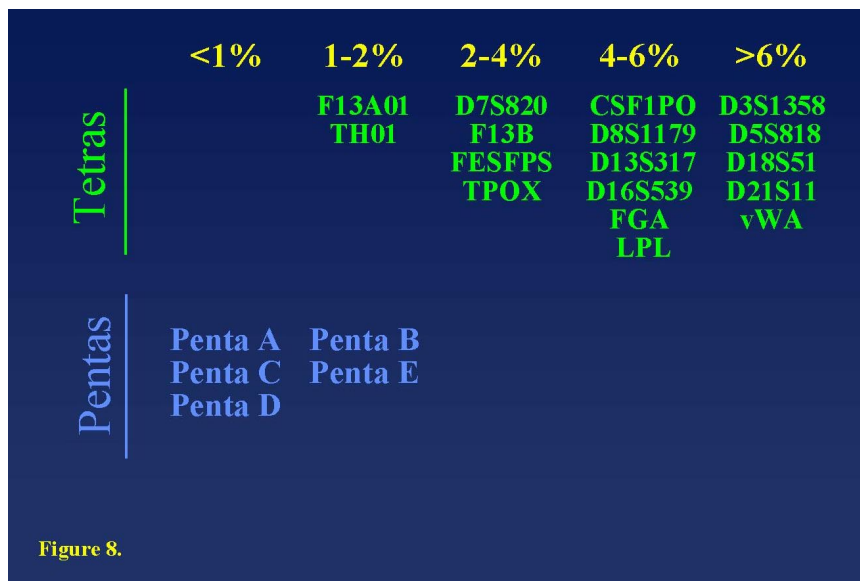
	<1%	1-2%	2-4%	4-6%	>6%
Tetras		F13A01 TH01	D7S820 F13B FESFPS TPOX	CSF1PO D8S1179 D13S317 D16S539 FGA LPL	D3S1358 D5S818 D18S51 D21S11 vWA
Pentas	Penta A Penta C Penta D	Penta B Penta E			

Figure 8.

Figure 8. Average stutter frequencies for several STR loci. The stutter contribution from each allele (determined as described in the figure legend to Figure 7) was used to calculate the average stutter for each locus. A summary of the data for all tested loci is presented. Loci are listed below their approximate average stutter defined by analysis of twenty DNA samples. Tetranucleotide loci are shown in the top portion of the figure and several pentanucleotide loci are displayed in the lower portion of the figure to allow easy visualization of the lower stutter contribution of the pentanucleotide loci.
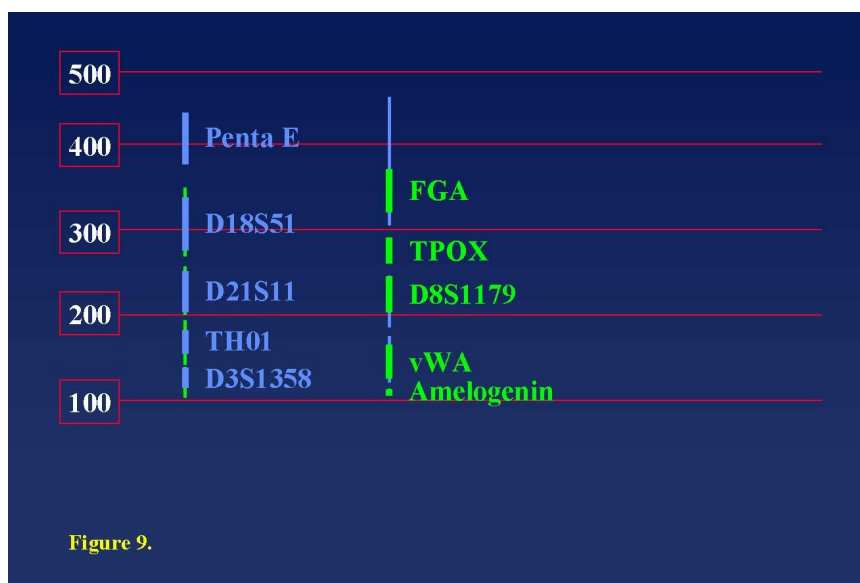

Figure 9.

