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IP-1
BIOTHREATS AND BIOCRIMES –
APPLYING MICROBIAL GENETICS AND FORENSICS ANALYSESD
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A number of bacteria, viruses, and fungi pose serious health risks to humans, animals, and plants, and the use of them as bioweapons can have serious consequences on human health, economic development, social stability, and political activities of nations worldwide. Terrorism can be defined as an attack or threat of an attack on the innocent to create fear, to intimidate, to inflict harm, and/or affect economic well-being. These acts have often been politically motivated, but may not always be so. The use of pathogenic agents as weapons has been documented for more than two millennia using crude to sophisticated approaches.
The potential of biological weapons being used is greater than any other time in history. The technology is attainable by individuals or small groups of individuals rather than just state-sponsored institutions. Further exacerbating the threat of use of bioweapons is that an attack can be easy to carry out and difficult to prevent. The anthrax letters attack of 2001 has brought to the forefront the need to enhance our capabilities for forensic attribution. To effectively combat bioterrorism and biocrimes the field of Microbial Forensics has been developed.
Microbial Forensics can be defined as a scientific discipline dedicated to analyzing evidence from a bioterrorism act, biocrime, or inadvertent microorganism/toxin release for attribution and deterrence purposes. Building a robust microbial forensics program is a challenging task; there are many aspects to consider, and because of the dynamic nature of molecular biology, it is a continuously evolving process. Unique identification of a microorganism may never be possible, because of the clonal nature of many microorganisms and, on a case-by-case basis, lack of population and phylogenetic data. The ultimate goal of attribution, however, is identification of the persons who committed the bioterrorist act or biocrime, intentionally or inadvertently. Therefore, traditional analyses, as well as microbiological and matrix analyses will be employed in investigations. Examples of biocrimes show how epidemiologic and genetic tools assist in differentiating between intentional and natural outbreaks. New high resolution techniques (e.g., electrospray ionization mass spectrometry) are becoming available that enable enhanced capabilities and have applications as well for human identification. Evidence interpretation issues specific to microorganisms are considered to include clonality, diversity, and population dynamics.
Lastly, a Scientific Working Group on Microbial Genetics and Forensics (SWGMGF) has been established to orchestrate the tasks required to establish a national microbial forensics program. These include: quality assurance and control guidelines, criteria for a national strain repository, criteria for a microbiology database, criteria for focus of research efforts, validation criteria, non-DNA based assays (e.g., immunoassays, isotopes, host response, background incidentals, matrix components), and population genetics. The current deliberations of the SWGMGF will be presented.

IP-2
DNA IDENTIFICATION FROM EXTREME SAMPLES
SUCH AS SINGLE CELLS AND DIFFICULT SAMPLES:
USES INCLUDING FORENSICS
AND COUNTER-TERRORIST APPLICATIONS.
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DNA identification by STR profiling is an extremely powerful method for forensic identification with power of discrimination in excess of 1 in 10 billion. Although several commercial profiling systems have become established and are extremely powerful, they require many hundreds of cells to maintain the necessary high rates of reliability and accuracy. STR profiling systems have also been applied to low cell samples such as cigarette butts and from cells left on pens, car keys, etc. However, these systems either still require similarly large amounts of DNA for high reliability, or as cell numbers decrease have fewer STR markers therefore markedly decreased discriminating power and reliability. In 1997, the first forensic identification of single cells was published1. These techniques have now been optimised and improved to provide:

1. Higher accuracy – 10 billion to 1 from single cells
2. Demonstrated use on forensic samples such as clothing, paper etc
3. Improved processing to several thousand samples per day. Our recent DNA analysis software can now current process in excess of 7 million genotypes per day.

These techniques are therefore currently being applied to a variety of difficult forensic samples including isolation and forensic profiling of:

1. Single sperm from rape cases, particularly rape with multiple assailants;
2. Single cells from samples which have too few cells for conventional profiling;

3. Single cells from samples contaminated by blood or other cell types;
4. Single cells from archived cases.

The forensic analysis of difficult samples, such as single cells, can also be undertaken using SNPs (single nucleotide polymorphisms) and mitochondrial analysis.
The application of these analysis techniques for counter-terrorist purposes such as document security and anti-counterfeiting will also be discussed.

References:

1. Findlay I., Frazier R., Taylor A, Quirke P., Urquhart A. Single cell DNA fingerprinting for forensic applications. Nature 389, 555-556.

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IP-3

STR PROFILING STRATEGY AND THE DEVELOPMENT OF NATIONAL DNA DATABASES IN EUROPE – A SUCCESS STORY

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The introduction of multiplex PCR systems has greatly facilitated the rapid typing of samples and computer-based storage of results in large DNA profile databases. Since 1995, more and more European countries have introduced national DNA databases for storing DNA profiles from unsolved crimes and known offenders.
The existing databases have been created within the framework of the respective national legal systems. Therefore, an obvious heterogeneity exists among the European DNA database systems regarding the catalogue of offenses leading to a database entry of an offender's DNA profile, and the criteria for a removal of an entry, the procedure for obtaining reference samples, the possiblity of long-term storage of these samples, as well as the criteria for database searches. Fortunately, significant progress has been made regarding the harmonization of typing systems within Europe. The European DNA Profiling (EDNAP) Group has initiated a series of scientific collaborative exercises for the evaluation of new DNA typing methods and systems. As a result, a number of loci were recommended suitable as common European systems. To coordinate and standardize operational processes of DNA typing in casework within the police laboratories as well as in the laboratories providing this service for the police, the DNA Working Group of the European Network of Forensic Science Institutes (ENFSI) has been founded four years ago. Based on the initial EDNAP exercises, and on recommendations by ENFSI, seven systems have been defined as the European standard set of loci – THO1, VWA, D21S11, FGA, D3S1358, D8S1179 and D18S51 (ESS loci). Furthermore, the Interpol DNA Monitoring Expert Group has adopted these loci as Interpol Standard Set of Loci (ISSOL). These loci now form the core of all existing criminal DNA databases in Europe, and will serve as a "common language" to facilitate the exchange of DNA profiles across national borders. The growing number of database records has led to a steadily increasing number of database hits helping to solve crime cases. From existing databases it becomes evident that the number of hits strongly depends on the rules for entering offender profiles. This has led to recent changes in database legislation in some European countries.

