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# A rapid screening method using DNA binding dyes to determine whether hair follicles have sufficient DNA for successful profiling

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ABSTRACT

We report a simple screening method to assess the viability of successful DNA profiling from single hair follicles. A total of 48 hair samples (shed and plucked) were collected from male and female donors and the root tips (0.5 cm) were stained using one of three DNA binding dyes (EvaGreen™, Diamond™ Nucleic Acid Dye and RedSafe™) at 20× concentration. The hairs were subsequently viewed under a Nikon Optiphot fluorescent microscope to count the approximate number of nuclei in one plane of view. The hairs were then processed using either (1) a DNA extraction kit (QIAmp® Mini Kit) and then amplified using the AmpFLSTR® NGM™ kit, which amplifies 15 short tandem repeat (STR) loci plus the gender marker amelogenin, or (2) by direct PCR amplification using the same DNA profiling kit. Diamond™ dye had the lowest background signal and plucked hairs treated with this dye produced full DNA profiles when amplified directly and was chosen to screen a further 150 mixed hair samples. These hairs were separated into one of five categories (1, >100 nuclei; 1.5, 50–99 nuclei; 2, 1–49 nuclei; 2.5, no nuclei but high fluorescent signal; 3, no nuclei and very low fluorescent signal) from which 60 of the hairs were chosen to undergo direct amplification using the NGM™ kit. It was found that there was a direct correlation to the category designation and the ability to obtain a DNA profile up-loadable to the Australian DNA Database. Approximately 91% of category 1 hairs resulted in either a full or high partial (12–29 alleles) profile by direct PCR whereas about 78% of category 3 hairs exhibited no amplification. The results show that this method can be used to predict successful STR amplification from single hair follicles. It is a rapid, sensitive, cheap, non-destructive and easy to perform methodology applicable for screening multiple hairs in order to aid forensic investigators in predicting hairs that will yield DNA results.



## 1. Introduction

Hairs are encountered frequently during a forensic investigation with telogen hairs the most commonly found as many humans shed approximately 75–100 hairs per day [1]. It has been estimated that 95% of hairs collected at a crime scene are identified as telogen hairs [2], typically lacking a follicular tag, as opposed to anagen hairs which are in the active growth stage, requiring force to be removed from the scalp and hence often contain cellular material [3]. Microscopy is the primary technique used for determining whether cellular material suitable for DNA profiling is adhering to the hairs. Recent studies have reported the usefulness of staining hairs with various dyes to visualize the nuclei within the hair root. These dyes include haematoxylin as this binds to chromatin present within DNA and histone complexes and stains the nuclei a dark violet [2,4]. DAPI is a minor groove binding dye that has also been used to stain hairs so as to visualize the number of nuclei present to determine viability for STR profiling [5,6]. DAPI has a relatively low binding specificity to DNA, as it has a positive signal when in the presence of detergents and other compounds. DAPI only has approximately a 20-fold increase in fluorescent signal when in the presence of DNA [7,8]. Hoechst 33258 dye has also been used to label the DNA within hair follicles *in situ*. However this method is time consuming requiring overnight staining with the DNA dye and the whole process takes several days [9].

Nucleic acid binding dyes have been used for a range of purposes such as flow cytometry, gel electrophoresis and DNA quantification. There are a range of different types of binding dyes that have various mechanisms which affects their level of fluorescence enhancement; SYBR Green I (SG), which is an intercalating dye as well as having electrostatic and extended groove interactions, has an approximate 1000-fold fluorescent enhancement when in the presence of DNA [10]. This compares

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with ethidium bromide, another well-known intercalating dye, which only has a 20–100-fold increase in fluorescent enhancement [11]. Recently available dyes with reported high fluorescence in the presence of DNA have been engineered to be less toxic and mutagenic than both SG and ethidium bromide such as GelGreen™ and Diamond™ Nucleic Acid Dye.

EvaGreen™ (EG) is a DNA binding dye which has been manufactured for its use in real-time PCR. The dye has also been used for DNA quantification, quantitative PCR and high resolution melt curve analysis [12]. The dye was found to have approximately a 70 times increase in fluorescent enhancement when in the presence of DNA [12], which is much lower than SG, however, another study stated it had similar DNA detection limits to SG [13]. This compares with a recent study [14] reporting that EG had a fluorescent enhancement of 602% compared with SG at 2544% [14]. It was found that EG was stable with its use in real-time PCR and has a relatively low PCR inhibition [12]. The manufacturer states that this dye is impermeable to cell membranes making it an unlikely stain for nuclei within hair follicles, which will be reviewed in this study.

RedSafe™ (RS) is a DNA binding dye used in gel electrophoresis primarily to replace ethidium bromide due to its toxic and mutagenic nature. This dye was found to detect down to 1 ng of DNA and be capable of permeating the cell membrane [15]; such capability makes the stain suitable for biological samples such as hair shafts.

