Combinatorial DNA barcoding for security applications

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by

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Abstract

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Forensic tagging has been widely used in recent years – from the use in large companies or institutions, to preventing large scale cable theft to private home owners who want to protect their property. Such tagging can be done by applying a solution containing a unique DNA code to devices, doors or valuable objects. Another method for forensic tagging is a spraying device which also deploys a unique code onto intruders. This code can then be decoded and used to create a link between criminal and the house/institution where the device was deployed or the solution applied to property. The market for such forensic applications within the UK is largely dominated by SmartWater® and SelectaDNA®. However, both companies base their decoding method on the time-consuming and expensive technique of sequencing. In collaboration with the security company Spyral®, we have developed a combinatorial system which exploits the advantages of Real-Time PCR (qPCR). Synthetic oligonucleotide constructs were designed, consisting of primer pair amplification sites and a target site, the latter to which a fluorescent probe can bind. The original approach for the target site detection involved HyBeacons® which are already extensively used in forensic science. After encountering many difficulties using these probes, the experimental approach was changed and TaqMan® probes were used which are already well-established for qPCR experiments. Having established a stable detection system using TaqMan®, it was shown that, despite limitations, mixtures containing several oligonucleotides could be decoded. The final series of experiments showed a positive outcome for deployment and subsequent recovery of the oligonucleotides.

Acknowledgement

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BHQ®	Black Hole Quencher
EDTA	Ethylenediaminetetraacetic Acid
FAM™	6-Carboxyfluorescein
HT	Hybridisation Target
HB	HyBeacon®
JOE™	6-Carboxy-4',5'-Dichloro-2',7'-Dimethoxyfluorescein
MGB	Minor Groove Binder
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
qPCR	quantitative PCR
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeat
TAMRA™	5-Carboxytetramethylrhodamine
TBE	Tris-Borate EDTA
TMAC	Tetramethyl Ammonium Chloride
TMT	10 mM Tris

Chapter 1: Introduction

1.1 Underlying Concept

Burglaries still pose a problem to the police, despite their decreasing numbers (Table 1). With often rather inventive methods, offenders avoid being apprehended in possession of evidence when burglarising commercial premises as well as in cases of cable theft from train tracks or telephone network installations. This often leaves the police unable to place offenders at the crime scene, despite having reasonable cause to suspect their guilt, and companies unable to track down their stolen property. A technical solution that tags offenders at the crime scene, could address this aspect of commercial property theft.

Table 1.1: Trends in CSEW incidents of crime from 1995 to year ending September 2014, with percentage change compared to the numbers of Oct-13 to Sep-14[1]

England and Wales

Offence group	**Oct-13 to Sep-14**	**Oct-12 to Sep-13**	Jan-95 to Dec-95	Apr-01 to Mar-02	Apr-05 to Mar-06	Apr-10 to Mar-11	Apr-11 to Mar-12	Apr-12 to Mar-13	Apr-13 to Mar-14
			Number of incidents (000s):						
Domestic burglary	**789**	**856**	**2,389**	**1,405**	**1,024**	**1,032**	**922**	**888**	**785**
%		-8	-67	-44	-23	-24	-14	-11	1
Other household theft	**777**	**857**	**1,570**	**967**	**812**	**938**	**1,120**	**1,017**	**777**
%		-9	-50	-20	-4	-17	-31	-24	0
Vehicle-related theft	**878**	**1,035**	**4,266**	**2,465**	**1,674**	**1,172**	**1,199**	**1,020**	**934**
%		-15	-79	-64	-48	-25	-27	-14	-6
Bicycle theft	**398**	**393**	**660**	**356**	**421**	**515**	**443**	**452**	**376**
%		1	-40	12	-6	-23	-10	-12	6
CRIMINAL DAMAGE	**1,393**	**1,639**	**3,300**	**2,601**	**2,643**	**2,133**	**1,999**	**1,739**	**1,451**
%		-15	-58	-46	-47	-35	-30	-20	-4
Unweighted base - number of households	*34,513*	*35,267*	*16,310*	*32,755*	*47,768*	*46,728*	*45,998*	*34,851*	*35,339*
ALL CSEW CRIME	**7,027**	**7,920**	**19,109**	**12,366**	**10,581**	**9,446**	**9,345**	**8,487**	**7,333**

1. **Adapted from data from the Office for National Statistics licensed under the Open Government Licence v.3.0.** *(http://webarchive.nationalarchives.gov.uk/20160105160709/http://www.ons.gov.uk/ons/publications/re-reference-tables.html?edition=tcm%3A77-373433)*

In recent years, the forensic product market has approached this problem by introducing the concept of forensic tagging of property. Hereby, the property is tagged with a uniquely coded material (often a DNA-containing suspension) that identifies the object and its location and will tag (by active or passive transfer) any person removing it. Once an item has been stolen, it is easier for the police to follow the trace left by the tagged object and offender, particularly if the coded material can be made visible on the intruder, for example using UV light. If the offender is able to dispose of the stolen items before being apprehended, the transferred material will provide the police with evidence placing them at the deployment scene. Market leading companies, such as SmartWater® or SelectaDNA®, have specialised in forensically tagging items with coded materials to deter criminals, and also assist recovery. However, these applications of forensic tagging mostly involve pre-marking items that may be stolen, rather than marking them (and the criminal) at the time of the burglary. Spyral® is approaching the problem differently by creating a device combining a camera, a motion sensor and a spray containing a unique combination of DNA constructs which is dispersed onto the intruder and property (Fig 1). In doing so, the offender is not only uniquely connected to the stolen property but also to the location of burglary, thus creating an evidentiary connection that will support prosecution.

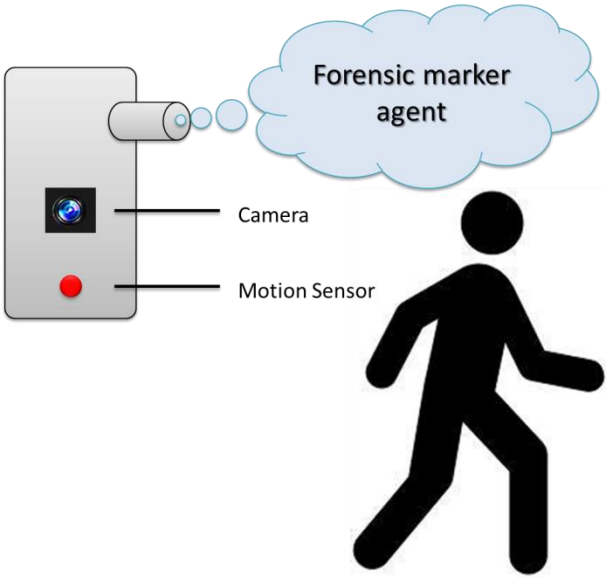


Figure 1.1: Spyral® security device The device is comprised of a motion sensor, a camera and a pressurised chamber containing the marker solution that is dispersed into the room once the device is triggered.

Furthermore, the device can be connected to security monitoring services or the police via wireless LAN, such that intrusion and device activation will be directly reported to the police, enhancing response times. This is a significant advantage for the

detection of the marker because the intruder has less time and opportunity to eliminate the evidence and potentially increases the chances of apprehending the intruder at or near the crime scene. It is likely that these features (automatic reporting, collection of evidence placing the intruder at the scene and the deployment of a unique marker) will greatly enhance the deterrent effect of such forensic tagging systems.

Both, SmartWater® and SelectaDNA® have developed a spraying device. SelectaDNA® offers several products for marking objects and interiors, e.g. mini kits for private individuals to apply to their own property and a gel or grease for larger areas. Furthermore, a spraying unit and an aerosol can be acquired. The aerosol is the closest equivalent to the product planned by Spyral®. It contains a unique code, as well as a UV-light-visible marker, which indicates the intruder's exposure to the marking agent. Once the unit is activated by a triggered alarm, the aerosol is deployed. However, this deployment can only take place once; a customer would always need to have several aerosol bottles in stock in case of multiple activations. The information given on their websites does not specify how the unique code is deciphered or whether each bottle contains a separate unique code. This can, however, be found in the respective patents.

SmartWater® is the leading crime deterrent company in the UK. Similar to SelectaDNA® it offers property marking and spraying devices. The spray devices include UV light marker and plant DNA (SelectaDNA®) or synthesised DNA (SelectaDNA® and SmartWater®) to create unique codes which are subsequently identified via sequencing (Cleary, 2003; Knights *et al.*, 2013).

The underlying idea for all these products is a unique code which links an intruder doubt-free to either the burgled premises or the stolen objects. The approach taken by Spyral® is to link an intruder to a burgled house. The more houses are equipped with the device, the more unique codes need to be generated. The synthetic DNA strands generated by other companies will eventually grow larger in size with increasing demand to continue exclusive codes. As will be shown in the following sections, due to the sequencing decoding method, larger sizes can significantly increase cost and time factors. In contrast, Spyral®'s approach of a cheaper and faster combinatorial system will be able to generate enough unique codes to cover all 1.75 million postcodes in the UK (Office for National Statistics, http://www.ons.gov.uk/ons/guide-method/geography/beginner-s-

guide/postal/index.html, Adapted from data from the Office for National Statistics licensed under the Open Government Licence v.3.0) and can easily be extended.

1.2 Experimental Approach

Thanks to Sir Alec Jeffreys FRS who identified the potential of tandemly repeated "minisatellites" in the human genome to be individual-specific genetic fingerprints, and the subsequent development of short tandem repeat (STR) DNA profiling, forensic science has been able to use DNA to solve criminal cases for 30 years (Gill *et al.*, 1985; Jeffreys *et al.*, 1985). Biological samples removed from crime scenes were analysed in laboratories and the genetic fingerprint obtained could then be matched to an individual. Originally, these analyses were done by southern blotting but today STR profiles are obtained via Polymerase Chain Reaction (PCR) and capillary electrophoresis (CE) (Butler *et al.*, 2004; Butler, 2005).