IP-4

THE FALLIBLE MTDNA DATABASES

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Under certain circumstances mitochondrial DNA may be the molecule of choice for forensic purposes. The key questions concerning a mtDNA sample taken from a victim or suspect are that of identity and geographic or ethnic affiliation. In order to be able to provide answers in the form of match probabilities, mtDNA databases of numerous populations are required for comparison. However, sampling and sequencing strategies, sample handling, and the sequencing process itself were notoriously poor and flawed, as testified by numerous publications in leading forensic journals. Databases typically refer to either ”race” (e.g. ”Hispanic”, ”Caucasian” etc.) or nationality, neither of which is meaningful or relevant for the origin of matrilines. Often ”random” sampling is alluded to, which in reality means that sampling was performed in ignorance of recent immigration history (and resulting social stratification) and regional differences of (former) ethnic groups. Sample sizes are rather small (<250) and sequencing barely goes beyond the popular first and second hypervariable segments of the control region.
There is no reason to keep forensic databases separate from those in the fields of human genetics and molecular anthropology. Any claim of ”high quality” of forensic databases in contrast to other databases should be received with much scepticism. The analysis of complete mtDNA sequences has now revealed that one cannot guarantee that mtDNA databases are error free. Although the latter should be the goal, ”it is not practical, and it is probably not technically feasible” – as Corinna Herrnstadt and her colleagues have recently stated. It would thus be helpful for workers to be open to the possibility of error and to maintain database errata for their work. When forensic databases are not publicly accessible sequence by sequence for critically examination, then we have to count with errors that are simply hidden away. There are various ways to examine the quality of a data set a posteriori, which are based on phylogenetic analysis, the knowledge about the positional mutation rate spectrum, and an increasing amount of published mtDNA information, the ultimate reference being the emerging worldwide phylogenetic tree of complete mtDNA sequences.
Thus, in the analysis of mtDNA in a forensic case, one is confronted with data sets for comparison that greatly differ in quality and information content. Consequently, providing a single match probability is inadequate in view of this complex fuzzy context.

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IP-5

SNP TYPING FOR FORENSIC APPLICATIONS

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The SNPs represent the most abundant form of genomic sequence variation (one SNP every 200-300 bp), they have low mutation rates, and are suitable for analysis using high throughput technologies. All these characteristics make these markers very useful for a variety of different applications such as forensic genetics, anthropology, clinical genetics, and pharmacogenomics and pharmacogenetics.
Approximately 50-70 autosomal SNPs are needed to give a discrimination power that is equivalent to short tandem repeat multiplexes that are currently in use for forensic applications.
Whether SNPs will replace STRs as the primary method of choice is a matter of conjecture at present but they have some promising charac-

teristics: The potential of massive throughput may result in economic advantages specially for large criminal DNA databases. Also, SNPs are better able to analyze highly degraded DNA because the distance between primer binding sites can be designed very short. In addition SNPs are also promising in paternity testing because of the low mutation rates.
Selecting potential SNP candidates for forensic applications requires consideration of criteria such as the validation status of the SNP, the degree of linkage between SNPs, the polymorphism levels of SNPs in the major population groups, the local sequence characteristics flanking the DNA site and the technology used for genotyping.
Mining of public and private SNP databases can yield numerous suitable loci with a high level of validation and different private and public consortiums are validating sets of SNPs for forensic applications. In addition efforts of standardization have been carried out both in Europe and USA.
mtDNA and specially Y chromosome SNPs are also very interesting and the latter has been successfully used in sexual crimes. Different panels and strategies for mtDNA and Y chromosome SNP typing have been developed.
There are many different alternative methods for SNP typing. Real-time PCR (TaqMan or LigthCycler), DNA microarrays, MALDITOF Mass Spectrometry, Multiplexes based on primer extension (minisequencing) and conventional sequencers (SnapShot), systems based in OLA (Oligonucleotide Ligation Assay) and Pyrosequencing, among others. Each of these methods has advantages and disadvantages which will be described in detail in this talk.

IP-6

APPROACHING AND AVOIDING DNA MIXTURES

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DNA mixtures offer many problems. The starting point for analysis, and often the ending point, is Gill's well-known dictim to avoid mixture calculations whenever possible. When evaluation of a mixture is unavoidable, some sort of numeric assessment of the evidential strength is necessary. In deciding on a calculation, care and objectivity are necessary to avoid overstating the strength of the evidence against the suspect.
Given a DNA mixture of which the types are clearcut and which include the alleles of a suspect, it is straightforward to quantify the evidence against the suspect with a likelihood ratio – per Weir – comparing how well alternative hypotheses explain the presence of the observed alleles. Another method of calculation, the "exclusion" method, sometimes seems easier and is usually also an acceptable approach for such a situation, notwithstanding the theoretical shortcoming that it ignores part of the information about the suspect's types.
However, clearcut mixtures may be more the exception than the rule. What of the common situation that some of the alleles of some of the contributors to a mixture may be absent or only ambiguously present? When many alleles are faintly present, possibly masked by stutter or difficult to distinguish from noise, which of them should be considered for purposes of calculation? One extreme is to admit every bump as a possible allele, but if no one is excluded this is too conservative to be useful. The opposite extreme is to follow a policy that alleles below a standard threshold of signal strength are ignored, but partial allelic dropout at a few loci can "exclude" a suspect notwithstanding that the evidence at other loci may intuitively be compelling that he contributed. So neither extreme approach is satisfactory. And the in-between approach of using a flexible threshold is tantamount to framing the suspect. It is quite a quandary. The ideal would be a Bayesian approach that neither assumes an allele occurs nor assumes it doesn't occur in the mixture, but rather takes a probabilistic view: If the allele is – or is not – present, what is the probability to see what we see? Implementing this approach is difficult, but needs to be done. In the meantime, there is the dictim of Gill.

