B188 The Introduction of Massively Parallel Sequencing (MPS) of Mitochondrial DNA (mtDNA) Into Forensic Laboratory Practice in Croatia

Marina Korolija, PhD, Zagreb, MD, CROATIA; Viktorija Sukser, MS, Forensic Science Centre "Ivan Vucetic", Zagreb 10000, CROATIA; Sara Rožic, MS, Forensic Science Centre "Ivan Vucetic", Zagreb, CROATIA; Lucija Barbaric, PhD, Forensic Science Centre "Ivan Vucetic", Zagreb 10000, CROATIA*

Learning Overview: After attending this presentation, attendees will be acquainted with the challenges of introducing advanced technology into newly established forensic research laboratories. Attendees will learn about some basic principles of massively parallel mtDNA sequencing, data analysis, and actual relevance of mtDNA results in a forensic investigation.

Impact on the Forensic Science Community: Given that mtDNA targeted MPS results are globally still scarce, this presentation will impact the forensic science community by contributing substantially to forensic genetics and other scientific fields, such as molecular anthropology and medicine, by extensive and high-quality analysis performed in Croatian population.

Massively parallel sequencing (MPS) has enabled faster, deeper, and wider than ever insight into miracles carried by genomes of living creatures. Sometimes these organisms take part in cases of forensic interest, leaving genetic material as a trace of their physical presence. DNA sequences of complete human mitochondrial genomes, although of great forensic value, were difficult to access until the advent of MPS technology. Time has finally come to dive deeper into the fine structure of human mtDNA phylogenetic tree and a phenomenon of heteroplasmy, thus pushing the limits of current mtDNA forensic applicability. The authors therefore established, optimized, and validated sequencing of mtDNA genome from reference samples, with an initial goal to establish Croatian population database. Extensive database of 299 samples will serve for haplotype comparison and evaluation in future forensic casework. In that context, present efforts strive towards the implementation of MPS mtDNA analysis into routine practice.

Long-range PCR-based approach was chosen for mtDNA enrichment in samples of total genomic extracts from buccal swabs. The whole circular, 16 569 bp long mtDNA molecule was amplified by two sets of primers that overlap in the non-coding, control region (CR). Sequencing library preparation was performed by fragmentation of long PCR products, followed by the addition of oligonucleotides for binding sequencing primers to library fragments, binding fragments to a flow-cell and barcoding them (NexteraXT, Illumina). Sequencing was performed on Illumina MiSeq platform by multiplexing 48 samples per sequencing run, using Illumina MiSeq sequencing chemistry (Reagent kit v2, 300 cycles) and standard flow-cell. Data analysis was performed by both Illumina BaseSpace mtDNA applications, and an in-house bioinformatics pipeline, which is based on experimental and sequencing error modelling for the detection of minor alleles in samples with heteroplasmy. The latter approach is still in its developmental phase. Haplogroups were assigned to samples by importing variants (differences from rCRS) into HaploGrep2 (v2.1.0), a web application based on comprehensive human mtDNA phylogenetic tree (Phylotree, Build 17).

Average coverage per sample across eight sequencing runs amounted 6103±1111, which is well above the predicted value of 4633 for the sequencing chemistry used. Macro-haplogroup distribution in Croatian population is as follows: 36% H; 23% U; 8% J and T, each; 6% HV and K, each; 4% V; 2% N1, I and W, each; 1% R0, X and D, each; 0.3% A. Total of 185 haplotypes are assigned to 299 analyzed samples. However, in cases when several samples share the same haplotype, the necessity of a more refined haplogroup classification within Phylotree is evident. Regarding point heteroplasmies (PHs), as much as 27.8 % of samples exhibit minor alleles at frequency ≥10% (approximate detection limit of Sanger sequencing). In 4.3% of samples, there are two or more PHs present, and the most common PH (16093Y) appears in 3.3% of samples. Detected PHs are mostly transitions, distributed compactly in approximately 1.1 kb long CR (30 of them) and more sparsely distributed throughout approximately 15.4 kb of the mtDNA coding region. Low-level (<10%) variant detection is still being optimized.

In conclusion, with accurate information on sequencing library in terms of quantity and quality, it is possible to outperform manufacturer-specified output of the flow-cell and sequencing chemistry, without compromising the quality of the run. Apart from cost-effectiveness, it improves sensitivity of minor allele frequency detection. Distribution of macro-haplogroups in Croatia matches that found in Europe. Haplotypes determined in this study will enable further branching of global mtDNA phylogenetic tree, especially in the most common European haplogroup H. Optimized protocol for whole mitogenome sequencing and heteroplasmy detection, along with Croatian mtDNA database, is going to be used in routine forensic practice in Croatia, primarily in human identification, when standard Sanger sequencing of mtDNA CR does not provide sufficient power of discrimination.

Mitochondrial DNA, Massively Parallel Sequencing, mtDNA Forensic Database