Diamond™ Nucleic Acid Dye's (DD) mechanism for DNA interaction is as an external binder. This dye has been found to be less toxic and mutagenic than ethidium bromide [16,17]. Due to a different mechanism of interaction compared with SYBR Green I it may have a decrease in intensity when in the presence of DNA. However it was found that the dye was as sensitive as SYBR® Green down to 0.5 ng of DNA in gel electrophoresis [15]. It was also found to permeate the cell membrane, allowing an interaction with genomic DNA. Due to the dye's cell permeability it may be a useful dye in staining biological samples for nuclei visualization.

Successful direct amplification of human hair roots has previously been undertaken to amplify single nucleotide polymorphisms (SNPs) [18] and the amplification of short tandem repeats (STRs) [19]. The application of direct PCR aims to reduce contamination and loss of DNA that can be experienced when undergoing DNA extraction [20]. Using direct PCR has shown to generate higher profile peak heights and less allele drop-out compared to using a DNA extraction process [21]. A study has previously shown that there was around a 60% loss of DNA during the extraction process with a minor increase when DNA binding dyes were present [22]. The amount of DNA that was lost when DNA binding dyes were present depended on the binding mechanism of the dye [22]. STR profiles have been generated directly after nuclear staining of the hair follicles with DNA binding dyes, RS, EG, DD and SG and showed that only SG had allele drop-out [23].

This study aims to review the use of three DNA binding dyes RS, EG and DD that are more sensitive than dyes used previously for nuclear staining of hair follicles using fluorescent microscopy and undertaking both DNA extraction and direct amplification. One dye, Diamond™ dye, was selected to screen 150 mixed hair samples and determine their viability for direct STR amplification.

## 2. Materials and method

### 2.1. Hair sample collection

Shed and plucked hairs were collected (48 in total) from both male and female donors varying in colour and age. Shed hairs (150 in total) were then collected from both male and female donor participants that varied in age and colour.

### 2.2. Nucleic acid binding dyes preparation

EvaGreen™ (Jomar Diagnostics P/L, SA, AUS) and Diamond™ Nucleic Acid Dye (Promega, NSW, AUS) were diluted to a working solution of 20× (1 in 500 dilution) in sterile water. RedSafe™ (Scientific, NSW, AUS) was diluted to a working concentration of 20× (1 in 1000 dilution) in sterile water.

### 2.3. Fluorescence microscopy and nuclear hair staining

The hair roots were removed (0.5 cm) from the shaft and placed onto glass slides. The dyes (1 μL of 20×) were then applied to the roots and a coverslip was placed on top to reduce contamination and aid in visualization of the nuclei.

Once the selected dye was applied to the hair root it was visualized under a microscope (Nikon Optiphot) using a B2A emission cube. The hair root image was then captured using a Nikon camera set at an exposure time of 1/20 s or 1/50 s at 40× or 100× magnification. The number of nuclei that were present in the image was recorded. As a hair root is three-dimensional and the image was only taken of one plane of view it would be expected to have a higher number of nuclei than visualized, however to attain standardization only one focal plane across the hair root was captured and nuclei recorded. Images were taken before and after staining to determine the level of auto-fluorescence.

Plucked and shed hairs were stained individually with DNA binding dyes and subjected to either DNA extraction then amplification, or were amplified directly. Four hairs for plucked and shed hairs were analyzed for all three dyes for both extraction and direct PCR. A total of 48 hairs were analyzed.

### 2.4. Extraction and PCR amplification

Selected stained hair roots that showed good fluorescent signal were placed into a 1.5 mL tube and extraction of the DNA was undertaken following the QIAmp® Mini Kit protocol (QIAGEN Vic, AUS) that used both dithiothreitol (DTT) and proteinase K in the digestion process of the hair, with an incubation time of 1 h at 56 °C. The DNA was eluted into 50 μL of AE buffer. After extraction the samples were quantified using the Qubit® 2.0 Fluorometer using the Qubit® dsDNA HS Assay Kit in triplicate (ThermoFisher Scientific, Vic, AUS) following the manufacturer's protocol. If the sample was quantified below 0.5 ng/μL the sample was submitted for real-time PCR (RT-PCR) analysis using the Investigator® Quantiplex RT-PCR kit (QIAGEN Vic, AUS) in triplicate following the manufacturer's protocol. The samples were then amplified using 10 μL of the sample (diluted to 1 ng/μL if required) which was placed into a 0.2 mL thin walled tube containing 10 μL of PCR master mix from the AmpF®STR NGM™ kit (Life Technologies) along with 5 μL of primer mix to make up a final volume of 25 μL. The amplification was conducted using GeneAmp® System 9600 (Life Technologies) thermal cycler following the manufacturer's protocol. A standard cycle number of 29 was used throughout the study. The NGM™ kit amplifies 15 STR loci plus the amelogenin locus.

### 2.5. Direct PCR amplification and conditions

The stained root fragment was placed into a 0.2 mL thin walled tube containing 10 μL of PCR master mix from the NGM™ kit along with 5 μL of primer mix and 1 μL of AmpliTaq Gold® DNA polymerase (Life Technologies). A further 9 μL of sterile water was added to make up a final volume of 25 μL. The amplification was conducted using GeneAmp® System 9600 thermal cycler using the manufacturer's protocol. A standard cycle number of 29 was used throughout the study.