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OP-1

GENETIC IDENTIFICATION OF NECROPHAGOUS COLEOPTERA

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Genetic analysis in Forensic Entomology is a useful tool for determining necrophagous flies and other insects at species level. To gain data, in many cases e.g. parts of the subunit I of the gene of the cytochrome oxidase is analysed. This region of the mitochondrial genome has been examined in a wide variety of insects, including many forensic relevant flies. However, little data exist which allow species determination by genetic means of different beetle families which may be of forensic interest.
This insect groups has specific properties which make their usage in Forensic Entomology difficult. Until now for determination of beetles specific knowledge of morphological details of larvae and/or adults, which are easier to determine, are indispensable. Rearing of beetle larvae found at the corpse to the adult stage is in general difficult and needs more time due to elapsed development time of beetles. Therefore species determination by genotyping is very useful also for beetles.
For genotyping DNA extraction PCR and subsequent sequence analysis from a part of the COI gene from different necrophilous silphids, histerids, clerids and dermestids was performed. Sequence data were compared inter- and intraspecific. By this it became obvious that some beetle families can exhibit a high intraspecific variation which may hamper molecular identification for forensic purposes.

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OP-2

SUDDEN INFANT DEATH AND HELICOBACTER PYLORI INFECTION - ANALYSIS BY REAL-TIME QUANTITATIVE PCR -

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Recently, a hypothesis was proposed that *H. pylori* infection could be a cause for sudden infant death syndrome (SIDS). We investigated this postulated association by examining formalin-fixed paraffin-embedded gastric tissues of a retrospective cohort of 94 SIDS and 5 control cases. As a control group we examined 109 saliva samples of living healthy infants. The presence of *H. pylori* was inferred from a very sensitive PCR with silverstain detection and further evaluated by a newly developed *H. pylori* specific real-time PCR assay. This assay routinely had a detection limit of 30 copies of *H. pylori* DNA, even in the presence of excess human DNA, and was first validated on mucosal biopsy samples of patients with known *H. pylori* infection. Only in 3 SIDS cases *H. pylori* could be detected by PCR. Real-time PCR allowed to estimate the abundancy in 2 of these as about 1 copy

H. pylori DNA per ng human genomic DNA. To confirm these results independently, the same samples were immunohistochemically tested with a *H. pylori* specific antibody. *H. pylori* could only be detected in 1 SIDS tissue. As a result we conclude that SIDS is not significantly associated with a *H. pylori* occurrence.

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OP-3

GENOTYPING OF ACP1, GC AND PGM1 VIA SNP-ANALYSIS BY ALLELE SPECIFIC PCR

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This paper describes an attempt of genotyping the human haemogenetic polymorphisms red cell acid phosphatase (ACP1), group specific component (GC) and phosphoglucomutase 1 (PGM1). The method is based on allele specific PCR primers detecting single base exchanges responsible for the differing substrate specifities. These primers vary in length or fluorescent dye to indicate the allelic sequence. PCR products are separated and visualized on an ABI 310 Genetic Analyzer using standard STR conditions. All primer binding sites were chosen to produce fragments ranging from 60bp to 200bp to enhance sensitivity in case of degraded DNA.
The alleles ACP1*A/*B differ by a single base exchange in exon 3 from ACP1*C. ACP1*B/*C show a single transition in exon 4 compared to ACP1*A.
One single base exchange in exon 11 codes for the subspecifities GC*1S and GC*1F, a second for the differing specifities GC*1 and GC*2.
All these alleles can be differentiated by two sets of four allele specific forward and reverse primers respective for ACP1 and GC.
Genotyping of PGM1 is similar in principle: Exon 4 reveales a single transition dividing PGM1*2 and PGM1*1. Another base exchange, located in exon 8, is responsible for the subspecifities PGM +/-. As both exons are divided by about 20kpb, 2/1 and +/- properties are detected independently by two separate amplicons. For this reason PGM1 2+1- and 2-1+ show identic fragments in respect of length and color.
About 200 blood samples from routine paternity cases, all tested with classic serological methods, were genotyped as described above and showed the results expected.

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OP-4

USER-FRIENDLY DNA TESTING PROGRAMS FOR PATERNITY

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DNA profiling has proven to be a powerful technique for paternity and kinship determinations. Calculations for many of the paternity problems can be done by applying formulas given in the literature. In practice, we also encounter complicated paternity problems which are very difficult to handle, e.g. cases that the alleged fathers cannot be typed but their relatives can, and missing person problems in which relatives of the missing persons are typed. Based on the ideas of Bayes Theorem, conditional probability and pedigree analysis, four computer programs have been developed for handling various common paternity and kinship determinations for (a) alleged fathers, (b) alleged fathers but without DNA typing, (c) incest cases, and (d) missing persons. These programs are respectively named as (a) EasyPA, (b) EasyPAnt, (c) EasyIN and (d) EasyMISS, and the family of programs is called EasyDNA. Both civil paternity and criminal paternity can be dealt with by the programs. The programs handle the calculations of complex paternity and kinship problems by enumeration, and employ the pull-down manual for data inputting. The programs are very user-friendly. Users can easily save the results for checking and/or reporting purposes. For example, in program (a) alleged father, it can handle the calculations for the standard trio case: mother-child-alleged father, and the motherless paternity case: child-alleged father, with or without DNA typing for the relatives of the mother. It can also handle the alternative hypothesis that, for example, the true father of the child is a relative (such as brother, half-brother, father etc) of the alleged father. In this presentation, we are going to explain the theory and describe features of the computer programs.

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OP-5

DNA ANALYSIS ON MATERIAL FROM BARRELS OF FIREARMS

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In cases of shots with close contact it is possible to find blood and tissue deposits deep inside the barrels of firearms. This has been shown in Rostock by endoscopic examination where we found morphologic aspects of biological material. The question was if we could detect the presence of human DNA.
In suicides with firearms the barrels have been systematically examined by endoscopy and the adhesions were morphologically evaluated. Then a swab was taken from the depth of the barrel with a cotton wool wad humidified with steril aqua bidest. The extraction was verified again by endoscopic examination. The extraction of DNA from the specimen was made by a modified Chelex-method. In addition the specimen was purified with a Qiagen- Kit. DNA-Typing was performed with Multiplex-Kit (MPX-2, Serag). The analysis was performed with a sequencer ABI310 (Applied Biosystems). The extracted DNA-profil was then compared to reference material which was taken within the autopsy. The validation of the results was shown by simultaneous analysis of swabs from used and endoscopic "clean" barrels. As expected in this swabs no human DNA could be found.
Results:

- From swabs taken from barrels extensiv DNA-profiles could be detected so that an individual typing of the trace was possible.
- Traces which could be easily detected morphologically (blood remainants and tissue deposits) contained DNA-concentration enabaling standard DNA-analysis according to DAD-criteria.
- DNA-gain and typability correlate with the morphologic endoscopic finding.
- Not in all cases with close contact shots utilisable material could be extracted.