Furthermore, human identification can also be done by sequencing specific regions in the genome. Since first described in 1977 (Sanger *et al.*, 1977), the method of DNA sequencing is nowadays used in many different ways, most prominently to sequence the entire human genome (Mardis, 2008; Lander *et al.*, 2001). Nevertheless, despite vast technical improvements in cost and throughput the process itself is still laborious and slow for the 3 billion basepair (bp) human genome. While Next generation Sequencing (NGS) technologies have revolutionised the cost and rate of acquisition of DNA sequence data, only the nanopore sequencing systems of Oxford Nanopore Technologies (single molecule sequencing) promise sufficiently rapid data generation that their use in DNA detection in the field is feasible (Feng *et al.*, 2015; Berglund *et al.*, 2011). Also the low accuracy of these pre-commercialised systems is not compatible with the evidentiary demands of forensic DNA analysis (Bandelt & Salas, 2012).

Synthetic DNA can however be used to generate many different unique codes, which can be detected by PCR amplification and subsequently identified by sequencing. The previously mentioned companies SmartWater® and SelectaDNA® are using such synthetically created long DNA chains in their systems (Cleary, 2003; Brown & Reichert, 2010). Previous studies have also used multiplex PCR as a faster method to detect multiple genotypes simultaneously (Leneuve *et al.*, 2001; Zarlenga *et al.*, 1999). In multiplex PCR several primer pairs are used and has the potential to amplify over 10 regions in a genome. First described in 1988 (Chamberlain *et al.*, 1988) it is now used in a wide range of applications, including forensic analyses (Edwards & Gibbs, 1994).

Furthermore, with DNA sequencing it requires at least 24 hours to obtain results and it is costly relative to a PCR-based detection process which produces results within a few hours, materials are cheaper and potentially can be done "in-house", i.e. samples do not have to be sent off to an independent laboratory. It is thus desirable for a fast and affordable system to be able to use a straight forward approach such as PCR. The system described in this project is using quantitative PCR (qPCR) as a detection method with a combinatorial DNA-based marker system.

Due to their being four potential nucleotides to use in a synthetic oligonucleotide, these can be generated with unique codes or tags that scale to the 4^{th} power of their length (n) e.g. 4^4= 256 tags for a tetranucleotide. The combination then needs to be detected by DNA sequencing which is too time consuming and costly. By contrast, using a combination of limited numbers of pre-designed sequences, enables a combinatorial system to approach the diversity of DNA sequence tags. This system in which PCR simultaneously amplifies and decodes the tag present, would be far more rapid to decode and avoids DNA sequencing.

For example the pilot combinatorial system described here includes oligonucleotides comprised of 12 primer annealing sites and three different target regions, flanked by the primer annealing sites (Figure 2).The primer annealing sites only work in pairs, i.e. forward and reverse sites cannot be randomly mixed with one another. Both components combined, primer annealing sites and target regions, in all possible variations, result in 36 different oligonucleotides. The outer primer annealing sites are identifiable via PCR, whereas the intramolecular target regions can be distinguished with the help of hybridisation probes, containing fluorescent dyes, detectable via qPCR. The original approach was to combine primer annealing sites and target regions via PCR, thus those fragments are henceforth called constructs.

The target is the annealing site for the hybridisation probes. Oligonucleotides were designed with a target and a pair of primer annealing sites whereby the target sequences are complementary to one of three different hybridisation probes. There are 12 primer annealing sites complementary to 12 forward and reverse primer pairs which may be randomly combined with any of the three targets resulting in 36 constructs. For a given primer pair, one, two or all three targets can be combined in 7 different ways, differing in the target combination present (T1, T2, T3, T1+T2, T1+T3, T2+T3, T1+T2+T3)

(Figure 2b). On top of this, multiple primer pairs can be used in one mixture, e.g. for two primer pairs the combined output of each reaction can generate $7^2 = 49$ codes, scaling up to 7^{12} (1.38×10^{10}), unique codes determined by the combination of targets in each of 12 PCR reactions. This number is large enough to easily cover all 1.75 million postcodes within the UK and by simply adding one or even two more primer pair annealing sites, the system can grow up to 6.8×10^{11} unique codes.

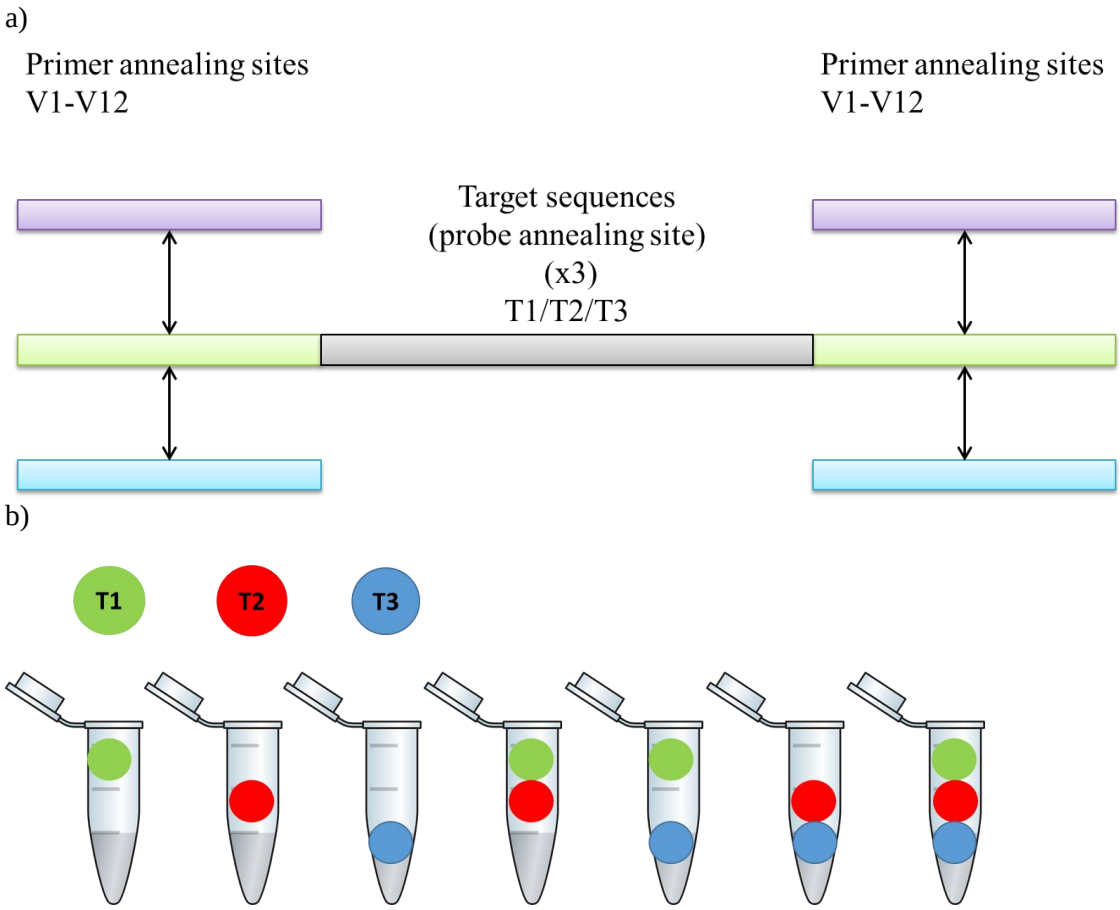


Figure 1.2: Combinatorial system for constructs
a) The diagram depicts a simplified version of the constructs. The 36 individual constructs are comprised of a target region to which the fluorescent probes will bind and two flanking primer annealing sites. There are three different targets and 12 different primer annealing sites. The arrows indicate exchangeable parts of the constructs. b) The three targets, each represented by a coloured dot, can be present in the mixture individually or groups, resulting in a total of seven different combinations. The 36 constructs, consisting of the primer pair annealing sites and the target region, can be combined in such a manner. When adding the factor of exchangeable primer pair annealing sites, this combinatorial approach can generate a vast number of unique combinations which are then easy to decode.

In the pilot project all 36 constructs were built via PCR using the "mega primer" approach. This involved extending oligonucleotide chains via PCR, resulting in fusing the core regions with the desired primer annealing sites (Sarkar & Sommer, 1990; Kammann *et al.*, 1989)**.** These constructs are 112 bp long, consisting of the primer annealing sites and the target. The target is defined as the HyBeacon® binding site (18 – 20 nucleotides) with 20 nucleotides flanking each side (Brown *et al.*, 2002). The synthesis of those constructs by PCR takes several steps and although inexpensive, is time consuming, and requires validation by cloning and sequencing the constructs. To simplify the construct production and decrease synthesis costs, the nucleotides between primer annealing sites and HyBeacon® binding sites were removed, resulting in approximately 60 bp fragments. The resulting synthetic constructs include one of the three target sequences, and vary in their length by one nucleotide; targets 1, 2 and 3 are 58, 59 and 60 nucleotides, respectively, in length. However they share the combinatorial properties of the original constructs and are decoded in the same way.