Figure 9. Locus configuration of the PowerPlex^{TM} 2 System. Each locus is displayed using thick lines to represent common alleles and thin lines to indicate rare alleles. The approximate size ranges of alleles are indicated by comparison with the lines labeled as multiples of 100 bases. Loci in the first column are labeled with fluorescein, while those in the second column are labeled with carboxy-tetramethyl-rhodamine (TMR). (Information regarding rare alleles was a generous contribution of Rebecca Sparkes, Forensic Science Service, personal communication. Not as many samples have been analyzed and described for the TPOX and Penta E loci. Thus, there are no thin lines indicating very rare alleles which may be associated with these loci.). The internal lane standard is labeled with a third dye, carboxy-X-rhodamine (CXR). The pentanucleotide locus, Penta E, is included in this system.
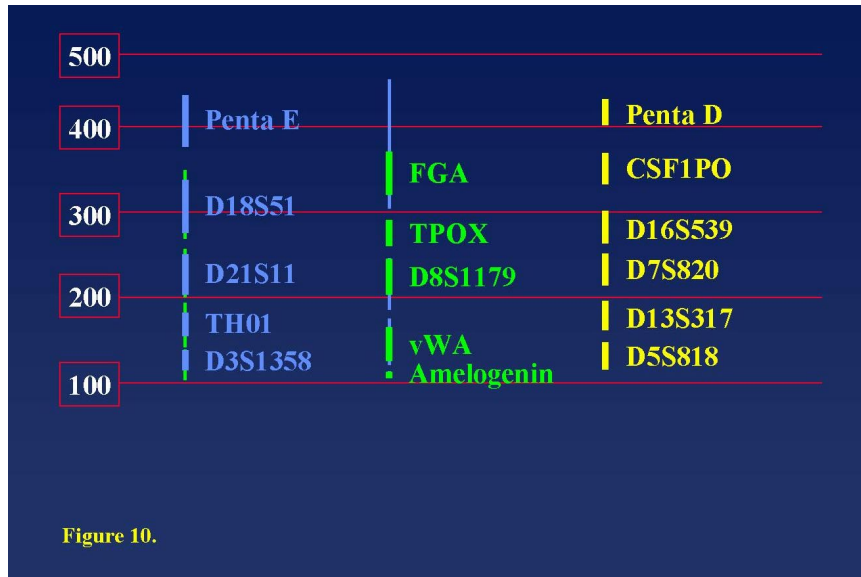
Figure 10. Locus configuration of the PowerPlex[TM] 16 System. Loci are represented as described in Figure 9. Six additional loci, shown in the rightmost column, are labeled with a third dye. The pentanucleotide loci, Penta E and Penta D, are included in this system. (Not as many samples have been analyzed and described for the loci in the rightmost column. Thus, there are no thin lines indicating very rare alleles which may be associated with these loci.)
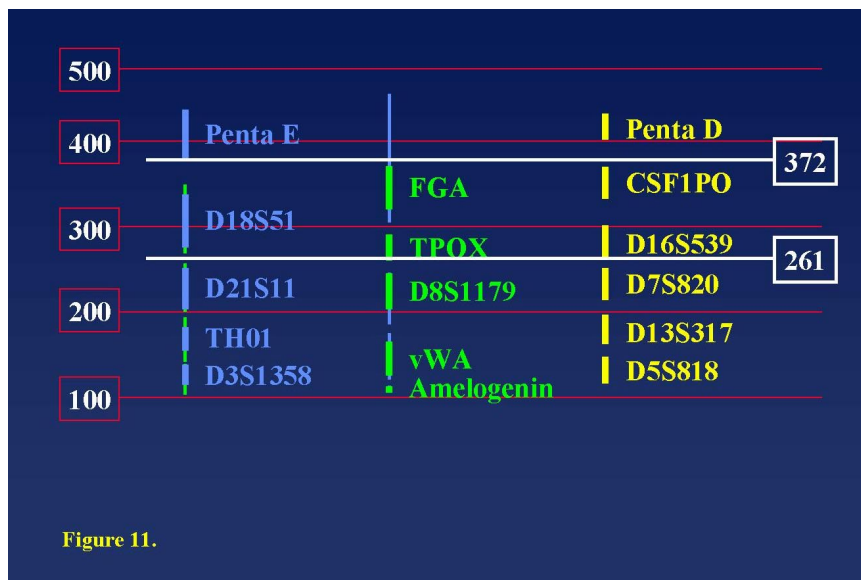

