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A new multiplex-PCR comprising autosomal and y-specific STRs and mitochondrial DNA to analyze highly degraded material

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ABSTRACT

The analysis of short tandem repeats is one of the most powerful tools in forensic genetics. Forensic practice sometimes requires the individualization of samples that may contain only highly degraded nuclear DNA, mitochondrial DNA or PCR inhibitors that hamper DNA amplification. We designed a new multiplex PCR with reduced size amplicons (<200 bp), providing a double sex determination (amelogenin plus two Y-STRs), the detection of two autosomal markers and the amplification of mitochondrial specific fragments from the hypervariable region I (HVI). Additionally, a quality sensor was developed to check for the presence of any PCR inhibitors.

The new multiplex PCR shows a reproducible detection threshold down to 25 pg and gives signals even out of highly degraded materials. All signals are reproducible and reliable as it could be shown in comparison to results from commercially available STR multiplex-PCRs. In no case DNA fragments were detectable using any other assay when the quality sensor was not detectable.

There was a good correlation between detection of mitochondrial specific fragments in the multiplex-PCR and success of subsequent sequencing of HVI region. The same could be shown for STR analysis: Most samples successfully analyzed in our PCR yielded at least a partial STR profile using a commercial STR kit.

We present an assay that allows an easy, reliable, and cost efficient evaluation of DNA sample quality combined with a first rough sample individualization and sex determination suitable for forensic purposes. This assay may help the forensic lab personnel to decide on further sample processing.

1. Introduction

Analysis of short tandem repeat markers (STRs) has become one of the most powerful tools for DNA typing in forensic casework [1–3]. It allows a reliable identification of individuals, the classification of biological traces, and it is also a crucial method in anthropological research [3,4]. The development of so-called multiplex PCRs and the automated detection of fluorescent-labeled PCR products after capillary electrophoresis allows a fast and reliable detection and evaluation [5,6].

In many identification cases however, the DNA is highly degraded, present in only minute amounts or destroyed in such a manner that only mitochondrial DNA is left. This can be the case, e.g. in mass disasters with development of high temperatures [7,8] or suicidal burnings [9,10]. Minute amounts of DNA may also be found in highly putrefied bodies [11,12], formalin-fixed tissues [13,14] or hair shafts [15].

In addition, the presence of PCR-inhibitors that in many cases are co-extracted during the DNA isolation process may substantially hamper PCR amplification. Such PCR inhibitors may, e.g. be present in skeletal remains buried in the ground (for example humic acids from the soil, fatty acids from the bone itself or DNA from bacteria or fungi from the soil environment) [16–18].

It is very often a long lasting, time consuming and costly process to obtain reproducible and reliable typing results. In many cases, a genetic analysis fails completely. So far great efforts have been made to develop fast and simple assays to assess DNA quality and quantity prior to amplification [19–21]. Despite the application of so-called mini STR PCRs that amplify fragments smaller than 200 bp [22–24], DNA typing of problematic materials might not be successful, leaving the sequencing of mitochondrial DNA as an alternative method for identification [25,26]. Furthermore, in

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many cases it is unclear whether a negative PCR result is caused by PCR inhibitors or simply by too small amounts of DNA. Various methods (e.g. real time PCR) to detect PCR inhibitors [27] or detect human specific DNA fragments [28,29] have been utilized to optimize analysis of problematic forensic trace material.

In this study we developed a PCR that amplifies the gender determining amelogenin [24], the y-STRs DYS390 [30] and DYS391 [31], and the autosomal STR-loci TH01 and vWA [both 23] as a pretest to check for DNA quality and amplifiability. Two different-sized (280 bp and 439 bp) fragments from the mitochondrial HVI region [32] are co-amplified to determine whether a sample contains enough mitochondrial DNA for sequencing analysis. An additional quality sensor was developed to check for the presence of PCR inhibitors and test PCR efficiency.

2. Materials and methods

2.1. Samples and DNA isolation

For PCR positive and contamination controls and for dilution experiments, a commercially available DNA was used (Human Genomic DNA female and male DNA, Promega, Germany).

For quality control, DNA from 100 male and 50 female individuals was extracted from saliva swabs or blood samples using the Spin Swab kit (Invitek, Germany) or the Nucleo Spin Blood Quick Pure kit (Macherey-Nagel, Germany), respectively, according to the manufacturers' instructions. Additionally, 127 samples from different biological materials that were part of our routine case analyses were investigated. See Table 1 for further description.