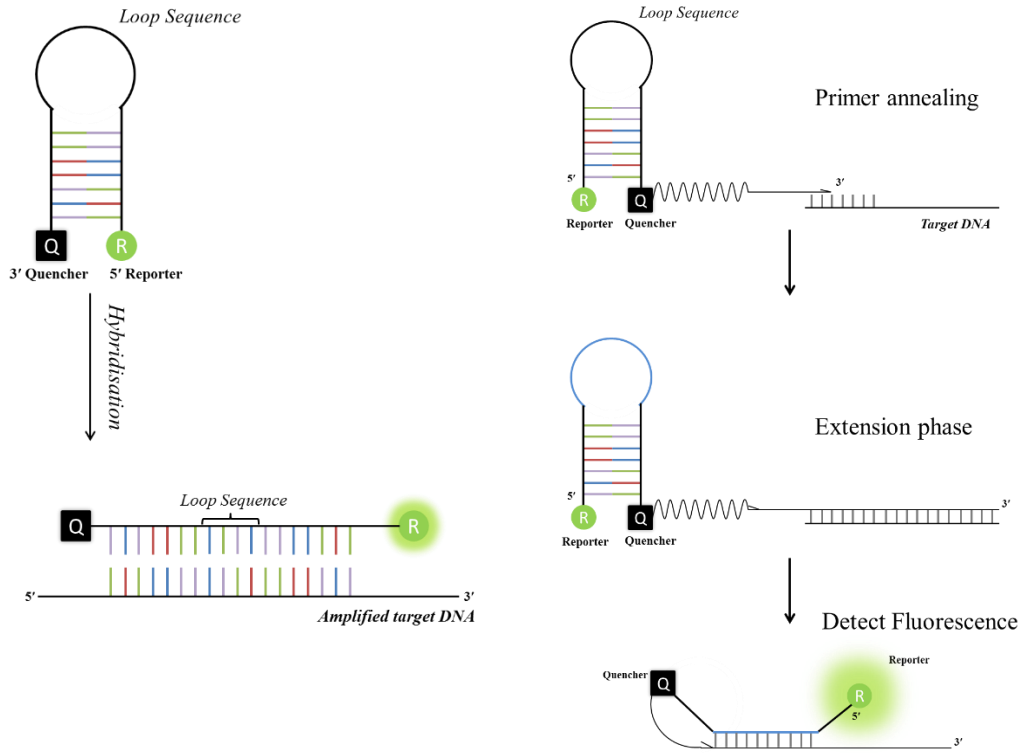
The aim of this was to show that it was possible to detect the presence of the three different target regions combined with the 12 primer annealing sites in as few steps as possible. This was achieved by combining the amplification and detection of the constructs using qPCR, with fluorescent detection of target sequences. The different fluorescent detection systems suitable for forensic applications with the respective PCR conditions are explained in further details.

1.3 HyBeacons®

Fluorescent tags have been used in Molecular Beacons and Scorpion primers to identify sequence change mutations in patient DNA (Thelwell *et al.,* 2000). Molecular Beacons possess a quencher and a fluorescent reporter, which becomes active only when the quencher is spatially separated from the fluorescent reporter (Fig 3a). Scorpion primers have a molecular beacon incorporated within them that simultaneously acts as a PCR block (Fig 3b) (Tyagi & Kramer, 1996; Whitcombe *et al.,* 1999). However, the interactions between fluorophore and quencher molecules can vary greatly, causing residual fluorescence that can lead to false-negative or false-positive results (Zhang *et al.*, 2001). In the early 2000s, David J. French *et al*. developed a new form of Molecular Beacon that does not possess a significant secondary structure named HyBeacons® (French *et al.,* 2002; French *et al.,* 2001). HyBeacons® are short single-stranded molecules with one or two internal fluorescent reporters and a 3′ phosphate which prevents the oligonucleotides from acting as primers in PCR. The molecules always show a small quantity of fluorescent emission, but when forming a double-stranded DNA molecule with the target sequence, the fluorescence emission significantly increases. HyBeacons® do not need a quencher moiety, as interactions between the dye and the nucleotide bases within the molecule when it is single stranded inhibit fluorescence. These interactions are destroyed upon hybridisation to the target region. This is thought to occur by exclusion of the fluorophore from the proximity of the nucleotide bases, when they base pair in the double helix, as illustrated in Figure 3c (Marks *et al.,* 2005; French *et al.,* 2008). This change in fluorescent emission enables the detection of the target DNA sequences by qPCR, by measuring fluorescence after every annealing step (Fig 3c).

a) Molecular beacon

b) Scorpion primer



c) HyBeacon®

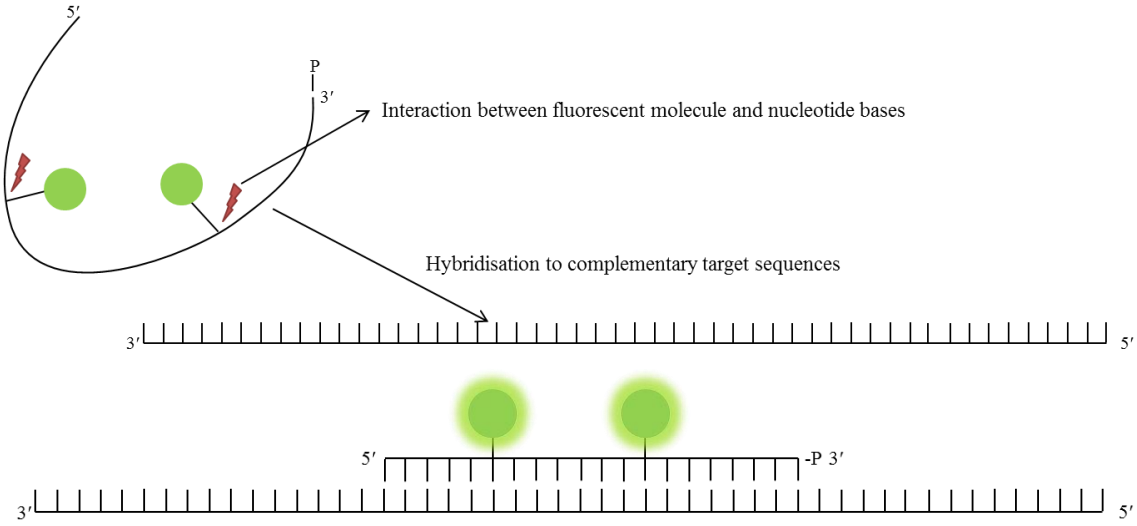


Figure 1.3: Molecular Beacons, their application and HyBeacons®
a) Molecular Beacons are comprised of a 3′ quencher, a 5′ fluorescent reporter and a hairpin. The beacons are only active when the quencher is spatially separated from the reporter after denaturation and binding to its target sequence.
b) Scorpion primers incorporate a molecular beacon and contain self-complementary sequences that protect the loop sequence that binds to specific target sequence.
c)HyBeacons® are labelled with dual fluorescent reporters that are naturally quenched by the probe itself, i.e. as long as the probe is in a single-stranded state the interactions between the nucleotide bases and the fluorescent molecules inhibit light emission. Through binding to the probe's target sequence, these interactions are lost and thus the dyes can emit light, which is then measured.

Detection of target DNA sequences through their interaction with complementary HyBeacons® can also be performed by melting curve analysis. The melting temperature (Tm) is dependent on the complementarity of HyBeacon® and target sequence; it decreases with increasing mismatches between the sequences. Originally, HyBeacons® were created to identify single nucleotide polymorphisms (SNPs) in genomic DNA (French *et al.*, 2001). Development of the systems now enable SNP analysis directly from saliva, buccal swabs and blood samples, without DNA extraction, making them an ideal tool for fast analyses of DNA variants, and an alternative to Molecular Beacons or Scorpion primers (French *et al.*, 2002).

HyBeacons® are intended for allele discrimination and DNA quantification, performed by endpoint analyses, i.e. melt curve determination (French *et al.*, 2001), and are currently used in the ParaDNA® system. This system allows rapid analysis of the quality and quantity of DNA within body fluid or forensic samples. This is achieved by amplification and melting curve analysis of two standard forensic Short Tandem Repeat (STR) loci (D16S539 and TH01) as well as the amelogenin locus, used for sex determination. Positive results using ParaDNA for the mentioned criteria indicate that full forensic DNA profiling is viable from the input samples. To date, ParaDNA® is the only pre-screening tool of this kind on the market (Liu, 2014).

1.3.1 Fluorescent reporter dyes

For experiments investigating several targets as in this project, it is crucial to choose fluorescent dyes that are within the qPCR machine's detection capability and are compatible with one another, i.e. their spectra do not coincide. The three fluorescent dyes selected for this project are FAM™ (6-Carboxyfluorescein), JOE™ (6-Carboxy-4',5'-Dichloro-2',7'-Dimethoxyfluorescein) and TAMRA™ (5-Carboxytetramethylrhodamine). These three dyes were chosen because their emission spectra do not overlap greatly, enabling the discrimination of their presence alone and in any combination. In addition the qPCR instrument used in this study, and commonly used in Forensic DNA laboratories (Applied Biosystems 7500/7500Fast Real-Time PCR) is able to excite and detect these fluorescent dyes (Fig 4).

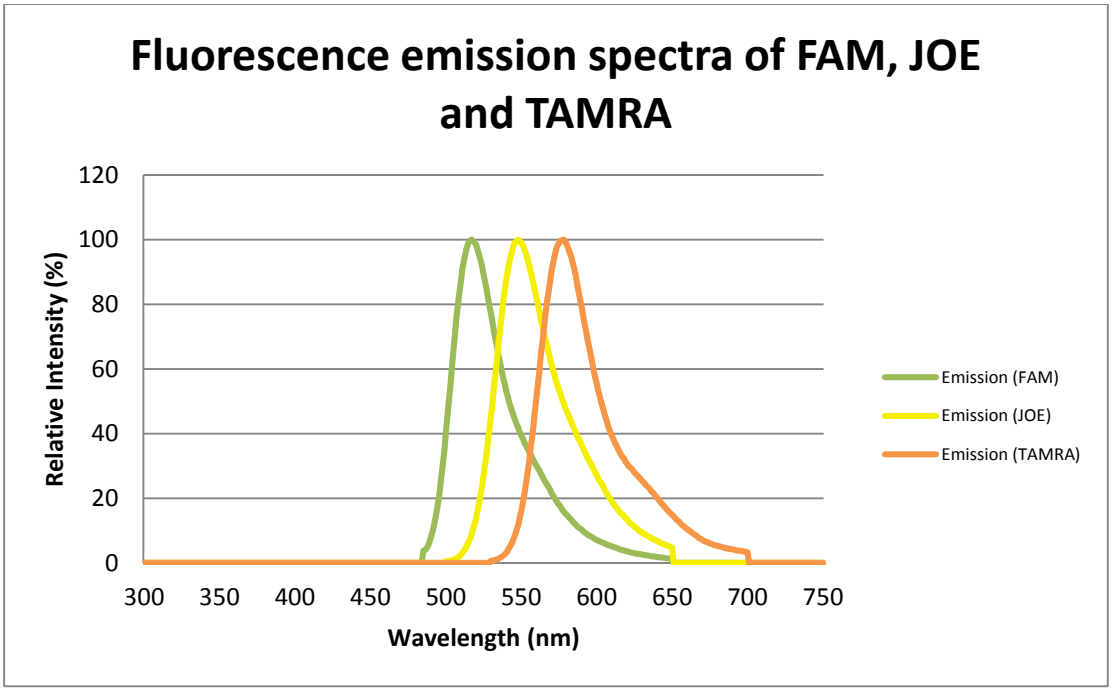


Figure 1.4: Fluorescence emission spectra of fluorescent dyes
The fluorescent dyes used for the three hybridisation probes were FAM™, JOE™ and TAMRA™. Values are taken from the atdBio website http://www.atdbio.com/content/33/FAM-fluorescein-HEX-JOE-ROX-TAMRA-TET-Texas-Red-and-others and the Fluorescence Spectra Viewer by Thermo Fisher Scientific Inc. (http://www.thermofisher.com/uk/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html) .