Figure 11. Inclusion of small alleles in configuration of the PowerPlex[TM] 2 and PowerPlex[TM] 16 Systems. In the PowerPlex[TM] 16 System, twelve CODIS STR loci generate amplification products completely below 372 bases. Only the rare FGA alleles (less than 2% of those observed) are larger and these are all separated from one another by four bases allowing easy assignment. Many of the Penta E and Penta D alleles, all separated from one another by 5 bases except for extremely rare microvariants (1 in 800 or less), are included above 372 bases. Eight of the CODIS STR loci produce amplification products completely contained below 261 bases, even for rare alleles. The PowerPlex[TM] 2 System has been designed with similar focus on small amplification product length providing compatibility use of degraded DNA samples.
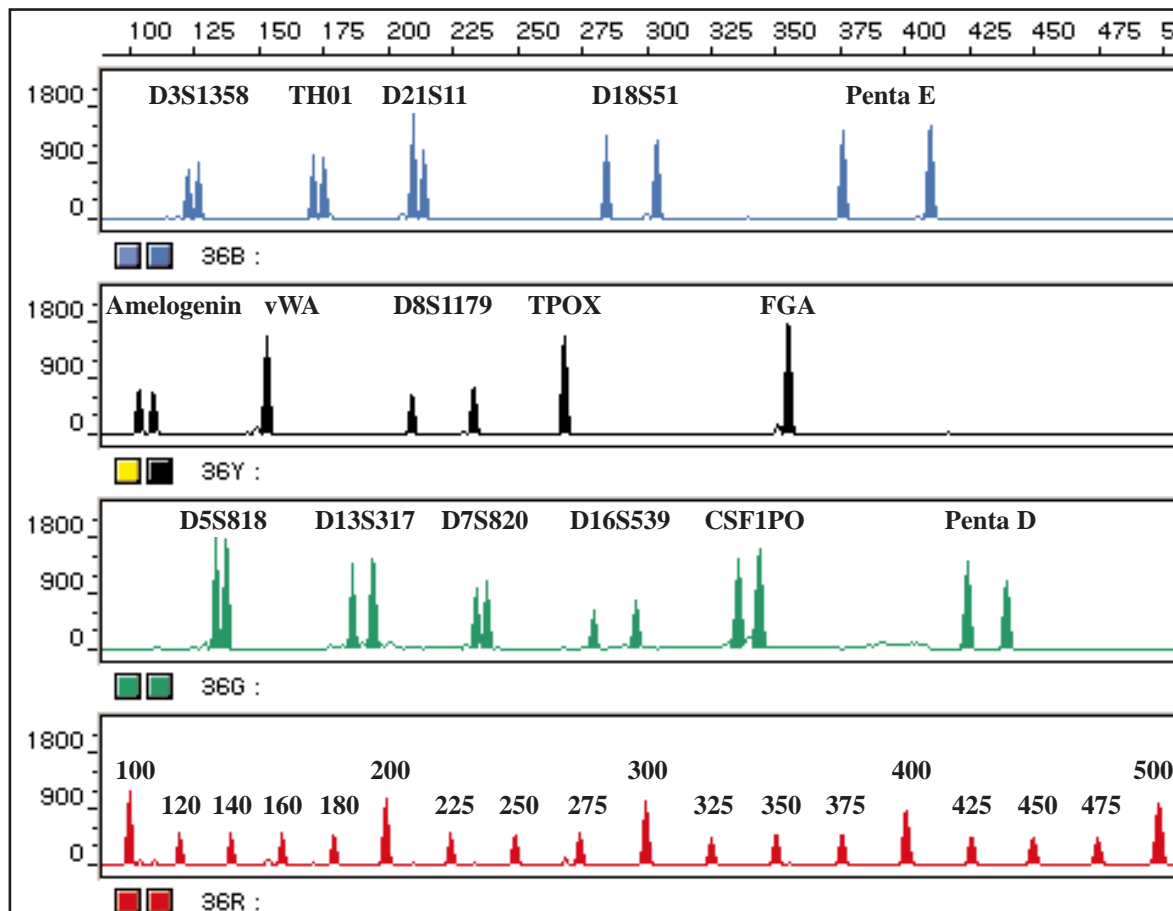
Figure 12. Prototype of the PowerPlex^{TM} 16 System. All 16 loci amplified in a single amplification reaction were analyzed in a single capillary using the ABI PRISM® 310 Genetic Analyzer. The fluorescein-labeled loci (D3S1358, TH01, D21S11, D18S51, and Penta E) are displayed in blue, the TMR-labeled loci (Amelogenin, vWA, D8S1179, TPOX, and FGA) in black, and the R6G-labeleled loci of this prototype (D5S818, D13S317, D7S820, D16S539, CSF1PO, and Penta D) are displayed in green. The fragments of the prototype ILS-500 size marker are labeled with CXR and are shown in red.