OP-6

STR PROFILES FROM ULTRA-LOW LEVELS OF DNA USING A MODIFIED CAPILLARY ELECTROPHORESIS PROCEDURE. AN ALTERNATIVE TO LOW COPY NUMBER (LCN) ANALYSIS?

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Orchid Cellmark has introduced the ABI PRISM 3100 Genetic Anayzer into routine forensic casework during the last year. It was noted during the initial validation studies that the machine produced slightly higher SGMPlus peak heights using the recommended injection conditions than were obtained from the same PCR sample analysed using an ABI PRISM 377 DNA Sequencer. Further studies have shown that by modifying the injection voltage and time it is possible to obtain significant increases in signal strength.
The PCR reaction in our laboratory has a volume of 25µl however only 1ml of this can be loaded on to a 377 gel when mixed with internal lane standard and loading buffer. The electro-injection mechanism of the capillary electrophoresis analyser makes it possible to concentrate the PCR product on the capillary without any significant loss in resolution. We have also investigated the effects of reducing the number of charged species present in the products of the PCR reaction (de-salting). Low molecular weight negatively charged species such as PCR primers that are present compete with the PCR products when the high loading potential is applied to the capillary. Reducing the levels of these ions through microfiltration or dialysis increases the proportion of DNA PCR product available for loading to the column and consequently increases the sensitivity of the system.
Examples from casework will be shown that illustrate the effects. Initial analysis of the blood from a murder victim's clothes sample using 377 gel produced a profile matching the victim. There were however indications of very minor profile from another individual. Re-analysis using the modified capillary procedure enabled a clear profile from another individual to be designated from the mixture.
The use of this technique is being actively investigated as an alternative to the LCN. It has the advantage that the number of PCR cycles remains the same and the risk of obtaining erroneous results through external contamination is reduced.

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OP-7
AUTOMATED DNA EXTRACTION FROM FORENSIC TRACES

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DNA fingerprinting has become a valuable tool in police casework. Therefore, forensic DNA laboratories are facing a significant increase in samples from crime scenes. To manage this higher quantity of samples automation of DNA extraction from forensic traces became an appropriate tool.
Due to the higher sensitivity compared to the magnetic-bead technology, a silica- membrane based system was chosen by the DNA laboratories of the federal states Northrhine-Westfalia (LKA Düsseldorf) and Berlin (LKA Berlin). DNA from cigarette ends, blood or salvia traces as well as buccal swabs are isolated with the MACHEREY-NAGEL *NucleoSpin, Multi-8 Trace* kit on several automation platforms, e.g. Tecan Genesis or PerkinElmer Multiprobe II.
By dealing with forensic traces cross-contamination is the most critical issue. This is prevented by using a flexible 8-well-strip technology, which allows to use an alternate pattern of extraction strips and dummy strips. Supported by a special pipetting scheme contact between different traces and used consumables is avoided.
The MACHEREY-NAGEL *NucleoSpin, Multi-8 Trace* kit allows concurrent preparations of 48 forensic samples and the subsequent PCR setup in less than 2 hours. DNA quality and quantity are as high as known from manual extractions.

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OP-8
THE DEVELOPMENT OF TWO NEW QUANTIFICATION KITS USING REAL-TIME PCR TECHNOLOGY

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Although the largest volume of forensic DNA STR analysis is carried out on samples collected for intelligence database purposes, the biggest challenge facing those who would provide systems for such analysis is being able to generate products capable of performing efficiently on the more compromised casework samples as well as the fresh and clean samples collected for a database.
Currently, many forensic laboratories use various quantification assays in order to quantify their DNA samples in preparation for short tandem repeat (STR) analysis. The quantification of DNA samples is used in human identification applications such as forensic testing, offender databasing, and paternity testing. Since these quantitation assays are used on a regular basis by forensic laboratories, it is important that they are accurate, reliable, reproducible, and easy to use. Applied Biosystems is developing two human and higher primate specific DNA quantification kits based on the fluorogenic 5' nuclease assay technology. The kits are designed to determine the amount of total amplifiable human DNA and total amplifiable human male DNA. The kits will contain all reagents necessary for the amplification, detection, and quantification of the DNA targets using an ABI PRISM® sequence detection system.
This paper will present an overview of the two new quantitation assays under development along with a review of Real-Time PCR. This will be followed by preliminary validation results performed at Applied Biosystems for species specificity, precision, mixture samples, and STR analysis and will discuss examples of how this technique, combined with subtle modifications to the AmpFLSTR® protocols can be used to improve results on even the most compromised of forensic samples. Recent developments in analysis methods and critical factors to be considered when analyzing low amounts of DNA will also be discussed.
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OP-9

AGE ESTIMATION OF DRIED BLOOD STAINS BY QUANTITATIVE MEASUREMENT OF RNA DEGRADATION USING DUPLEX RT-PCR

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The estimation of the age of dried stains is an unsolved problem in forensic science. Based on the increasing use of molecular techniques we addressed the question whether nucleic acid degradation itself could be used as age indicator. Whereas DNA seems to be stable in dried stains, RNA is believed to be rapidly degraded. Messenger-RNA offers a unique advantage for quantification of the fragmentation level: the poly-A-cap. In degraded RNA preparations the average size of cDNAs synthesized using oligo-dT-primers is smaller than in intact samples so that the sequences located near the 5'-end (standard) will be over-represented compared to intact samples when simultaneous amplification of both fragments is performed.
We have developed a semi-quantitative duplex RT-PCR amplifying the house-keeping gene ß-actin with several primer sets which allows differential quantification of RNA degradation levels. Using laser-induced capillary electrophoresis for visualization of amplification products, we have investigated 115 bloodstains stored for up to 15 years. The distribution of the peak area quotients of standard and target sequences was closely correlated with the age of the samples. Further statistical analysis showed that the position of primer binding sites along the cDNA determines the range of significant differences between samples of various ages: primer sets which amplify closely related sequences located near the 5' end allow differentiation of age differences of several years whereas primer sets with greater distance between the amplified fragments recognize smaller age differences in more recent samples.
Our data show, that mRNA suitable for RT-PCR can be isolated from samples stored for at least 15 years. Although RNA degradation is a non-linear, variable process, the determination of the storage interval and hence the age of blood stains seems to be possible with a precision of ± 1-2 years or less when appropriately selected primers are used.

