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Development of HyBeacon® probes for specific mRNA detection using body fluids as a model system

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ABSTRACT

HyBeacons are linear oligonucleotides which incorporate fluorescent dyes covalently linked to internal nucleotides. They have previously been used with PCR and isothermal amplification to interrogate SNPs and STRs in fields as diverse as clinical diagnostics, food authentication, and forensic DNA profiling. This work explores their use for the identification of expressed gene sequences through mRNA profiling. The use of mRNA is becoming increasingly common in forensic casework to identify body fluids on evidence items, as it offers higher specificity and fewer false positives than current chemical presumptive testing methods. The work presented here details the development of a single-step one-tube RT-PCR assay to detect the presence of body fluids of forensic interest (saliva, blood, seminal fluid, vaginal fluid and menstrual blood) using HyBeacon® probes and melt curve analysis. Each assay shows a high degree of specificity to the target body fluid mRNA suggesting there is no requirement to remove genomic DNA prior to analysis. Of the five assays developed, four were able to detect between 10 and 100 copies of target cDNA, the fifth 1000 copies of target. The results presented here demonstrate that such an approach can be optimised for non-expert users and further areas of work are discussed.

1. Introduction

Recent research has shown that HyBeacon probes offer a flexible and robust approach to nucleotide sequence detection across a variety of applications [1–8]. The versatility of the probe comes from the design which is specific to a complimentary target sequence. When hybridised to single-stranded target DNA they emit greater amounts of fluorescence than when un-bound. Detection is performed using melt curve analysis, and the temperature at which the probe dissociates from the target is determined by the degree of complementarity between the probe and the sequence to which is it bound, and can easily span a range of 30 °C for the detection of mismatched or partially complementary target sequences [4,5]. This DNA based approach has allowed scientists to compare and match samples in a large number of applications ranging from Single Nucleotide Polymorphism (SNP) analysis (Fig. 1A) in food and medical diagnostic applications [7,8] to Short Tandem Repeat (STR) profiling (Fig. 1B) in the analysis of forensic samples [4,5]. However, the detection of expressed gene products such as mRNA sequences, rather than DNA, is becoming increasingly important in a variety of fields. Research looking to measure and detect mRNA expression patterns in tissues often have a health focus such as disease diagnostics [9,10], susceptibility [11] and treatment [12], although there are other non-health applications which have no need to identify gene mutations or expression level and are simply concerned with the provenance of a biological sample.

Body Fluid Identification (BFID) forms part of the field of forensic genetics [14]. Investigations can often require activity level or cell source information which can allow the linking of a downstream DNA profile to a body fluid (and therefore an individual or action), or to allow discrimination between two versions of an event [13,14]. Currently this information is mainly acquired using chemical tests (such as the Kastle-Meyer test for blood [15]) or microscopy (in the case of the

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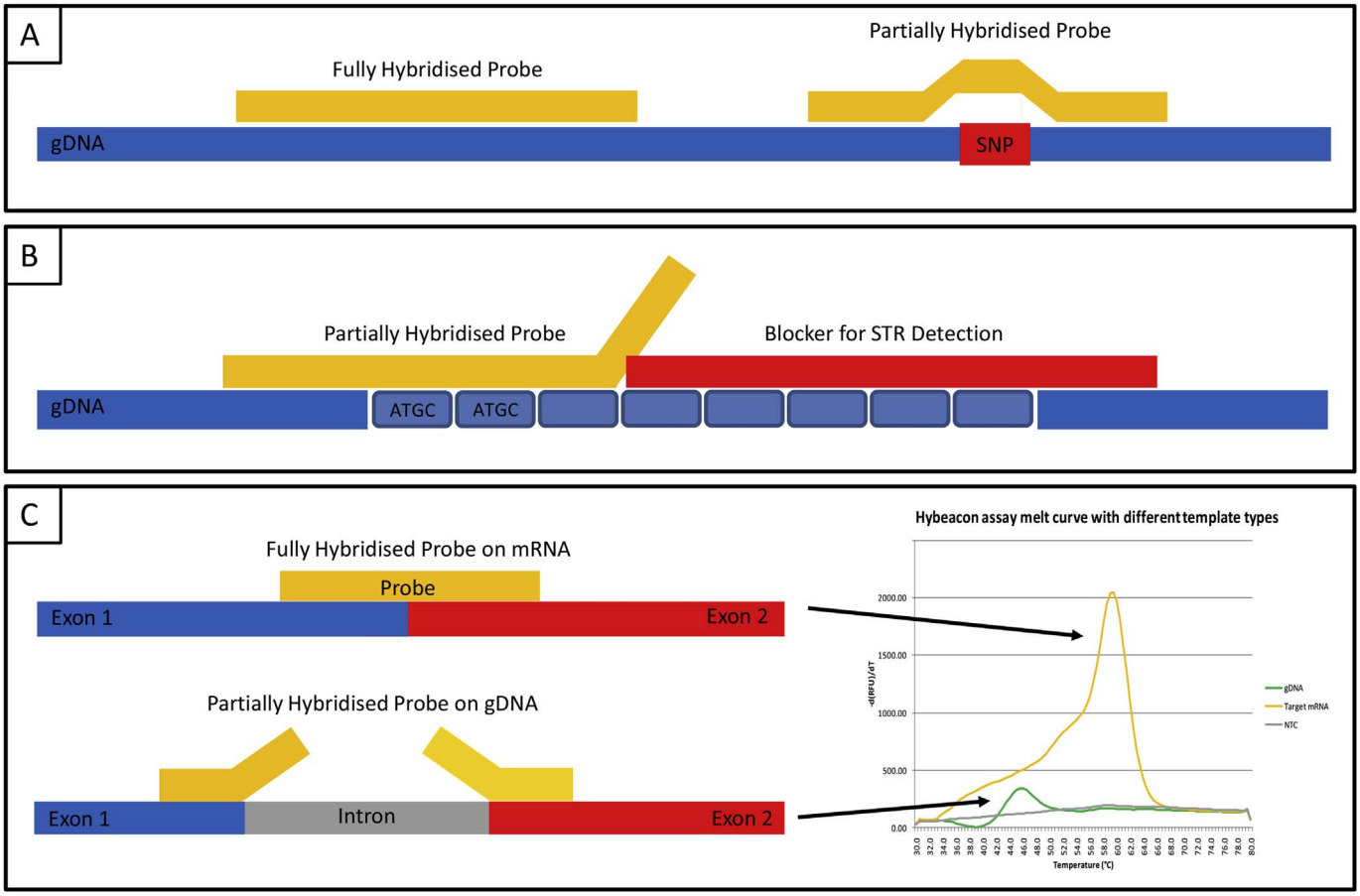