1.4 TaqMan®

TaqMan® probes are short DNA hybridisation probes used in PCR detection of specific DNA sequences. The principle of detection relies on the 5′-3′ exonuclease activity of *Taq* DNA polymerase that liberates a reporter molecule when the probe is degraded. The technique was first described by Holland *et al.*, in 1991 using radioactively labelled reporters. The labelled probes were degraded by the *Taq* polymerase into smaller pieces which could then be distinguished from non-degraded probes. This method was further developed to utilise fluorescent dyes (Holland *et al.*, 1991; Gelfand *et al.*, 1993; Lee *et al.*, 1993). A short probe (18-22 nt) which is complementary to a target sequence is designed, containing a 5′ fluorescent reporter and a quencher at its 3′ end. The proximity of the quencher and fluorophore prevent emission when the probe hybridises to the target sequence. Extension of a primer upstream of the probe leads to the 5′-3′ exonuclease activity of *Taq* DNA polymerase removing nucleotides at the 5′ end of this double-stranded molecule. This releases the reporter from proximity to the quencher and a fluorescent signal can be detected (Holland *et al.*, 1991; Lee *et al.*, 1993). In this system, the reporter is a fluorescent molecule and the quencher can either be a dye, such as TAMRA™ or Dabcyl, or a so-called dark quencher, e.g. Black Hole Quencher®, but the principle of the technique is the same (Fig 5). Until recently, TaqMan® probes were protected by patents (EU) (Gelfand *et al.*, 1993).

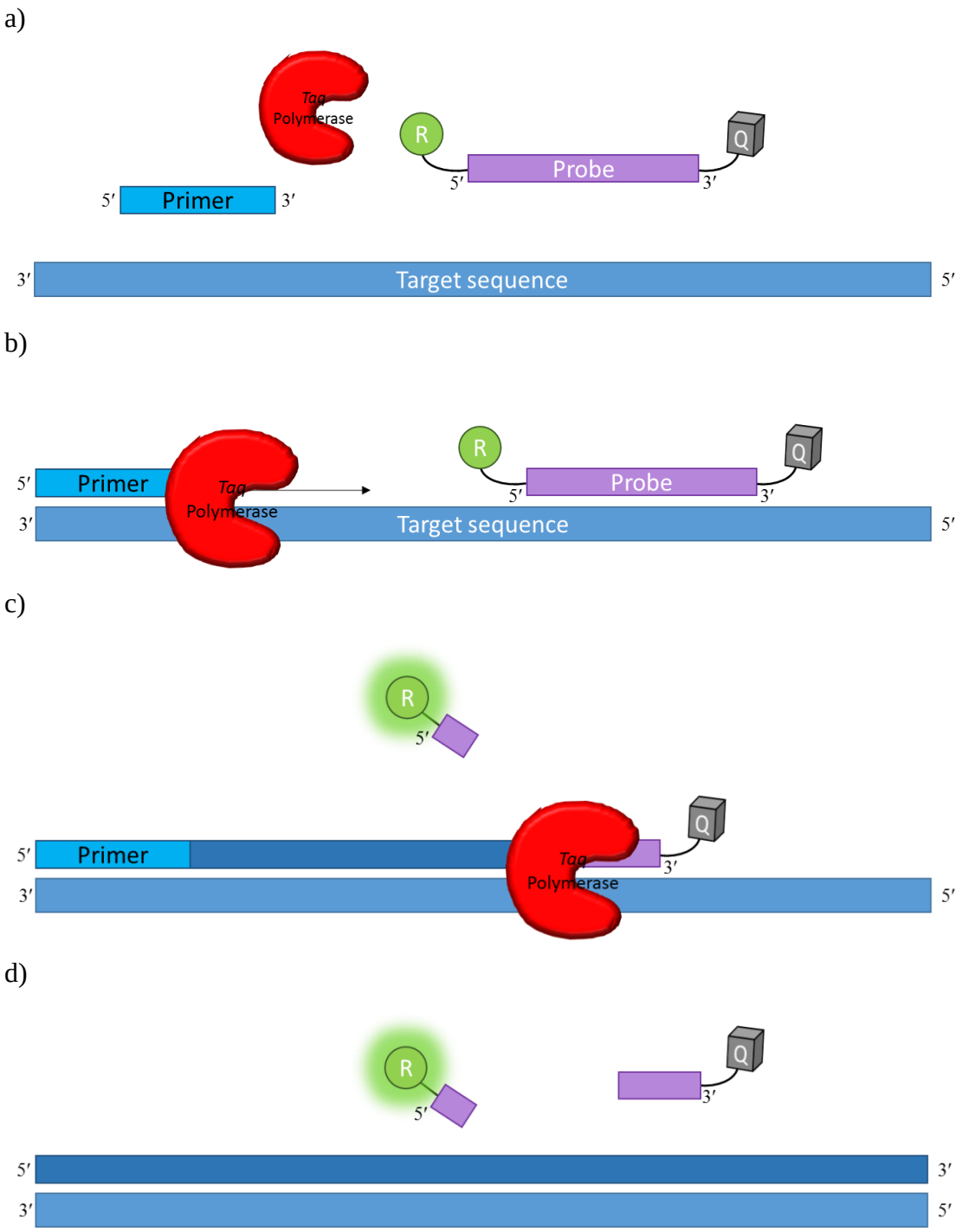


Figure 1.5: Principle of TaqMan® probes target sequence detection
a) The TaqMan® probe is added to the PCR mix and is complementary to the target sequence. It comprises a 3′ quencher and 5′ fluorescent reporter. b) When primers anneal to their complementary sequence, so will the TaqMan® probe. c) During the elongation step, when Taq DNA polymerase synthesises the complementary strands, the enzyme will degrade the probe starting at its 5′ end. As the nucleolytic cleavage is at the phosphate backbone neither the fluorescent dye nor the quencher is affected by the enzyme activity. d) Once the quencher is separated from the reporter, the latter can emit light when excited by a laser at its excitation frequency.

1.4.1 Quencher

As described, single-stranded DNA detecting probes generally comprise a 5′-fluorescent reporter and a 3′-quencher. The latter can be fluorescent itself (usually TAMRA™ or Dabcyl) or be a "dark" (non-fluorescent) quencher (Johansson & Cook, 2003; Yang *et al.*, 2009; Livak *et al.*, 1995). The principle of these quenching mechanisms is based on FRET (Fluorescence Resonance Energy Transfer), i.e. electron-donating and –accepting. In the probe's "dark state", quencher and fluorescent reporter are in close proximity and the quencher absorbs the reporter's fluorescence. When the probe binds to a target sequence, the resulting conformational change (Molecular Beacons or Scorpion Primers®) or enzymatic cleavage (TaqMan®) removes the quenching effect and a signal is transmitted (May *et al.*, 2003). The problem with fluorescent quenchers is the possible overlap of both reporter and quencher emission spectra, resulting in high background noise. The advantage of using dark quenchers consequently is that there is no acceptor fluorescence (quencher) and only the donor fluorescence (reporter) is measured. There are several dark quenchers on the market, such as Black Hole Quenchers® (Johansson & Cook, 2003), Epoch Dark Quencher™ (Daum *et al.*, 2004) and minor groove-binding quenchers (Afonina *et al.*, 2002). However these molecules are protected by patents and require licensing agreements for commercial use.

In order to fully exploit all advantages of qPCR and secure the best possible outcome, not only the fluorescent reporter probes need to be carefully chosen but also the DNA polymerase. Hot start enzymes are commonly used in forensic laboratories (Butler, 2005) and in view of the nature of the presented project, all experiments should be designed as closely to forensic standards as possible.

1.5 “Hotstart” *Taq* DNA polymerases

The polymerase chain reaction (PCR) is an important tool in all molecular biology laboratories, especially in forensic settings (Butler, 2005; Saiki *et al.*, 1988). Hot start PCR was introduced to reduce mis-priming and primer dimerisation during the amplification of low copy number targets, as *Taq* DNA polymerase shows some non-specific activity below the commonly used extension temperature at 72 °C. Originally, a hot start PCR meant that all reagents for the reactions are only mixed when a certain temperature was reached, i.e. by opening the reaction tubes during the process and risking contamination (Chou *et al.*, 1992). This obstacle was overcome by adding a “wax vapour barrier” between the reagents, which would melt at a specific temperature, and result in a temperature-dependent mixing of all PCR reagents (Chou *et al.*, 1992).

An alternative approach is to modify the DNA polymerase to inhibit its activity prior to the initial denaturation step of the PCR. This can be achieved by binding the enzyme to specific antibodies which act as thermolabile switches; the polymerase’s activity is inhibited at low temperatures by the antibody binding to the enzyme’s active site, and at high temperatures the active *Taq* polymerase is released when the antibodies are denatured, e.g. Platinum®*Taq* (Sharkey *et al.*, 1994). Another alternative is a chemical modification of the enzyme that blocks the enzyme’s activity at lower temperatures. The modification is removed when the polymerase is exposed to higher temperatures, e.g AmpliTaq®Gold (Birch & Laird, Walter J., Zoccoli, Michael A., 1997; Birch, 1996). A third alteration could be an additional non-antibody protein which acts as thermolabile blocker, eg. Paq5000™ Hotstart. This protein binds to a primed polynucleotide template and thus blocks the elongation by the DNA polymerase. When exposed to higher temperatures, the thermolabile blocker is inactivated and amplification can ensue (Borns, 2007).