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OP-10

COMPREHENSIVE CATALOG OF STATISTICAL FORMULAE, ALGORITHMS AND SOFTWARE - STEP TOWARDS GOOD STATISTICS PRACTICE IN FORENSIC GENETICS

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Statistical evaluation of forensic DNA evidence requires using a lot of formulae. So there is an urgent need for the comprehensive catalog of carefully approved formula as well as corresponding algorithms and software. The arguments in favor of this are as follows.
Some old and new formulae are disseminated among different journals which may be not familiar to forensic scientists. Moreover, some of them are published initially with errors which are reproduced in subsequent sources. Some older formulae are in rare use or forgotten. An example can be the useful Chakraborty's formula for the sample size required to reach the representativeness of the reference population samples. It is cited very rare and as a result too many published population data appear to be sparse ("unsaturated') and insufficient. Analogously, there is a lot of useful software which are not well-known to the users. Unfortunately, however, it appeared that sometime calculations provided by software are dubious. For example, one should keep in mind that for the cases of mixed stains GRAPE software computes incomplete set of the required likelihood ratios. Corresponding information is still not compiled in special manuals or reviews and does not find worth practical application.
It seems there is no agreement between formulae and procedures used in different countries. For instance in Russia for paternity testing a rather archaic approach is still in use. It is based on the conservative estimate for the paternity index, namely: the product of reciprocals of $p_i(2 - p_i)$, where p_i is the frequency of i-th allele. It is obvious that the estimate has undesirable statistical properties: it is biased, insufficient, and inefficient and has limited application for the "idealized" populations in Hardy-Weinberg equilibrium only. Nevertheless, the procedure is one and only dictated by Instruction on parentage testing approved by the Ministry of Health.
Recently several new statistics were derived for complicated scenarios such as DNA mixture analysis, different degree of kinship, absence of one parent, mutations etc. Several new formulae, algorithms were proposed and software created for testing Hardy-Weinberg equilibrium, linkage disequilibrium, population stratification and differentiation, for the estimation of F-statistics (fixation indices, coefficient of coancestry, etc.) and so on. Most of them implement exact nonparametric approaches and modern Bayesian ideology and methodology. Their realization requires sophisticated computational algorithms and facilities. In this respect some new problems are raised, e.g. the problem of convergence for the procedures based on Markov chain Monte Carlo algorithms.
One of the most desirable goals of statistical analysis is to present the initial (raw) data in compact, parsimonious and vivid form without loss of significant information they contain, i.e. "to separate the grain from the chaff", or "to filter the signal of the noise", and so on.
In this respect the problem of population differentiation can be considered form the opposite point of view as a problem of defining the pattern of similarity in corresponding contingency table. In statistical terms it is a problem of multiple comparisons or multiple hypotheses testing between and within different parts (blocks) of the table. Widespread approach realized in several software consists in a pairwise comparison between categories (rows or columns) in a table. The problem, however, is what kind of statistics, adjustments for the multiplicity of comparisons, critical significance level and/or degrees of freedom (when chi-square-like statistics are used) are needed for the adequate solution of the problem.
We (follow after Z. Gilula and A. M. Krieger, 1989) have demonstrated that so-called "chi-square reduction" can be used as one of the most reasonable measures of the homogeneity within given blocks of categories. As a result the original large population data set can be transformed into more compact (collapsed) table in which collapsed blocks appeared to be internally homogeneous. Not only classical chi-square statistic (and/or corresponding randomization procedures) can be used for this purpose as well as for most of the above analysis. Now about 20 new more effective competitors of chi-square are known which applicability has been demonstrated.
One should keep in mind that there are no universal formulae and unique test statistic for the above computing. We believe that it should be good statistics practice to compile and apply many of them and to investigate the consistency between obtained results
We have prepared and will present a catalog of formulae, algorithms and available software which are useful for the statistical evaluation of forensic DNA evidence and population data analysis.

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OP-11
SANCT – METHODOLOGY AND SOFTWARE FOR THE STRUCTURAL ANALYSIS OF FORENSIC POPULATION DATA

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One of the most desirable goals of statistical analysis of large databases is to reveal some kind of statistical structure and present it in compact parsimonious form. Dozens and hundreds samples of different ethnic origins on genotype and allele frequencies for the loci commonly used in forensic genetics are accumulating over the world. Some samples are regarded as belonging to the same or closely related ethnic groups. In such cases it seems to be reasonable to collapse (combine) them into internally homogeneous blocks (clusters). Resulting data set of enlarged samples could be regarded as more representative reference samples. However, before such collapsing their homogeneity should be controlled with statistical tests. In statistical terms the problem is equivalent to the defining the structure (or similarity pattern) in contingency tables which in turn is a sequence of multiple hypothesis rejections and acceptances. Ideally all possible combinations of categories (rows and/or columns) in a given contingency table should be constructed and analyzed. This approach, however, is impracticable: even for the tables of moderate size the total number of all possible grouping of categories becomes astronomical. For example, in case of a set of 25 population samples it is larger then 10^{18}. Another approach could be based on pairwise comparisons between categories. It is implemented in several software like Arlequin, GENEPOP and others, which provide computing the modern powerful exact nonparametric tests. Unfortunately, the tests implied are two-sample tests, which, strictly speaking, require some kind of adjustment for the multiplicity. Other software use different genetic distances, which also are two-sample statistics. At the same time commonly used adjustments for multiplicity such as Bonferroni or Sidak corrections (as well as numerous their modern improvements) or so-called 'simultaneous test procedures' appeared to be inadequate for large tables. They can lead to contradictory false conclusions: too often neither or few categories can appear to differ significantly when original table is extremely heterogeneous.

The idea of collapsing, that is combining the homogeneous (similarly distributed) classes (categories) during the statistical analysis of discrete data, is an old one. For a long time it is in wide use to solve the problem of sparseness of data tables. For example, it is commonplace to combine classes with small expected frequencies when analyzing discrete data with the chi-square-like tests. Corresponding archaic rules of thumb for the applicability of such statistics (e.g., such as to combine classes with expectations less then 5) now should be replaced with more universal diagnostics proposed by Simonoff and Tsai and Mudholkar and Hutson. The main procedure implemented in SANCT software consists in a stepwise collapsing of the most similar pair of categories. Several stopping rules are realized and can be compared. Further collapsing is interrupted when one of the following statistics becomes significant: reduction, distance, unadjusted P-value for the current collapsing block, Sidak adjusted P-value for the current collapsing block, Fisher's P-value combination test and integral heterogeneity for all collapsing blocks. Their comparison confirm that the test of internal homogeneity integrally within all revealed blocks of similar categories (population and/or allele or genotype) based on the Chi-square Reduction Principle is one of the most adequate.

