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Research paper

Improved amplification results following episodes of failure to amplify at the Amelogenin Locus using PowerPlex® ESI 16 Fast System

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ABSTRACT

In 2012 the Israel Police DNA Casework laboratory adopted the 16 STR PowerPlex® ESI kit for routine use. The Promega Company updated this kit and developed the PowerPlex® ESI 16 Fast System in which all autosomal primer pairs remained identical to the original set, except at the amelogenin site. The master mix was improved and optimized which allowed for direct, faster and more robust amplification. Prior to implementing the PowerPlex® ESI 16 Fast System in our lab, we conducted a preliminary assay where 213 casework samples were amplified using the new kit. These samples had previously been extracted by one of two extraction kits employed by our lab. (the PrepFiler ExpressTM and PrepFiler BTATM Forensic DNA Extraction Kits). The amplification results from these samples were compared to samples amplified using the original PowerPlex® ESI 16 kit. Multiple incidents of failure to amplify at the amelogenin locus were noted using the new system with the recommended protocol at a rate of 13% (28 of 213 samples). Experiments were performed to understand whether these amplification failures could be a result of primer binding site mutations, extraction method reagents and/or inhibitors. The conclusions reached following these experiments, in conjunction with consultation with the manufacturer, led to the trial of a modified amplification protocol where the suggested annealing temperature was reduced by 2 degrees. To evaluate the efficiency of this altered protocol, a comparison study was undertaken where 88 additional casework samples were chosen and amplified using both the modified 58°C and the recommended 60 °C annealing temperatures. We concluded that the most effective method in our laboratory for achieving a consistent and balanced amplification at the amelogenin locus was to reduce the annealing temperature from the manufacturer's recommended 60 °C to 58 °C. This modification resulted in a reduction of the failure to amplify at the amelogenin locus from 13% (28/213) to 1.1% (1/88) without any observed changes to the autosomal STR amplification results.

1. Introduction

A call by the European Forensic community for the development of superior next-generation STR genotyping systems resulted in the development of a number of novel commercial kits combining increased discrimination power, improved performance and better separation of amplified products [1,2]. With these new kits available, the Israel Police DNA Casework lab adopted Promega's PowerPlex® ESI 16 kit for our routine casework load, with the occasional use of PowerPlex® ESX 16 for the elucidation of ambiguous results [3–6].

Within a short time of releasing these new multiplex kits, Promega offered upgraded, and improved versions of their PowerPlex® ESI and PowerPlex® ESX systems for faster amplification on casework specimens and reference samples. These new FAST kits promised comparable typing results as those achieved with the original PowerPlex® ESI and PowerPlex® ESX systems but with a 75% reduction in cycling time [7]. An overall amplification time of less than one hour provides a true advantage for high throughput forensic laboratories.

In an ongoing effort to maximize productivity and effectively streamline our processes, the Israel Police DNA Casework lab commenced a validation protocol prior to the implementation of the new PowerPlex® ESI 16 FAST kit as our default kit for routine casework. As the autosomal primer pair sequences in the new kit were reported to be unchanged from the original kits, we expected to continue to observe balanced and completely concordant profiles as

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we had seen using the original PowerPlex® ESI kit. What we did observe though, was a surprisingly high percentage of failures to amplify or reduced amplification at the amelogenin site in comparison to amplification results from the same samples using the original PowerPlex® ESI kit. This surprising phenomenon had not been reported in the developmental validation where over one thousand samples had been tested using the suggested protocol [7]. Regarding the amelogenin primer pair sequences in the upgraded version, it was reported that no sequence changes had been made other than the addition of three bases to the 5′ end of the unlabeled primer in order to facilitate improved adenylation, and the removal of one base from the 5′ end of the labeled primer in order to prevent artifacts [7]. Other than the amelogenin anomalies seen when amplifying with PowerPlex® ESI 16 FAST kit, all other loci in the achieved profiles provided comparable, balanced and robust results as promised.

Gender determination of forensic samples is of utmost importance in criminal cases. A failure to amplify at the amelogenin locus may occur as a result of mutations or deletions at the specific primer binding site [8]. Other parameters known to influence the success of PCR amplification may be inhibition or degradation [9]. Physical properties of the PCR process can also have an influence on amplification success. Annealing temperature is known to affect the bonding strength of primers to their specific regions on the DNA strand and cycle number may also affect PCR results [9].

In this article we present our observation of an unexpectedly high rate of failure to amplify at the amelogenin locus when using the PowerPlex® ESI 16 Fast System's recommended parameters. We present our process of understanding this result, and our recommendation of a 2 °C reduction of the annealing temperature to provide consistent amplification results.

2. Materials and methods

Results presented in this article originated from evidentiary DNA casework samples and positive control DNA (2800 M) supplied with the PowerPlex® ESI 16 Fast Systems (Promega).