1.6 Patents

Additional to the forensic background of this project, the commercial association needs to be considered as well. SmartWater® and SelectaDNA® have their products protected by patents, i.e. no other company can use their products and systems without a licence, agreed to by the owner of the patent. Spyral® is aiming to bring the product to market and it is thus desirable to protect their product by patent as well.

In the UK a patent can last up to 20 years as long as renewal fees are paid every year. Patents can be acquired for products or processes that contain new functional or technical aspects. They explain how inventions work, how they are made and/or what they are made of. While applying, the invention should not have been publicly discussed or been published because this may result in the patent being declined (Contains public sector information licensed under the Open Government Licence v3.0 http://www.ipo.gov.uk/).

Several different patents exist which relate to products similar to the invention developed here. Two of them are directly related to this project. The first one relates to the principle of marking an intruder, hereafter named DNAMARK, and is owned by Crime Solutions (Brown *et al.*, 2002). The second is an alternative to the use of HyBeacons® using simple oligonucleotide chains to introduce a length variant in all oligonucleotides and their detection via capillary electrophoresis (Wetton & Hopwood, 2011). This patent was owned by the Home Office, and lapsed with the closure of the Forensic Science Service (FSS). DNAMARK describes a device that can be installed in commercial properties and in private houses to identify an intruder by spraying a solution onto the intruder and taking their picture. The marker system is comprised of a primer pair capable of amplifying the deployed double-stranded DNA fragment which can be detected via ultraviolet or infrared light. The marker is similarly constructed to the Spyral® design. It contains a target, two primer regions and two additional sequences that separate the target from primer regions.

Chapter 2: Material and Methods

2.1 PCR-built construct validation

2.1.1 Construct extraction from plasmid

The 112 bp constructs already existed in pGEMT plasmids. The fragments were extracted from the plasmids by amplification. Two primers annealing in the flanking plasmid-DNA (RBM13F and RBM13R) were used and the resulting PCR products were purified, quantified and henceforth used as experimental input. PCRs were set up in 10 µl volume with 1x PCR buffer (45 mM Tris HCl, 11 mM NH_4SO_4, 6.7 mM β-mercaptoethanol, 113 µg/ml BSA, 4.5 mM MgCl2, 4.4 µM EDTA and 1 mM dNTPs), with the primers at a final concentration of 0.5 µM and the *Taq* polymerase (KAPA BIOSYSTEM, Inc., Wilmington, MA) at a final concentration of 0.02 U/µl. For each reaction 20 ng plasmid DNA was added. In order to confirm the success of the reaction, the samples were run out on 3% (w/v) LE agarose gels (Lonza) containing 0.5 × TBE buffer (Tris-Borate, EDTA). DNA was visualised via gel electrophoresis, under UV transillumination by incorporating ethidium bromide at a concentration 0.5 ug/ml in both the gel and the running buffer. Only 1µl of the PCR product was used for this procedure because the reactions were further used. All sequences can be found in the appendices.

2.1.2 PCR purification

The PCR products were purified according to the modified Zymoclean™ gel extraction procedure. 300 µl of 6 M NaI (kept at 4 °C) were added to the PCRs, mixed and the total volume was then added to a Zymoclean™ column. To bind the solution to the column, the samples were spun at 10 000*g* for 1 minute and the flow through was discarded. Subsequently, 500 µl of Solution E (50% (v/v) Ethanol, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA) were added and after an incubation time of 5 minutes at room temperature, samples were spun at 16 000*g* for 1 minute. The washing step was repeated, followed by a final spin to dry the column. The columns were put in clean tubes and the DNA was then eluted in 6 µl preheated (at 65 °C) TMT (10 mM Tris-HCl pH 8.8). Samples were left at room temperature for 1 minute before being spun at 16.000*g* for 1 minute. The last step was repeated twice to obtain a final volume of approx. 12 µl.

2.2 Allele-specific Oligonucleotide Hybridisation

5 × loading dye (30% (v/v) glycerol, 0.5 × TBE, 0.02 % (w/v) bromophenol blue) and 5 × the reaction volume of denaturing solution (0.5 M NaOH, 2 M NaCl, 25 mM EDTA) were added to a 10 µl PCR. DNA was transferred to a nylon membrane (MAGNA Nylon Membrane Filters), lying in a dot blotting apparatus, attached to a vacuum pump (Fig 6). Subsequently, 2 × SSC (saline sodium citrate buffer) was added to neutralise DNA. Finally, the membrane was dried at 80 °C for 10 minutes and cross-linked in a CL-1000 Ultraviolet Crosslinker UVP. UV crosslinking immobilises the DNA to the nylon membrane through covalent binding of DNA to membrane.

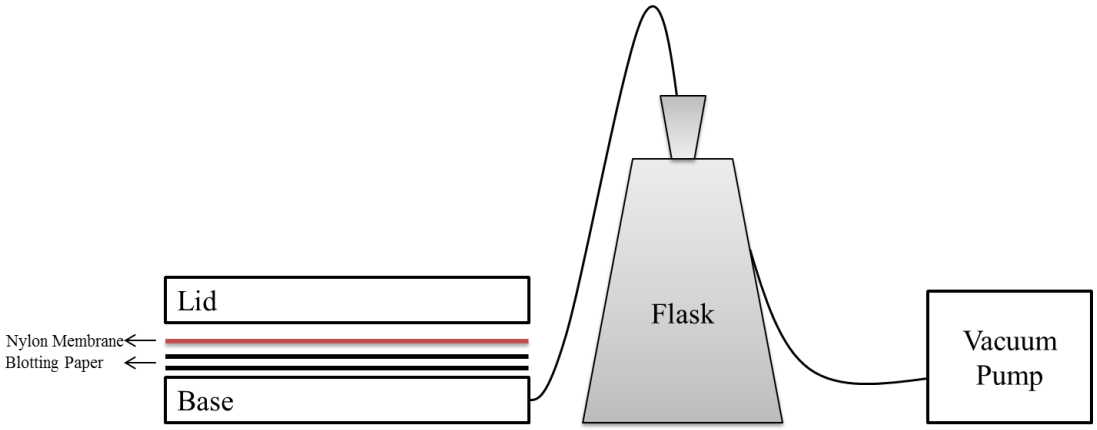


Figure 2.1: Dot blotting apparatus
The dot blotting apparatus is connected to a vacuum pump via a flask which collects all liquid waste. The apparatus has a base and a lid. Between those two components the 3mm paper and the nylon membrane are placed, the membrane on top. The samples are loaded onto the membrane via holes found in the lid, arranged to fit a 96-well plate.

Three oligonucleotides matching the targets served as labelling oligonucleotides, HT1, HT2 and HT3 for target1, 2 and 3, respectively. Before use, the probes (1x T4 buffer, 3.5U T4 polynucleotide Kinase, 1.4µM oligonucleotide, 0.12µl γ^{32}P-ATP [0.04Mbq]) were left at 37°C for 1 hour or more to label. The reaction was stopped by adding 20µl Kinase stop solution (25 mM diNa EDTA, 0.1% SDS (w/v), 10 µM ATP).

Hybridisation and washing steps were performed in Thermo® hybridisation bottles using Thermo-Hybaid hybridisation ovens. Membranes were pre-hybridised in 3ml TMAC hybridisation solution (3M TMAC, 0.6% SDS (w/v), 1 mM diNa EDTA, 10 mM Na phosphate pH 6.8, 5x Denhardt's solution (1% Bovine S erum Albumin (BSA), 1 % Ficoll, and 1 % Polyvinylpyrrolidone (PVP)), 4 µg/µl yeast RNA) for 5-10 minutes

at 53°C. After adding a competitor ASO, to repress mismatched hybridisation, the actual radioactive labelled ASO was added to the membrane and hybridised for at least one hour at 50°C. Membranes were washed twice in 3 ml TMAC wash solution (3M TMAC, 0.6% SDS (w/v), 1mM diNa EDTA, 10 mM Na phosphate pH 6.8) for 5 minutes at 50°C, followed by a final wash of 10-15 minutes with 5 ml TMAC wash solution at 53°C. The membranes were then rinsed several times with 3x SSC before put into a cassette with a phosphorimager screen and exposed overnight at room temperature. Data were analysed by scanning the phosphorimager screen with the Typhoon 9400 (GE Healthcare, Life Sciences).

2.3 HyBeacon® approach

2.3.1 Oligonucleotide design

Primers

Varying regions flanking the target core had been previously designed by Nurul Hamidi, a former postgraduate student of Dr Richard Badge who built the PCR-based constructs. As already shown, the first three primer pairs have been successfully established for the PCR. The subsequent 9 primer pairs were ordered according to the given sequences. All primer sequences can be found in Tab. 2. All primer pairs were designed to have similar features with melting temperatures ranging between 51 and 55°C. Stocks were kept at 100 µM with two dilutions of 50 µM. The primer sequences were designed with the help of primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and a Perl script (by Nurul Hamidi), generating a group of randomised sequences.

Table 2.1: Primer pairs designed for constructs
All primers have similar melting temperatures to ensure they all work under the same PCR conditions.

