

A NEW APPROACH TO QUANTIFICATION /NUCLEAR AND MITOCHONDRIAL DNA OF BIOLOGICAL TRACES BY ABI PRISM® 7700 SEQUENCE DETECTION SYSTEM

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We have developed a real-time quantitative PCR assay to measure the concentration of DNA extracted in forensic biological traces. Its rapid and sensitive results were obtained in less than 4 hours. This real-time quantification can be performed on the ABI Prism® 7700 Sequence Detector using the fluorogenic probe in a TaqMan® assay. We suggest a protocol that will be able to detect and quantitate human DNA and optimize PCR conditions (as the number of cycles) of DNA analysis in forensic caseworks.

Introduction

PCR amplification is influenced by the quality and quantity of DNA extracted by the biological trace. Lack of amplification is due to the presence of PCR inhibitors, such as heme compounds in DNA extracted from bloodstains, highly degraded DNA or insufficient DNA quantity. An accurate quantification of human DNA determines if there is a sufficient amount of DNA for the second step of the DNA analysis: the PCR amplification.

A number of important questions need to be answered before forensic application of this new approach can be contemplated. First, what is the "sufficient" amount of DNA to obtain a full DNA profile without allelic "drop-out" or a lack of amplification of some loci. Second, the PCR condition, particularly additional cycle number of PCR, could be changed to avoid detection of low quantities of DNA. Third, it is necessary, when there are low copy numbers of DNA, to recommend a RFU threshold.

We have addressed these issues by developing a real-time quantification by TaqMan® PCR assay for measuring the concentration of DNA extracted from biological traces following PCR amplification by commercial forensic kits. This technique permits continuous monitoring of the progress of an amplification reaction, which is essentially a combined thermal cycler/fluorescence detector. The ABI Prism® 7700 Sequence Detection System is able to measure the fluorescent spectra of the 96 amplification wells continuously during DNA amplification, and the data are analyzed onto a PC computer with Windows NT® 4.0 operating system.

Materials and Methods

Sample

DNA was purified from 35 forensic samples (20 saliva, 10 hairs, 5 bloodstains) by DNA IQ™ method. We have used as positive control DNA the human DNA 9947A in the amplification kit for the nuclear DNA and the Standard Reference Material (SRM) 2392 by NIST for the mitochondrial DNA.

Real-Time Quantitative PCR

We have used the same couple of primer used in PCR amplification of forensic sample and particularly the primers designed from the published amelogenin gene sequence (Nakahori Y. *et al.* Genomics 9:264-269): 5'-CCC TGG GCT CTG TAA AGA ATA GTG-3' (Amel-A Forward) and 5'-ATC AGA GCT TAA ACT GGG AAG CTG-3' 10µM (Amel-B Reverse) and a labelled fluorescent TaqMan® probe 5'-(VIC) TCT TTA TCC CAG ATG TTT CTC AAG TGG TCC TGA-3' 5µM (Amel-X), 5'-(FAM) ATT CTT CAT CCC AAA TAA AGT GGT TTC TCA AGT GG -3' 5µM (Amel-Y). We have used the couple of primers used in PCR amplification of the region HV1 for mitochondrial DNA 5'-CAC CTG TAG TAC ATA AAA ACC CAA TCC-3' (HV1 Forward), 5'-AGG GTT GAT TGC TGT ACT TGC TT-3' (HV1 Reverse), and a labelled fluorescent TaqMan® probe 5'-(VIC) CAT CAA AAC CCC CTC CCC ATG CTT-3' 5µM (HV1).

TaqMan® amplification reactions were set up in a reaction volume of 50µL (except TaqMan® probes and amplification primers) supplied in a TaqMan® PCR Core Reagent kit (Applied Biosystems). TaqMan® probes and PCR primers were custom-synthesized by PE Applied Biosystems. Each reaction contained 25µL of 2X Universal MasterMix; 300nM of each amplification primer; 100nM of the corresponding TaqMan® probe. We have used 10µL of extracted DNA for amplification.

Each sample was analyzed in triplicate. A calibration curve was run in parallel and in triplicate with each analysis. Identical thermal profiles were used for PCR amplification by commercial forensic kit. Thermal cycling was initiated with a 2-min incubation at 50°C, to allow the uracil N-glycosylase to act, followed by a first denaturation step of 11 minutes at 95°C and then 50 cycles of 94°C for 1 minute and 59°C for 1 minute.

Amplification data collected by the ABI Prism® 7700 Sequence Detection System and stored in the PC computer were then analyzed by use of the Sequence Detection System software developed by Applied Biosystems.

Results

Precaution against PCR contamination was taken. Aerosol-filter pipette tips were used for handling of liquids and separate areas were used for the preparation of amplification reactions. Multiple negative water blanks and PCR blanks were, included in every analysis. To determine DNA quantity we serially diluted the positive control human DNA 9947A (0.1 ng/µL human genomic DNA), in water, consisting of the DNA concentration from 1000pg to 0.5pg (1000pg-500pg-100pg-50pg-10pg-5pg-1pg-0.5pg).

The aim of this new approach to DNA quantification is to obtain a “real” DNA quantification and some information on the PCR amplification. The choice of the couple of primer of the X-Y homologous gene amelogenin (Sullivan *et al.* 1993 BioTechniques vol.15,n.4,636-641) links our experience on PCR amplification of DNA extracted from biological evidence. In many cases, we obtain only incomplete DNA profile, but always the sex information. Then, probably, the size and the percent of success during an amplification of this couple of primer have directed the choice of these primers. Using the same couple of primers (amel-A e -B) that we use in the commercial forensic kit we obtained the following information: the amplification plot shows the corresponding progress of DNA amplification during the cycle number. It was expected that a reaction with fewer copies of DNA (LCN) required an increase of the number of cycles to produce a quantity of DNA. For example, it was necessary for 30-32 cycles to obtain a full or incomplete DNA profile.

In fact, if we amplify the DNA Control standard using 28 cycles we obtained a full profile up to 0.5-0.25 ng whereas I obtain, in some cases, a full profile up to 0.05ng using 32 cycles. In forensic casework, where I have very bad forensic evidence, using the information obtained from the amplification plot by ABI Prism® 7700 Sequence Detection System, we have obtained a full profile or “good” incomplete profile using 30-32 cycles.