Extraction of the DNA from casework samples was carried out using magnetic bead based extraction with either the PrepFiler Express™ or PrepFiler BTA™ Forensic DNA Extraction Kits (Life Technologies, Foster City, CA) [10] according to sample type, as determined by the operational protocols. The PrepFiler Express™ kit is designed for common forensic sample types, such as swabs and stains containing body fluids. The PrepFiler BTA™ extraction kit is recommended for challenged forensic sample types such as cigarette butts, tape lifts and other adhesive-containing substrates.

The extracted DNA was quantified using Quantifiler® Trio DNA Quantification Kit according to the manufacturer's instructions (Life Technologies, Foster City, CA).

Amplification was performed using the PowerPlex® ESI 16 Fast Systems (Promega) in accordance with the manufacturer's recommendations for 30 cycling using annealing temperature of 60 °C, in an Applied Biosystems® GeneAmp® PCR System 9700 Thermal Cycler (Life Technologies, Foster City, CA) [11].

Specimens were also amplified with a modified protocol with a 2 °C decrease of an annealing temperature to 58 °C instead of 60 °C.

Amplified products from all DNA samples were separated and detected by capillary electrophoresis on 3500×L Genetic Analyzers (Life Technologies, Foster City, CA). Prior to electrophoresis, 1 μl of amplified product or allelic ladder was added to 10 μl of deionized Hi-Di™ formamide (Life Technologies, Foster City, CA) with CC5 Internal Lane Standard 500 (Promega Corporation, Madison, WI). Samples were denatured at 95° C for 3 min, followed by a quick chill of 3 min. Electrophoresis was done using Performance Optimized Polymer (POP-4™) (Life Technologies, Foster City, CA) in a 36 cm array using recommended run parameters. Fragment analysis was performed using GeneMapper® ID-X software (Life Technologies, Foster City, CA). Detection thresholds for the PowerPlex® ESI 16 System (Promega) for determining heterozygous peak alleles for data generated was set at 300 RFU and homozygous allele calls were made at 1500 RFUs.

Detection thresholds for the PowerPlex® ESI 16 Fast Systems (Promega) for heterozygous peak alleles was set at 300 RFU and homozygous allele calls were made at 1000 RFUs.

3. Results and discussion

A total of 213 evidentiary samples were extracted using the PrepFiler Express™ and PrepFiler BTA™ Forensic DNA Extraction Kits. Overall, we observed a 20% reduction or failure to amplify at the amelogenin site in the samples analyzed.

Of these, 28 (13%) completely failed to amplify at the amelogenin locus. In 15 (7%) of these samples, amplification results at the amelogenin site showed significantly lower peak heights (RFU's) in relation to the rest of the profile (lower than 50% in comparison to the adjacent D3S1358 locus).

In our attempt to understand this phenomenon in our hands, we investigated a number of the possible causes which may result in a failure to amplify, and specifically at the amelogenin locus. These included:

- a possible mutation in the primer binding sites of the amelogenin locus, prohibiting complete amplification,
- reagents from the extraction methods that might interfere with the new kit amplification,
- carryover inhibitors remaining even after extraction in problematic samples.

In order to determine whether a mutation in the binding region of the primers caused the problem, we added a positive control DNA (2800 M) provided with the kits to 6 of the 28 samples where a complete failure to amplify had been observed. Out of these 6 samples, 3 (50%) showed continued problems in amplification at varying degrees at the amelogenin locus, even in the presence of the positive control DNA where no primer binding site mutation is known. One of these three samples continued to present a complete failure to amplify at the amelogenin locus even in the presence of the added positive control DNA. In the two additional samples the peak heights (RFU) of the expected amelogenin alleles were significantly reduced. In these three samples, all autosomal loci amplified as expected. Fig. 1 illustrates one of these events of failure to amplify at the amelogenin locus (Fig. 1). Expected mixture profiles of the known sample and positive control profile were observed at all other loci. The three remaining samples of the six tested, provided expected autosomal mixture results, and now normal XY alleles at the amelogenin site, showing a reversal of the failure to amplify.

In the three samples where continued failure to amplify occurred, even after spiking with control DNA, we concluded that the phenomenon was most likely not due to a mutation in the binding region of the primers of this locus, but rather as a result of some carry over products or inhibitors from either the extraction process or some inhibitor found on the sample/item of evidence itself.

In the three samples where the failure to amplify was reversed, sequencing or another molecular assay of these samples could be done to conclusively determine that a primer binding site mutation was not the culprit for these failures to amplify.

The next step was then to explore whether the problem originated specifically in some component of the extraction reagents (PrepFiler Express™ and PrepFiler BTA™ Forensic DNA