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Research paper

Inter-laboratory validation study of the ForenSeq™ DNA Signature Prep Kit

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ABSTRACT

The implementation of massively parallel sequencing (MPS) in forensic science revealed the advantages of the new method compared to the forensic benchmark in DNA-STR analysis, the capillary-electrophoresis (CE): Sequence information and the possibility to multiplex hundreds of markers in one multiplex PCR increase the discrimination power of a forensic (STR-) profile. The EU funded project DNASeqEx (DNA-STR Massive Sequencing & International Information Exchange) aims to evaluate MPS-based materials in their respective developmental stages using the two established platforms MiSeq FGx (Illumina) and Ion S5™ (Thermo Fisher Scientific). As part of this project, we present here an inter-laboratory validation of the Forenseq™ DNA Signature Prep Kit, focussing on STRs included in primer mix A. Our study comprises tests of concordance, reproducibility, sensitivity (1 ng, 500 pg, 250 pg, 125 pg, 63 pg, 31 pg) and mixtures (male-male and male-female at ratios of 1:1, 1:5, 1:10, 1:15, 1:20, 1:100, 1:500, 1:1000). Sequencing results found to be virtually concordant to CE results, to reference profiles and reproducible between duplicates and between both laboratories. We observed first locus drop-outs (LDO) at a DNA input of 63 pg (20 sample pool) and 125 pg (38 sample pool). Alleles were found to be well balanced at a DNA input of 250 pg or more. We found the kit to perform well on moderate mixtures (1:1–1:20).

1. Introduction

For many years traditional capillary electrophoresis (CE) has been the gold standard for the analysis of short tandem repeat (STR) markers in forensics. It is essentially based on fragment length measurement of PCR products. Now, the new approach of massive parallel sequencing (MPS) is becoming increasingly attractive as nucleotide sequences of the fragments are determined. Additionally, it enables multiplexing of hundreds of target markers including forensically relevant STRs and single nucleotide polymorphisms (SNPs).With the gain in information compared to CE, the complexity of data processing and interpretation as well as the vulnerability to external factors like instrument performance, laboratory environment, staff training and quality levels of reagents and consumables may increase as well. Due to the more frequent use of the MPS technology in many forensic laboratories there is growing need to go beyond well-established in-house validation studies. As mentioned above MPS may be prone to different conditions in different laboratories that might potentially influence the quality of the DNA profile. Therefore, it is important to start measuring – or at least assessing – the extent of inter-laboratory variability on significant key factors of MPS including sensitivity, reproducibility and concordance. Conformity of results originating from different laboratories has always been a crucial issue for successful implementation of a new technology in the workflow of forensic DNA analysis. To support forensic laboratories with the implementation of MPS, Illumina (San Diego, USA) offers an entire class of linked products to prepare (ForenSeq™ DNA Signature Prep Kit), analyse (MiSeq FGx sequencer) and interpret (Universal Analysis Software (UAS)) forensic DNA samples using the MPS methodology. This package seems to be suitable for an inter-laboratory performance comparison because the predefined instrumentation, procedures and settings constitute a suitable basis for evaluating influencing factors.

The ForenSeq™ DNA Signature Prep Kit combines forensic STRs and SNPs in one multiplex PCR to enable MPS on a forensic benchtop sequencer (MiSeq FGx). The UAS allows simple analysis of MPS data without high skill requirements in bioinformatics. It performs trimming of adapter sequences, demultiplexing of samples, quality filtering, alignment and allele-calling of STRs and SNPs and visualizes the results

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as bar plots (number of reads). Sequence information is given optionally and can be exported to an Excel file, which also contains coverage information, quality control indicators and threshold settings. Default analysis parameters like read thresholds and stutter filters can be adjusted by the user. The kit consists of two different primer mixes for library preparation, Primer mix A (PMA) serves mainly for human identification and contains 58 STRs (27 autosomal, 7 X- and 24 Y-chromosomal), Amelogenin and 94 iiSNPs (identity informative SNPs). Further, it allows performing population statistics using the UAS. Primer mix B (PMB) includes all targets present in PMA plus additional 56 aiSNPs (ancestry informative SNPs) and 22 piSNPs (phenotypic informative SNPs), for the inference of externally visible characteristics and the continental ancestry of a DNA sample.

Previous publications described several applications of the ForenSeq™ DNA Signature Prep Kit in the forensic field: identification of individuals from mass graves [1], population analysis [2–6], relationship analysis [7,8], analysis of degraded samples [9] and ancestry prediction [10]. One advantage of using MPS for the analysis of STRs is the possibility to multiplex more than 100 markers in one reaction instead of the restricted multiplexes available for CE analysis consisting of up to approximately 30 STR markers [11–15]. Therefore, a gain in discrimination power could be achieved [3,6,16–27]. Early validation studies of the ForenSeq™ DNA Signature Prep Kit performed tests of reproducibility and concordance, sensitivity, flow cell capacity, mixtures, species specificity, inhibited samples, casework samples and ancient samples [23,24,28–34]. Some groups presented results on the modification of the recommended protocols: Guo et al. [33] and Mehta et al. [35] tested quantitative polymerase chain reaction-based library normalization instead of the proposed bead-based normalization and Elwick et al. [36] compared the effect of various inhibitors on the ForenSeq™ DNA Signature Prep Kit with the influence on the HID-Ion AmpliSeq™ Library Kit and ID panel at the Ion PGM™ System (Thermo Fisher Scientific (TFS), Waltham, MA).