Another problem is a search of adequate statistical measure for the category similarity. With large tables, different statistics can lead to different results especially when data sets are sparse (unsaturated). Besides classical statistics like Pearson's chi-square (X^2) or loglikelihood ratio (Kullback-Leibler information), the number of new competitors is growing enormously. Among them are statistics developed by Freeman and Tukey, Neyman, Nass, Williams, Anscombe, Zelterman, Mudholkar and Hutson and several new families such as Cressie-Read power divergence statistics, blended weight Hellinger distances, blended weight X^2, etc. All the above-mentioned statistics are implemented in software SANCT and are realized in both, the asymptotic and exact versions. The last one requires to be run on powerful computing facility. The software is being developed using object oriented C++ and have been successfully run in different computer architectures and operating systems. Applicability, reliability and validity of this approach have been demonstrated with the analysis of the world population data compiled from DNA-PCR Databank: (http://www.uni-duesseldorf.de/WWW/MedFak/Serology/database.html), and several other publications as well as original data.

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OP-12
SELECTED ASPECTS OF DNA DATABASE FORMATION IN POLAND

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According to the resolution of European Council in 09.06.1997 candidate countries were invited to establish national DNA databases. In 1998 authorities of Polish Police made a decision to create the national DNA database involving STR data. Its principles were supported by functional templates of British and German systems. Accordingly, extent legislative, educational and organisational actions were undertaken. As a result of Police Headquarters authorities initiative the governmental project of the novel Police Bill now includes a paragraph that gives way to introduction and operation of DNA database in criminal prosecution. The database is allowed to collect and process information on genetic profiles of persons responsible of committing a violent or recurrent crimes, delivering a crime as a constant income source, involved in an organized criminal group, or underage offences. Furthermore, the system is expanding to establish DNA database for missing and unidentified persons. It is emphasised, that positive result of the database search does not bear evidential proof in terms of the penal code, but carries indication of an evidence source. In order to attain the quality of a criminal evidence a forensic genetics expertise testimony is needed.

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OP-13
PROPOSALS FOR THE FORMAT FOR POPULATION DATA BASES AND THEIR ANALYSIS
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"Open and show all your data", visualization and "statistification" or GSP (Good Statistics Practice) must be the main principles in data basing. Make all your data available to the users preferably online or under request from the authors. Show individual genotypes when feasible. Show not only relative frequencies (proportions) but absolute counts for both, genotypes and alleles. Compute allele proportions with at least four or even more significant digits. Present genotype counts in form of triangle matrix; such presentation visualizes the "saturation" of the data and permits to present important information on the partial fixation indices in compact form on the same matrix. To achieve the saturation, provide preliminary estimate of the required sample size using e.g., Chakraborty's formula: Nmin = ln [1 – (1 – a)^(1/r)]:[4 ln (1 – Pmin)] where Nmin is the minimum number of independent individuals to be analyzed, <a> is the probability of error, <r> is the number of alleles revealed by the system and Pmin is the minimum allele frequency. Nmin is influenced mainly by Pmin. For example, if Pmin = 0.01, <r>is from 2 to 25 and <a> from 0.001 to 0.0001 then the minimum sample size Nmin from 190 to 310 is required, and from 1900 to 3100 if Pmin = 0.001.
When data set (genotype or allele table) appear to be unsaturated (sparse) then different statistical tests and/or estimates can lead to contradictory results and conclusions. In such cases according to GSP different statistical methods (estimation and/or test statistics) should be used and their consistency should be investigated, so it is desirable to prepare input files in different formats suitable for the analysis with different software (Arlequin, Fstat, GDA, GENEPOP, GENETIX, PopGen, PowerMarker, PowerStats, TFPGA, etc.) and provide their availability to the interested users. Apply these (or analogous) software to estimate (with the highest permissible precision) and compare the exact P-values (e.g. for the test of Hardy-Weinberg equilibrium and/or linkage disequilibrium), F-statistics and other population genetic parameters. Keep in mind, however, that when using software like Arlequin, GENEPOP, HWE, FTPGA or others in which the MCMC (Markov chain Monte Carlo) algorithms are implemented, the convergence of MCMC should be controlled. Otherwise significantly different results (estimates and/or P-values) can be obtained from run to run. Moreover, it appeared that the above software compute corresponding standard errors (S.E.) for the P-value in different ways. To avoid confusion one could find nonparametric 99% confidence limits for the P-values. Namely, carry out eight independent runs of the program and regard the range of the obtained values as such limits. In parallel, the procedure permits to check the convergence of the MCMC algorithms implemented in the software applied. Usually estimations of exact P-values are calculated as a proportion of the simulated values of a given test statistic which are equal to or exceed the observed value. Obviously, closeness of the P-values over runs can be easily controlled through the use of homogeneity tests (e.g., chi-square) applied to counts of those simulated values. If counts are significantly heterogeneous among the runs then repeat the process with larger number of randomized permutations until homogeneity is reached. Moreover, when P is not equal to 0.5 its distribution is asymmetric and calculation of the above nonparametric confidence intervals for P should be recommended instead of S.E.
One of the pending problems of GSP in forensic genetics is to find the most adequate statistical methods for defining the similarity pattern in large databases. In statistical terms this is a problem of multiple comparisons and multiple hypothesis testing. To solve them the new statistical methodology, SPAN—Similarity Pattern ANalysis — and corresponding software, SANCT (Structural ANalysis of Contingency Table), is developing. About 20 different test statistics are implemented in this software. Its applicability, reliability and validity were demonstrated with the analysis of several large data sets compiled from Promega, DNA-PCR Databank: (http://www.uni-duesseldorf.de/WWW/MedFak/Serology/database.html), DB on Nuclear DNA (http://www.ertzaintza.net/cgi-bin/db2www.exe/adn.d2w/INPUT?IDIOMA=INGLES) and other published world population data as well as original data from Russia. During the work some corrections were reported to proprietors of the DBs. Evaluated similarity/dissimilarity patterns appeared to be rather reasonable and interpretable: samples from ethnically related populations have been combined into internally homogeneous blocks (clusters). Such enlarged statistically homogeneous sample blocks can be used for more precise and reliable estimations of allele frequencies and other intra- and inter- population genetic parameters. SPAN methodology and SANCT software as important parts of GSP provide an adequate and fruitful approach to the analysis of large data bases in forensic genetics. Rationally organized and visualized format for the data presentation and GSP have to become an inherent part of good data basing practice.