DNA from *Hydra vulgaris*, necessary to design the PCR quality control, was kindly provided by the Zoological Institute of the Christian-Albrechts-University in Kiel, Germany. The hydra DNA was extracted out of 30–40 individuals using a phenol–chloroform extraction method as described in Ref. [33].

2.2. Validation of the new multiplex PCR

In line with our routine case analysis and also for quality control, DNA samples were analyzed as follows: depending on DNA quantity and quality, different amounts of DNA solution were employed to multiplex PCRs using the AmpF*l*STRIdentifiler™ (Applied Biosystems, Germany), the Powerplex ES and the Powerplex Y kit (both Promega, Germany) according to the manufacturers' recommendations. Amplification of Y-specific STRs was only done with samples from male individuals or when the presence of male specific DNA was suspected, e.g. in vaginal swabs after a suspected rape.

For fragment analysis, 0.2 μl/sample of a size marker (LIZ500 for Identifiler and ILS600 for Powerplex PCRs) was used. Electrophoresis was performed on an ABIPrism 310. Allele assignment was done by comparison with commercially available ladders and determination of fragment sizes using the 310 Gene Scan 3.1.2 software. Peaks below 50 relative fluorescent units (rfu) were not analyzed in this study.

Amplification of mitochondrial specific fragments was done as described in [32]. A total of 40 samples (20 samples showing strong mtDNA-specific signals in our new multiplex-PCR, 20 samples showing no or only weak mtDNA-specific signal) were subjected to mtDNA amplification. Amplification success was analyzed on ethidium-bromide containing agarose gels using a gel imaging system (Geldoc™ EQ, Biorad). Additionally, 30 randomly chosen casework samples were quantified by real time PCR using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystem).

In every PCR positive and negative controls were included (defined amounts of DNA from a male and from a female individual, no template controls, and extraction negative controls). Only when those controls showed the expected results, also the data of the investigated samples were used for further analysis and interpretation. Thus, we were able to decide whether a total PCR failure in our samples, including the non-amplification of the quality sensor, was due to PCR-related problems such as a missing or non-working PCR reagent or PCR inhibition. When all controls were correct and the sample showed no signals at all in two independent amplifications, the sample was considered as containing a PCR inhibitor and was subjected to a cleaning procedure to remove potential PCR inhibitors.

2.3. Genetic typing using the newly developed screening PCR

The newly developed multiplex PCR includes a double sex determination with amelogenin [24] and two Y-specific STRs [30,31], two autosomal STRs [23], and two differently sized fragments of the mitochondrial HVI region [32]. Additionally, a DNA fragment specific for *Hydra vulgaris* is used as a quality sensor showing PCR efficiency and the presence or absence of PCR inhibitors (see Table 2 for further details). In this study, the samples were amplified in a 9700 GeneAmp PCR System (Applied Biosystems, Weiterstadt, Germany) according to the following program: 5 min 94 °C as initial step, 30 s at 94 °C denaturation, 1 min at 58 °C annealing, 2 min at 72 °C extension for 30 cycles and 10 min at 72 °C for final elongation. Fragment analysis was performed on an ABIPrism 310 with 1 μl PCR product plus 11.8 μl formamide and 0.2 μl of ROX500 per sample (all Applied Biosystems).

2.4. Preparation of the PCR quality control and inhibitor testing

A hydra-specific 344 bp fragment of the hydra actin gene was amplified out of 14 ng hydra DNA using the primer pair Actin34F and R (F: 5-aagctcttccctcgagaaatc-3 and R: 5-ccaaaatagatcctcc-gatcc-3) [34]. PCR products were separated on ethidium-bromide

Table 1
Different biological traces that were used in this study to test the new multiplex-PCR. Shown are the different tissues/materials (n = 127) that were routinely analyzed in our institute for case analysis and additionally employed to the multiplex-PCR.

	Number of samples	Condition/description
Toothbrush	10	Material for identification of putrefied bodies
Single Hairs	28	Mostly without roots, up to 30 years old
Bone	1	Burned victim
Soft tissue (aorta, kidney, spleen, skin, placenta)	27	Tissues from highly putrefied bodies for identification purpose
Paraffin-embedded tissues	9	Fetal tissues obtained after abortion, formalin fixed and paraffin-embedded
Doc feces and vormit	2	Material from dogs that have post-mortem eaten parts of their owners
Oral swabs	15	Taken from alleged perpetrators and the victims
Vaginal or anal swabs	19	Material taken after alleged sexual assault to detect male DNA traces
Skin abrasions	7	Material taken after alleged sexual assault to detect male DNA traces
Blood traces	6	Minute traces of blood
Unknown	3	Presumed bone fragment and blood stain