SYNTH NAME	SEQUENCE	Length nt	Tm °C	Primer pair combination
NH001	5′ CGTACGCGCCTTAAGTTTTC 3′	20	54	V1
NH002	5′ CGTTACCGACCCCTATAGCA 3′	20	53	
NH003	5′ GTGGACCGACTAGGGACAAA 3′	20	52	V2
NH004	5′ TAAATAGGTCGCCCACGTTT 3′	20	51	
NH005	5′ AGGTTACGTGGACTCCGTTG 3′	20	53	V3
NH006	5′ ATCTTGTCGGTACGGAGGTG 3′	20	53	
NH007	5′ GAATCCTAGGTCGACGCAAC 3′	20	53	V4
NH008	5′ CTCTTAGGTCCGCTCGATTG 3′	20	55	
NH009	5′ CAGGTCGCACGTAGTATCCA 3′	20	54	V5
NH010	5′ CTCCGGACGTTAGGACTTCA 3′	20	54	
NH011	5′ CACCGCCGATACTTAGTCGT 3′	20	54	V6
NH012	5′ TAACATCGGGCGTAGGTAGC 3′	20	54	
NH013	5′ CACCGAGTTAGACCGACCT 3′	20	54	V7
NH014	5′ GGGACCCGTCGGATTAGTAT 3′	20	52	
NH015	5′ GTCCGCCCGAGTCATAATAA 3′	20	52	V8
NH016	5′ ATCGGGCCGTTAGTAGGAAT 3′	20	53	
NH017	5′ TATAAGCGGCGTGGGATTAG 3′	20	53	V9
NH018	5′ CCTTCGCTCTACGTGAGTCC 3′	20	55	
NH019	5′ TTCGGACCTAGGCGTAGAAA 3′	20	53	V10
NH020	5′ AAGGGTCCCGCTAACGTATT 3′	20	53	
NH021	5′ CCCGATAGCACGTAGTCGAT 3′	20	55	V11
NH022	5′ CCGTGCGCGTTACTAAACTT 3′	20	54	
NH023	5′ TTAGCCTAAGCGGTCGAAAA 3′	20	53	V12
NH024	5′ CCAGGACCGTCGTAACAATC 3′	20	52	

Synthetic oligonucleotides

For all experiments using HyBeacon® probes, the original double-stranded 112bp long oligonucleotides were truncated to the shortest possible length, i.e. 58-60 nt depending on the length of the HyBeacon® annealing site (18-20 nt). This not only reduced manufacturing costs but also ensured a higher stability of the oligonucleotides after several thawing and freezing. For the first tests, oligonucleotides described in table 3 were ordered. These shortened oligonucleotides were ordered with ATDBio, School of Chemistry, University of Southampton, United Kingdom. A list of all designed oligonucleotides which are planned to be used for further experiments can be found in the appendix. The oligonucleotides were received at a very high concentration (between 200 and 1300 ng/µl), therefore they all were diluted down to a concentration of 20 ng/µl in 10mM Tris (TMT). For all qPCR assays, the oligonucleotides were further diluted down to 100 molecules/µl; this was achieved by creating several 1:10 dilutions until the samples reached the desired concentration.

Table 2.2: Sequences of all synthetic oligonucleotides
The sequences refer to synthetic oligonucleotides, ordered at ATDBio, Southampton. For first tests, only those constructs which already existed as a longer version were ordered with all targets. For all remaining primer pairs, only those combinations with Target1 (T1) were ordered. Target sequences are marked in red.

Name	Sequence	Length nt	Tm °C
V1T1	CGTACGCGCCTTAAGTTTTCCACTGTCAGTGTCTGACATGCTATAGGGGTCGGTAACG	58	74.5
V1T2	CGTACGCGCCTTAAGTTTTCTGAAGTCAGAGCTGGGAGTTGCTATAGGGGTCGGTAACG	59	75
V1T3	CGTACGCGCCTTAAGTTTTCAGGAGTGTAGCCTACCAGCATGCTATAGGGGTCGGTAACG	60	75.5
V2T1	GTGGACCGACTAGGGACAAACACTGTCAGTGTCTGACAAAACGTGGGCGACCTATTTA	58	73.8
V2T2	GTGGACCGACTAGGGACAAATGAAGTCAGAGCTGGGAGTAAACGTGGGCGACCTATTTA	59	74.3
V2T3	GTGGACCGACTAGGGACAAAAGGAGTGTAGCCTACCAGCAAAACGTGGGCGACCTATTTA	60	74.8
V3T1	AGGTTACGTGGACTCCGTTGCACTGTCAGTGTCTGACACACCTCCGTACCGACAAGAT	58	75.2
V3T2	AGGTTACGTGGACTCCGTTGTGAAGTCAGAGCTGGGAGTCACCTCCGTACCGACAAGAT	59	75.7
V3T3	AGGTTACGTGGACTCCGTTGAGGAGTGTAGCCTACCAGCACACCTCCGTACCGACAAGAT	60	76.2

HyBeacon® probes

The three HyBeacon® probes were also ordered with ATDBio, School of Chemistry, University of Southampton, United Kingdom. Each probe contained two intramolecular fluorophores, HBres0170 was labelled with FAM™ (6-carboxyfluorescein), HBres0359 with JOE™ (6-Carboxy-4',5'-Dichloro-2',7'-Dimethoxyfluorescein) and HBres0360 with TAMRA™ (Carboxytetramethylrhodamine). Table 4 gives sequences and location of the integrated fluorescent dyes of all three HyBeacon® probes.

Table 2.3: HyBeacon® probes ordered designed for the Spyral® Detection project

SYNTH NAME	SEQUENCE	Length nt	Tm °C	Relevant target
HBres0170	5'CACTG(FAMdT)CAGTG(FAMdT)CTGACAP3'	18	45.8	Target 1
HBres0359	5'TGAAG(JOEdT)CAGAGC(JOEdT)GGGAGTP3'	19	49.3	Target 2
HBres0360	5'AGGAG(TAMRAdT)GTAGCC(TAMRAdT)ACCAGCAP3'	20	52.5	Target 3

2.4 HyBeacon® based qPCR

qPCRs were performed using the Applied Biosystems 7500/7500Fast qPCR System (Applied Biosystems®, Warrington, UK). PCR reactions were set up in 20 µl with 1× KAPA *Taq* buffer A (750 mM Tris HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% (w/v) Tween 20, 15 mM $MgCl_2$), primers at a final concentration of 0.5 µM, all three HyBeacons® (Table 4) at 0.15 µM, 0.2 mM dNTPs and 0.02 U KAPA *Taq* polymerase (ANACHEM, Luton, UK). Negative controls contained 2 µl of PCR-clean H_2O (Sigma-Aldrich Co. LLC). For each reaction, 2 µl oligonucleotides diluted to 100 molecules/µl were added. After the initial denaturing step at 95°C for 30 seconds (s), the cycling stage followed comprised of 40× denaturation (95 °C 30 s), annealing (50°C 30 s) and elongation (67 °C 30 s) steps. Subsequent to the amplification, samples were denatured (95°C 30 s) and cooled (35 °C 30 s). Data were analysed with the qPCR Instruments - 7500 Fast System.

2.5 TaqMan® approach

2.5.1 TaqMan® probes and oligonucleotide design

TaqMan® probes require specific criteria for successful use; the probe's T_m should be 5-10 °C higher than the amplifying primers' T_m and cannot contain a triple-G residue at the 3′ end. To meet all these requirements, the oligonucleotides designed for the HyBeacons® system (58-60 nt) were found to be too short The original oligonucleotide sequences (112 nt) were thus re-evaluated and shortened to 82 nt for the TaqMan® based detection. These shortened oligonucleotides were ordered with ATDBio, School of Chemistry, University of Southampton, United Kingdom. For the initial TaqMan® testing, three different designs, all containing FAM™ as fluorescent reporter and detecting Target2 were planned and ordered (Table5).

Table 2.4: TaqMan® probes designed for the Spyral® Detection project
In order to ensure maximal efficiency for detection with TaqMan® probes, several designs for only one target were planned, all differing in Tm and length. Once the best conditions were evaluated, two more probes were designed for the remaining two targets.

SYNTH NAME	SEQUENCE	Length nt	Tm °C	Relevant target
MGB-NFQ	5'FAM AAGCAGTTGAAGTCA **MGB** 3'	15	64°C	Target2
MPTQ (BHQ1®) (Taqman2)	5'FAM ACCTCAGTCCAAGCAGTTGAAGTCAGA **BHQ1**3'	27	60.9°C	
NTTQ (BHQ1®)	5'FAM GAAGTCAGAGCTGGGAGTAG **BHQ1**3'	20	61.2°C	
Taqman1	5'JOE TACCACAAAATCTCACTGTCAGTGTCT **BHQ1**3'	27	57.7°C	Target1
Taqman3	5'TAMRA ACCCGACATGAGGAGTGTAGCCTA **BHQ1**3'	24	58.9°C	Target3

2.5.2 TaqMan® based qPCR

qPCRs were performed using the ABI® 7500/7500Fast instrument. PCR reactions were set up in 20 µl with 1× Ampli*Taq* Gold® Buffer I (150 mM Tris-HCl, pH 8.0, 500 mM KCl, 15 mM $MgCl_2$), primers at a final concentration of 0.025 µM, all three TaqMan® probes (Table 5) at 0.25 µM, 0.2 mM dNTPs and 0.02 U Ampli*Taq* Gold® polymerase (Life Technologies Ltd, Paisley, UK). Negative controls contained 2 µl of PCR-clean H_2O (Sigma-Aldrich Co. LLC). For each reaction, 2 µl oligonucleotides diluted to 100 molecules/µl were added. After the initial denaturing step at 95°C for 30 s, the cycling stage followed comprised of 40 × denaturation (95 °C 30 s), annealing/elongation (60 °C 1 minute) steps. Data were analysed with the qPCR Instruments - 7500 Fast System.

2.6 Deployment tests

For deployment tests a prototype device was constructed which could hold a total of 20 ml of solution. The distance between the deployment unit and the target was between 3-4 m. The device was comprised of a battery and a bracket for the cylinder containing the DNA solution. It was part of a larger framework which enabled a flexible position for the deployment. The framework with the deployment device was positioned on top of a table and the nozzle out of which the DNA solution was sprayed was at an approximate height of 180 cm (Fig 7a and 7b). The cylinder held up to 20 ml solution which was fully deployed in four sprayings. The target comprised of an A4 blotting paper sheet on which small paper discs, with a diameter of 6 mm, were evenly spread. DNA diluted in 75% (v/v) EtOH and 10 mM Tris, pH 8.5 was used for these tests (Fig 7c and 7d). 75% (v/v) EtOH was used to preserve the DNA in a stable and clean way for the period after preparation until the analyses subsequent to the deployment. This DNA solution was deployed in an environment spatially separated from the PCR analyses and any previous experiments involved in this project.