The EU funded project DNASeqEx (DNA-STR Massive Sequencing & International Information Exchange (HOME/2014/ISFP/AG/LAWX/4000007135)) aims at evaluating MPS reagents and instrumentation in their respective developmental stages for use in forensic casework and STR databasing. For this purpose, two established sequencing platforms, namely MiSeq FGx (Illumina) and Ion S5™ (TFS) were used within the participating laboratories: the National Institute of Toxicology and Forensic Science, Madrid Department (Spain), the Institute of Legal Medicine and Forensic Science, Charité – Universitätsmedizin Berlin (Germany) and the Institute of Legal Medicine, Medical University of Innsbruck (Austria). The Institute of Applied Genetics, Department of Molecular and Medical Genetics, University of North Texas Health Science Centre (USA) acted as consultant to the consortium. Within this project an evaluation of the ForenSeq™ DNA Signature Prep Kit has been carried out at two DNASeqEx-laboratories including tests to evaluate concordance, reproducibility, sensitivity and mixtures. DNASeqEx focuses on forensically relevant STRs, which is why the results of the SNP-markers are not evaluated here. To test for inter-laboratory reproducibility and concordance, all tests were performed in a uniform manner at the Department of Forensic Genetics at the Institute of Legal Medicine and Forensic Sciences at Charité in Berlin (BER) and the Institute of Legal Medicine, Medical University of Innsbruck (GMI).

2. Material and methods

2.1. Samples and DNA extraction

The following samples were used for this study: GEDNAP samples (German DNA Profiling, http://www.gednap.org), NIST (National Institute of Standards and Technology) Standard Reference Material (SRM) 2391c component A-F [37,38], NIST 2372 component A [39,40], control DNA 2800 M (Promega), control DNA 9947A (TFS) and control DNA 007 (TFS). Both NIST sample sets were purchased from NIST (Gaithersburg, USA) and were distributed between GMI and BER. NIST SRM 2391c includes three single-source DNAs (female component A, male components B and C), one mixed-source DNA (component D is a mixture of component A and C), female single-source cells spotted on 903 paper (component E) and male single-source cells spotted on FTA paper (component F). One 6 mm punch of the papers for each component (E and F) was incubated in 190 µl G2 Buffer (Qiagen) plus 10 µl Proteinase K [20 mg/mL] (Qiagen) on a thermomixer (Eppendorf, Hamburg, Germany) at 56° C and 300 rpm shaking overnight. DNA extraction was performed on a Biorobot EZ1 Advanced XL (Qiagen) using the EZ1 DNA Investigator Kit (Qiagen) and the "Trace Protocol". The extracted DNA was shared between both laboratories. NIST SRM 2372 component A is derived from a single male donor and served as a human DNA quantification standard.

GEDNAP samples were available at both laboratories because of previous proficiency exercises. GEDNAP samples 48 and 49 were purchased in 2014, GEDNAP samples 50 and 51 in 2015. The corresponding DNA extracts were stored at −20° C. All GEDNAP samples used in this study were single-source samples, either from a female (G48_PA, G48_PB, G49_PB, G49_PC, G50_PB, G51_PA) or a male (G48_PC, G49_PA, G50_PA, G50_PC, G51_PB, G51_PC) donor and are provided as 10 µl blood on a cellulose swab each.

BER: GEDNAP cellulose swabs were incubated in 500 µl ATL Buffer (Qiagen) plus 10 µl Proteinase K [20 mg/mL] (Qiagen) on a thermomixer (Eppendorf, Hamburg, Germany) at 56° C and 300 rpm shaking overnight. 200 µl of the lysate were used for DNA extraction, which was performed on the QIASymphonySP (Qiagen, Hilden, Germany) using the QIAsymphony DNA Investigator Kit (Qiagen) following the manufacturer's protocol. DNA was eluted in 60 µl H_2O and quantified with the Rotor-Gene Q (Qiagen) and the Investigator Quantiplex Kit (Qiagen) following the manufacturer's protocol.

GMI: GEDNAP cellulose swabs were lysed in 500 µl extraction working solution (10 mM Tris, pH 8.0; 10 mM EDTA, pH 8.0; 100 mM NaCl; 2% SDS) and 20 µl Proteinase K [20 mg/mL] for 1 h at 56 °C (Eppendorf Thermomixer 5436, shaking). Following to incubation, 350 µl of the lysate were mixed with 500 µl MTL buffer (Qiagen) and extracted fully automated using the EZ1 DNA Investigator kit (Qiagen) on a Biorobot EZ1 Advanced XL (Qiagen) following the manufacturer's protocol. The amount of genomic DNA was determined by means of a TaqMan real-time PCR assay targeting AluYb8 [41] on a 7500 Fast Real-Time PCR Instrument (TFS). Thermal cycler protocol consisted of an initial denaturation at 95 °C for 20 s which was followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/elongation at 60 °C for 30 s.

2.2. Analysis of samples

2.2.1. Library preparation and sequencing analysis

Library preparation, purification and normalization was performed with the ForenSeq™ DNA Signature Prep Kit according to the manufacturer's protocol [42], unless otherwise noted. DNA input amount was 1 ng/assay according to the manufacturer's recommendation, unless otherwise noted (sensitivity and mixture studies). All prepared libraries included one negative control (NC) and one positive control (PC) sample. BER used DNA Engine Dynal (Biozym, Hessisch Oldendorf, Germany) or the Mastercycler® nexus (Eppendorf, Hamburg, Germany) for PCR amplification, whereas GMI used GeneAmp PCR System 9700 (TFS). Prior to sequencing on the MiSeq FGx (BER and GMI) DNA libraries were pooled and loaded onto the sequencing cartridge (MiSeq FGx Reagent Kit, Illumina) according to the manufacturer's recommendations, unless otherwise noted [42,43].

2.2.2. UAS analysis

All MPS data analyses presented here were performed with the UAS applying the manufacturer's default settings, unless otherwise noted