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OP-14
FORENSICS GOES WEB - ASSESSMENT OF THE STATISTICAL SIGNIFICANCE OF A DNA RESULT BY MEANS OF WEB-BASED QUALITY-CONTROLLED POPULATION DATABASES: EMPOP (MTDNA) AND ENFSI STRBASE (STRS)
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The assessment of the statistical weight of a DNA result depends on the genetic properties of the DNA marker applied. Short Tandem Repeats (STRs) are the most frequently used autosomal markers in forensics. The significance of a match between an STR profile obtained from a stain and one obtained from an individual is usually reported on the basis of the frequency of this profile in a given population and is obtained by multiplying the expected frequencies of the individual STR genotypes. Uni-parentally inherited markers such as Y-chromosomal STRs and the mitochondrial DNA (mtDNA) Control Region require direct comparison to a relevant population database, as the regions of the genomes on which they are located do not recombine in the course of transmission and thus have to be regarded as single-linked molecules. There is a great variety of worldwide population data for all the three classes of forensic DNA markers mentioned above. A scientifically sound collation of these data in a quality-controlled way has been established for Y-chromosomal STR markers by the forensic group around L. Roewer in Berlin, and is posted in a user-friendly, interactive form on the internet (YHRD, www.ystr.org). Similar to this site, a mtDNA population database (EMPOP) and a pan-European (autosomal) STR-database have been created and will be offered to the forensic community via internet with the principal demand of the highest conceivable quality of the data.
Especially mtDNA population data have been challenged for their quality in recent publications, in which the authors demonstrate that current means of data collection, transfer and report are not providing sufficient safety in order to avoid errors. Within the EDNAP (European DNA Profiling) Group a web-based on-line mtDNA database (EMPOP) has been established with a concept for minimizing the

occurrence of erroneous data. EMPOP is conceived for the scientific community, in particular within the forensic field, freely accessible and open to the entire academic world. EMPOP is holding high quality mtDNA population data with a concept developed for minimizing the occurrence of erroneous data. This concept involves proficiency testing programmes in order to assess concordance of mtDNA analysis and data interpretation with the internationally recognized recommendations, an electronical data transfer to avoid clerical errors in the course of data transcription, the permanent linking of the raw data to the compiled sequences in order to enable conclusion of questionable positions, additional IT-controlled double analysis of the population data and sequence quality score values allowing for a better characterization of the quality of the database sequences.
The European Network of Forensic Science Institutes (ENFSI) has undertaken an extensive study collecting STR-data from 24 European populations (5700 profiles) using the AMPFLSTR SGM plus system (Applied Biosystems, Foster City, CA). This allele proportion (frequency) database – further referred to as the ´ENFSI STRbase´ - can be used to calculate match probabilities of DNA profiles from cosmopolitan Caucasian populations across all Europe, regardless of their specific country of origin. Differences in allele proportions between populations (Fst-values) are small, nevertheless, they cannot simply be ignored as the match probabilities of DNA profiles derived from a European database will tend to be lower than those derived from an appropriate cognate population database. In order to take account of both sampling error and population sub-structuring effects, various methods were applied including the Balding size bias correction, the Balding and Nichols Fst correction, a minimum allele proportion, an upper bound of a 95% confidence interval and a lower bound on the genotype match probability of 1 in 1 billion. The ENFSI STRbase has been installed on the internet, so that it can be used by European laboratories to enable the calculation of the match probability for any sample, using any adjustment factors they wish.

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OP-15

EXPANSION AND IMPROVEMENT OF THE Y-STR HAPLOTYPE REFERENCE DATABASE (YHRD)

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With the launch of the first Y chromosome-specific haplotype database for forensic use in the worldwide web at January 1, 2000 two important objectives are pursued:

(i) the generation of reliable Y-STR haplotype frequency estimates for minimal and extended haplotype profiles to be used in the quantitative assessment of matches in forensic casework (criminal investigation, kinship testing)
(ii) the assessment of inter-population stratification as far as reflected by Y-STR haplotypes

The initial phase of the online presentation of the databases (meanwhile an Asian and an US American repository have been added) went unexpectedly well with more than 100.000 visits and a lot of critical users who helped to improve this unique forensic web resource.
The number of laboratories and institutions which passed the QC tests and contributed population data increased steadily from 13 in early 2000 to 150 in May 2003.
To meet the demands of a rapidly growing number of laboratories performing Y-STR analyses in forensic or kinship/genealogical testing the YHRD has now been significantly enlarged to become a repository of quality assessed haplotype profiles from worldwide distributed reference populations. In May 2003, the YHRD has collected 18.038 Y-STR based haplotypes from 145 populations from Europe, Asia, the Americas, Africa and Oceania contributed by institutions mostly working in the forensic practice.
To improve the user-friendly presentation of the data and to allow fast searches and statistical extractions via the internet the database has now been re-programmed in Java allowing a number of new features to be added.
The increasing number of new polymorphic Y-STRs has prompted us to start collecting extended Y-STR haplotypes which include the 9-locus core ("minimal") set of markers plus up to 10 further loci.
The strategy of decentralized population sampling and centralized quality testing and databasing led to a dense coverage of larger geographic territories, e.g. of Europe. As revealed by Analysis of Molecular Variance (AMOVA) the Y-chromosome STR haplotyping is the method of choice to detect inter-population stratification. It is now quite evident, that the ethnicity of the donor of a stain could be detected by Y-chromosome haplotyping more reliably than with any other kind of DNA polymorphism.
We propose to discuss the future development of online haplotypic databases (mt-DNA, Y chromosome) with forensic applicability on the background of our 2 years usability experiences and recent developments in forensic genetics and (bio)informatics.
In conclusion: A scientifically approved, efficient and user-friendly database for the linearly and uniparentally inherited STR markers on the Y chromosome is only a mouse click away. The online availability of the database makes statistical calculations on basis of haplotypic data feasible for all experts working in the field of forensic or kinship diagnostics. A network of forensic analysts guarantees the quality and the timely update of the population data.