Fig. 1. Diagram showing different HyBeacon approaches for DNA identification A) SNP detection as used in medical and food authentication applications, B) STR detection with the use of a blocker oligo, and C) the proposed approach for detection and differentiation of gDNA and mRNA.

identification of sperm [15]) to identify a body fluid. However, these tests can be time consuming, require expert interpretation, use hazardous chemicals, and are subject to a number of false positives. In addition such tests are generally not human-specific, although antibody based tests that have fewer false positives do exist for some sample types [16,17]. Research in this field has seen a steady progression towards assessing and understanding the utility of messenger RNA (mRNA), DNA methylation profiling [18–20], micro RNA (miRNA) [21–23] and microbial markers [14] to confirm the presence of forensically relevant body fluids on evidence items, such as swabs from sexual assault kits [14]. Today mRNA detection is an extensively researched method and a number of mRNA markers for forensically-relevant body fluids, such as saliva, seminal fluid, blood, menstrual blood and cervicovaginal fluid (CVF) have been identified [24–30]. A selection of these identified mRNA markers has been tested through European DNA Profiling Group (EDNAP) exercises [31–35]. Increasingly, forensic laboratories are beginning to offer mRNA profiling as a routine part of casework services [36,37]. While these developments address the specificity and sensitivity issues of simpler detection methods, new issues arise out of the more complex lab procedures required to isolate mRNA and remove any contaminating genomic DNA (gDNA), generate complementary DNA (cDNA) through Reverse Transcription (RT), amplify the resulting cDNA, and then differentiate the fragments, usually accomplished by capillary electrophoresis (CE) or high resolution melting (HRM) [38].

The specificity of a HyBeacon probe to its complementary sequence and its detection using melt curve analysis may solve many of the processing issues currently encountered by laboratories performing this service, and also serve to demonstrate the wider applicability of HyBeacon detection to mRNA. Positioning the probe such that it spans an exon:exon junction in mature mRNA (see Fig. 1C) will allow differentiation between gDNA and mRNA. Where the intron is present in the gDNA, the probe will hybridise to a reduced number of nucleotides, resulting in a melt peak with a lower melting temperature (T_m) than one where the probe is fully hybridised to the target sequence. This allows the specific detection of mRNA targets in a sample where the gDNA is still present. The development of a one step approach to RT-PCR followed by melt curve detection, without further sample manipulation, further simplifies this process, increasing the usability for non-specialists.

The aim of this work was to develop a one-step RT-PCR process that would allow for the identification of body fluids from RNA extracted samples with gDNA still present, and that could be performed on a standard PCR and fluorescence detection platform. The single-step process would reduce the cost and time required for a result and would also allow for the RT and PCR steps to occur in a single tube, reducing the complexity of the process. The eventual scope of this work is to develop a simple system where a user can directly sample from a crime stain of interest and identify the body fluid present without any further manipulation of the sample or extract, similar to the generation of a DNA profile directly from evidence items using the ParaDNA Intelligence Test [4].

Here we present data on the initial assessment of the utility of HyBeacon probes for use in mRNA gene expression detection using the forensically relevant system of body fluid identification.