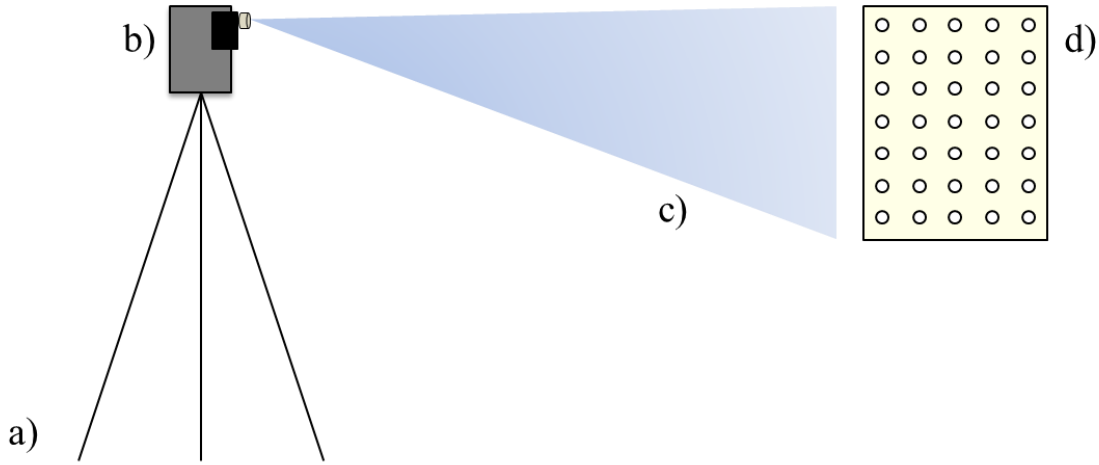


Figure 2.2: Diagram of prototype deployment device
a) The actual device is set on a tripod allowing liberty in movements of height and angle for deployment. b) The device holding the container with DNA solution, the mechanism initiating the deployment and a battery sits on top of the tripod. c) The solution can be shot four times, releasing an average of 5ml of solution. d) The target consists of 6mm large discs 3MM blotting paper. These discs were fixed onto an A4 blotting paper sheet in a pattern as is shown.

For the first deployment tests, ΦX174/*Hae*III DNA marker was used as DNA input to avoid possible contamination of the deployment "scene". Two solutions were prepared, one with and one without ΦX174/*Hae*III DNA; the latter was the negative control and deployed first. The DNA concentration of the solution was approximately 5 ng/µl; i.e. 30 µl of 200 ng/µl marker was added to 30 ml of EtOH-Tris-solution. Subsequently, the discs were first processed using the QIAcube® system to purify DNA. The fully automated process eluted the DNA in a final volume of 200 µl. The protocol used for this procedure was "Blood and body fluid spin protocol" with the QIAamp® DNA Mini Kit and can be found on the Qiagen website. QIAcube® protocols are generally used to purify DNA/RNA from tissue, blood or other body fluids. Additionally, 6-10 discs with DNA solution on it was chosen and incubated in 200 µl dH_2O for one hour at room temperature. The discs were then removed and the remaining liquid used as PCR input (1µl per reaction).

Chapter 3: Results

The first experiments involved PCR, gel electrophoresis and dot blotting of the existing DNA constructs. These were performed as a proof of principle to ensure not only that amplification with the primer pairs was efficient, but also that the constructs contained the intended target sequences.

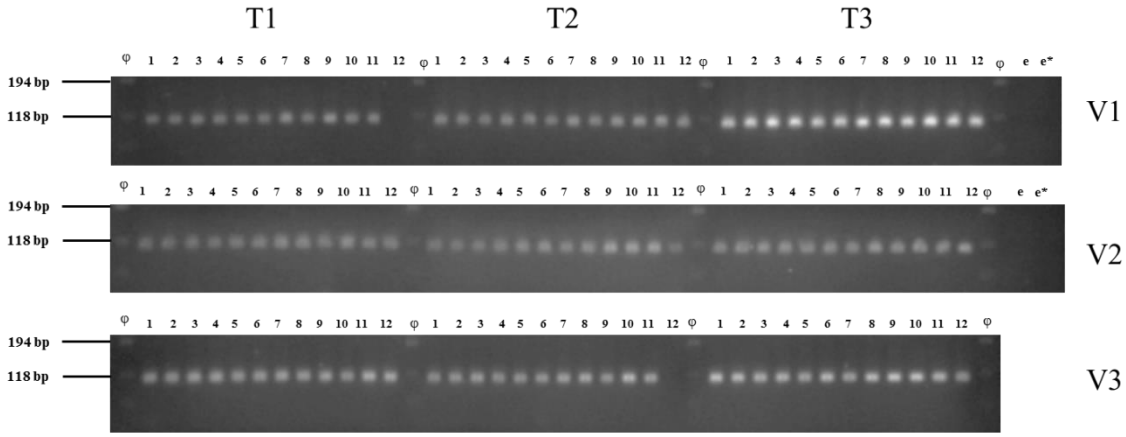
3.1 PCR-built construct validation

In the first experiments, the existing DNA constructs were tested for intactness. These molecules were originally built by Nurul Hamidi via mega primer PCR (Kammann *et al.*, 1989; Sarkar & Sommer, 1990). Nine of the originally planned 36 long constructs had been made (V1T1, V1T2, V1T3, V2T1, V2T2, V2T3, V3T1, V3T2, V3T3, where V1-3 correspond to primer annealing sites and T1-3 the core targets). These DNA fragments were maintained in pGEM®-T Easy plasmids. The construct molecules were amplified by PCR using primers which anneal close to the insertion site within the plasmids. The resulting PCR products were gel purified and used as input for subsequent PCR assays. These constructs were 112 bp long.

3.1.1 Optimisation of PCR conditions

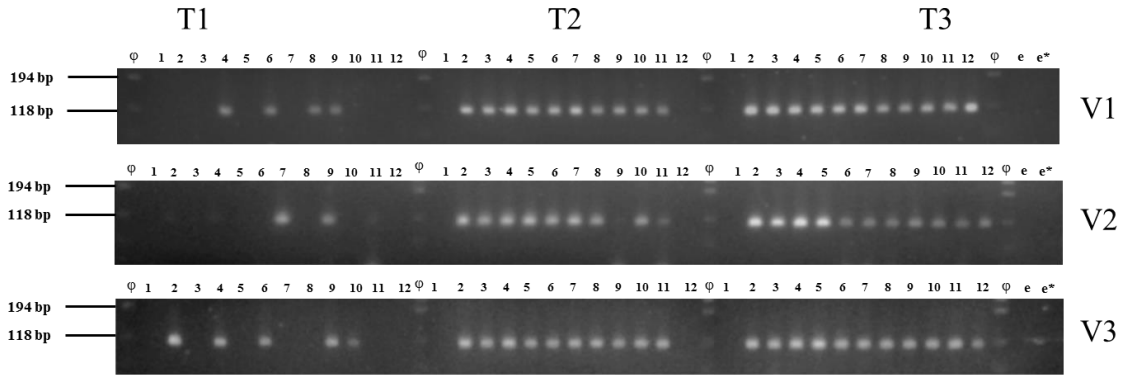
For all subsequent experiments, the PCR conditions were optimised by adjusting annealing and extension temperature. First, the annealing step was set to a temperature range between 48° to 63°C (Fig 8a); subsequently, the extension step was explored over a temperature range of 66° to 72°C (Fig 8b). From the gel electrophoresis, it was observable that the PCR efficiency for the annealing step was similar across the complete temperature range. The annealing temperature was therefore maintained at 50°C. The extension gradient showed strong efficiency up to 68°C, but above this temperature, amplification efficiency decreased. In order to create robust conditions, the extension temperature was therefore set at 67°C. These temperatures were henceforth used for all PCR assays.

(a) Annealing gradient



Well	1	2	3	4	5	6	7	8	9	10	11	12
°C	48	48.4	49.3	50.5	52.2	54.4	56.9	59	60.7	61.8	62.7	63

(b) Extension gradient



Well	1	2	3	4	5	6	7	8	9	10	11	12
°C	66	66.2	66.5	67	67.7	68.6	69.7	70.5	71.1	71.6	71.9	72

Figure 3.1: PCR optimisation using annealing and extension temperature gradients
All PCR products were fractionated on 3% (w/v) LE agarose gels with (ϕ) Phi X174 *Hae* III DNA molecular weight marker. "e" and "e*"indicates reactions lacking any input construct (negative control). These control reactions are differentiated between a negative control added in a pre-PCR environment (e), where the PCR master mix was prepared, and post-PCR environment (e*) where the constructs were added to the mastermix. (a) The annealing gradient was set between 48° and 63°C with all other conditions constant for the PCR. For each primer pair reaction, only amplifiable constructs were used as PCR input, i.e. only V1 constructs were amplified for the V1 primer pair amplification, etc. All reactions were performed in 4 replicates. (b) The extension gradient was set between 66° and 72°C with the same conditions of the optimised adjusted annealing temperature. As for the annealing gradient, only amplifiable constructs were used in 4 replicates (V1 constructs for V1 primer pair amplification, V2 constructs in V2 primer pair amplification, etc.).

3.1.2 Primer and target identification

To confirm the nature of each amplicon, three PCRs were performed, each with one of the three primer pairs V1, V2 and V3. Fragments of 112 bp were expected and therefore Phi X174 *Hae*III digested DNA was used as a molecular weight marker for gel electrophoresis. Only three of the nine constructs should amplify in each PCR, according to the presence of the primer pair binding site, i.e. all V1Tx constructs should amplify with the V1 primer pair but not with the V2 or V3 primer pairs, and so on. Figure 9 shows that the fragments were of the expected length and non-specific amplification was not detected by ethidium bromide staining.