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OP-16

SEARCHING FOR THE NEEDLE IN THE HAY STACK-EXPERIENCE WITH MITOCHONDRIAL RESTRICTION SITE SCREENING IN MASS YIELD OF STAIN HAIRS

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Mitochondrial restriction analyses are performed rather seldom in forensic applications although they have been known as a well-established method for a number of years. We conducted a population study comprised of 250 blood samples (i.e. samples from 100 Germans, 50 Russians, 50 Hungarians, and 50 Bantus from Cameroon) which revealed the existence of 89 variable restriction sites (VRSs), i.e. 51% of all polymorph sites in HV1 and HV2 were found. The sequences were checked for the existence of VRSs by means of a computer program considering 583 restriction endonucleases. In the course of a case study we used the rare transition at position nt16263 to detect hairs in the resulting *Mnl* I site of the victim in a sex offence. Basically, mitochondrial VRSs analyses are a suitable method to identify relevant and non-relevant samples in cases involving many traces. Efforts can be concentrated on the relevant traces then to achieve a high likelihood

of identity. In one of two case reported here, for all hairs having been successfully tested we were able to prove that they did not originate from the victim of the sex offence.
Regarding a second murder case, we were requested to identify hairs of the victim from a carpet, which were buried in the ground for over two years. One hundred and two hairs were checked using *Hae*III, *Hyp*CH4V and *Tsp*45I to select relevant hairs for the sequencing procedure. Finally three hairs which exhibited the wanted sequence were detected.

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OP-17

MULTIPLE AGE-DEPENDENT DELETIONS OF MITOCHONDRIAL DNA IN DIVERSE HUMAN TISSUES

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It has been reported that mitochondrial deletions accumulate with age in postmitotic tissues. Since the late 1980s these studies focused on a few single mitochondrial deletions, especially the 4977 bp deletion which has been studied in a lot of different tissues. In recent years, there have been some studies about extensively rearranged mitochondrial DNA (mtDNA) in skeletal muscle or brain tissue. Unfortunately, either only a few individuals or only one tissue were tested thus far. The aim of our study was to compare the incidence of many different deletions in human caudate nucleus, cerebellum, skeletal muscle, and heart.
Tissue specimens were taken at autopsy from 50 individuals. DNA was extracted, subjected to a long-range PCR, and analysed by Southern blots using DIG-labelled mtDNA probes. Upon screening for different deletions, the four tissues exhibited an age-related increase in number and variety of mitochondrial mutations. In heart and skeletal muscle, these deletions appear between the fourth and fifth decade of life. While in skeletal muscle many individuals revealed a distinctive array of rearrangements, in heart muscle a uniform pattern of deletions was observed, especially in older age. Cerebellar tissue showed deletions only in individuals older than eighty years. The spectrum of mtDNA mutations in caudate nucleus displayed no deletions below age twenty, a banding pattern of deletions smaller than 8 kb from age 20 to 50, and a heterogeneous array of rearrangements above the age of fifty years, similar to the pattern observed in heart and skeletal muscle. In these three tissues, some fragments of identical size were detected. This might indicate a systemic process.
The most important result of our study is that cerebellar tissue must contain defence mechanisms against oxidative damage. While in skeletal muscle it is suggested that the accumulation pattern is due to a lot of stochastic events, a uniform pattern is observed in human heart. Because mitochondrial deletions are induced by ischemic/reperfusion events, number and variety of deletions may help to clarify mechanisms of sudden cardiac death.

OP-18

PARTIAL SEQUENCING OF MTDNA VIA SNP- AND FRAGMENT LENGTH ANALYSIS

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This paper describes an attempt of partial mtDNA analysis by means of four primersets detecting sequence variation in HV1, HV2 and HV3. The method is based in principal on allele specific PCR primers detecting base exchanges combined with amplicons creating different fragment lengths in case of insertions or deletions. This approach does not need PCR generated mtDNA template – it is applicable to native DNA samples. Allele specific primers detecting base exchanges vary in length or fluorescent dye. PCR products are separated and visualized on an ABI 310 Genetic Analyzer using standard STR conditions. All primer binding sites were chosen to produce fragments spanning less than 140bp to enhance sensitivity in case of degraded DNA.
The following variations of mtDNA can be differentiated by four amplicons which partially may be multiplexed: 16311C; 16399G; 73G; 309.1C; 309.2C, 315.1C; 489C; 498d; CA deletion and insertions at 523.
HV1, 2 and 3 regions of 160 human mtDNA templates were sequenced using the BigDyeTerminator-Kit (Applied Biosystems). All corresponding original DNA samples where subsequently processed by the method described revealing the results expected by prior forward and backward sequencing.
This attempt of mtDNA sequence detection via SNP- and fragment length analysis characterized in 96 samples six major haplotypes with frequencies of about 16%, 13%, 12%, 7%, 6% and 6% respectively. The remaining 64 samples were divided into 34 more rare haplotypes with frequencies ranging from about 4% to less than 1%.
We regard this method advantageous for instance in forensic casework when mtDNA of numerous samples may be screened prior to final standard sequencing.

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OP-19

SNP GENOTYPING OF FORENSIC SAMPLES USING MALDI-TOF MS: THE NEW GENOLINK SYSTEM

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Because of their frequency in the human genome and their comparatively low mutation rate single nucleotide polymorphisms (SNP) are widely used for the detection of genetic variations. As SNPs have only 2 alleles compared to 5 or more in STR loci the analysis has to be performed with a higher number, especially for forensic approaches such as paternity tests or human identification in general. Keeping this in mind, SNP genotyping has the potential to be at least as discriminating as e.g. the analysis of highly polymorphic STR loci.
Here we present the detection of eight varying SNPs which might serve as forensic markers. They are located on different autosomes in non coding regions. The analysis was performed by GENOLINK, a SNP genotyping module taking advantage of the speed and high accuracy of a MALDI-TOF MS system to create a reliable and cost effective platform for functional genomic studies. GENOLINK is composed of a novel genotyping-method genoSNIP, a robotic system for automated sample preparation and a bioinformatic package. The genoSNIP method is based on extension primers containing a non-nucleoside