Figure 3.2: Test amplification of PCR-built 112bp constructs
All nine constructs were amplified with three different primer pairs. Only those with the respective variable sequences will amplify in the respective primer pair reactions, e.g. constructs with variable sequences V1 will amplify in reaction V1. The reactions were fractionated on a 3% (w/v) LE agarose gel with a (ϕ) PhiX174*Hae*III DNA molecular weight marker. "e" indicates reactions lacking any input construct (negative control).

To identify the different targets, PCR reactions were transferred to nylon membranes by dot blotting and used in allele-specific oligonucleotide hybridisation. This method is usually used for SNP genotyping but as target identification is based on the same hybridisation principle it was suitable for this purpose, i.e. identifying sequence differences in a combinatorial system, involving oligonucleotides composed specific for each target. Three hybridisation targets (HT) were designed to hybridise to the original target sequences; numbered respectively to the targets, i.e HT1 hybridised to target1, HT2

to target2 and so on. Figure 10 shows the pattern in which the membrane was loaded and the result after hybridisation. The sample group underlined in green had previously been amplified using the respective primer pair, i.e. V1 samples were amplified using V1 primer pair, V2 samples using V2 primer pair and so on. Prior to the hybridisation experiments, 1µl of the 10µl PCR reaction was used in gel electrophoresis in order to confirm successful amplification (data not shown). Red stars indicate the target which is supposed to amplify within that specific target hybridisation. It is clear that only those products containing Target1 show a strong signal when hybridised with the Target 1 specific oligonucleotide HT1; Target 2 and Target 3 show the same outcome with their respective HTs. Although some non-specific background hybridisation can be seen from the phosphorimager capture of the data, the genuine signals deriving from successful target hybridisation are clearly identifiable and generally display much stronger signals. The exception to this is the signal in sample V2T1 deriving from a V1 amplification (second row in HT1 target hybridisation) which came about most likely from a cross-contamination during hybridisation or when handling the PCR products during the process of creating the dot blot.

These experiments were mainly performed to confirm that all components of the original constructs were present in the stored DNA. However, in the worst case scenario that qPCR identification with HyBeacons® failed, these methods could have represented an alternative identification system.

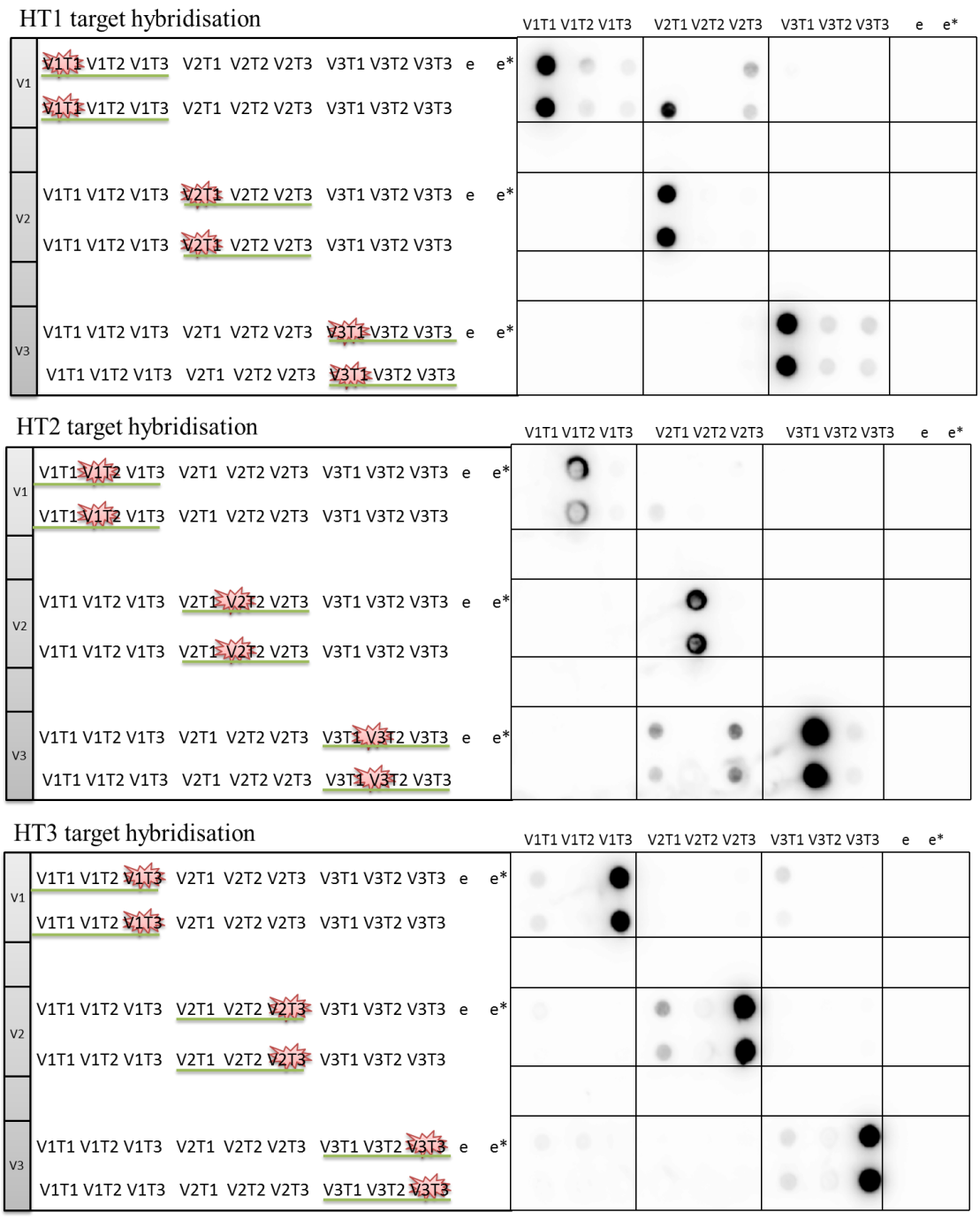


Figure 3.3: Target specific hybridisation
Reactions were transferred to a nylon membrane, crosslinked and hybridised with target specific oligonucleotides, labelled with γ^{32}P-ATP. Signals were captured using a phosphorimager screen, scanned with the GE Typhoon 9400 phosphorimager. The top line shows construct used as template in PCR with "e" and "e*" being the negative controls, "e" refers to a negative created in the PCR hood (i.e. PCR product clean environment) and "e*" refers to a negative created on the bench which is not PCR product clean. V1, V2, V3 on the left hand side indicate the primer pair used; HT1, HT2 and HT3 refer to the respective oligonucleotide probe.

3.2 HyBeacons®

HyBeacons® were originally created by David J. French to identify single nucleotide polymorphisms (SNPs) in the human genome but here were used to identify and distinguish between the three specific target sequences within oligonucleotide constructs (French *et al.*, 2001). The three probes utilise three different fluorophores; HyBeacon® res0170, containing FAM™ hybridises to Target1, HyBeacon® res0359, containing JOE™ hybridises to Target2 and HyBeacon® res0360, containing TAMRA™ hybridises to Target3 (Fig 11). Each probe was designed with specific spacing between the two intramolecular fluorophores (5-6 nucleotides), such that when the oligo is single stranded fluorescence is minimal. On binding to their complementary targets and with formation of duplex DNA, the fluorescence increases and can be measured by qPCR.

The original mega primer PCR-built constructs were 112 bp long. By removing redundant bases between the primer annealing sites and the target sequences which serve as HyBeacon® binding sites, the oligonucleotides were shortened to approximately 60 nucleotides. This was done to decrease costs when ordering those oligonucleotides from ATDBio Ltd. Along with the HyBeacon® probes (res0170, res0359 and res0360), these molecules were used in the qPCR experiments.

HBres0170:
Hybridising to V1T1

```
5′  CGTACGCGCCTTAAGTTTTC3′(V1 reverse Primer)
                     5′CACTGTCAGTGTCTGACA P3′
                       ||||||||||||||||||
3′ GCATGCGCGGAATTCAAAAGGTGACAGTCACAGACTGTACGATATCCCCAGCCATTGC 5′
```

HBres0359:
Hybridising to V2T2

```
5′  GTGGACCGACTAGGGACAAA3′(V2 reverse Primer)
                     5′TGAAGTCAGAGCTGGGAGT P3′
                       ||||||||||||||||||||
3′ CACCTGGCTGATCCCTGTTTACTTCAGTCTCGACCCTCATTTGCACCCGCTGGATAAAT 5′
```

HBres0360:
Hybridising to V3T3

```
5′  AGGTTACGTGGACTCCGTTG3′ (V3 reverse Primer)
                     5′AGGAGTGTAGCCTACCAGCA P3′
                       ||||||||||||||||||||
3′ TCCAATGCACCTGAGGCAACTCCTCACATCGGATGGTCGTGTGGAGGCATGGCTGTTCTA 5′
```

Figure 3.4: HyBeacons® binding to the target sequences
The three different HyBeacons® bind to three different target sequences within the oligonucleotides amplicons. HyBeacon® res0170 (HBres0170) which carries the fluorophore FAM™, hybridises to target 1, thus all samples labelled VxT1. HyBeacon® res0359 (HBres0359) containing JOE™ as fluorescent reporter, hybridises to target2, i.e. all samples labelled VxT2. Finally, HyBeacon® res0360 (HBres0360) with TAMRA™ as the fluorescent dye, hybridises to target3, therefore all samples labelled as VxT3. The nucleotides carrying the fluorescent molecules are shown in red. The HyBeacons® furthermore carry a 3′-phosphate to prevent any elongation from the probe's 3′ end.

The HyBeacon® probes are henceforth referred to as HyBeacon1/HB1 for HBres0170, HyBeacon2/HB2 for HBres0359 and HyBeacon3/HB3 for HBres0360.

3.2.1 First tests using HyBeacons® in qPCR experiments

Reactions were prepared in a pre-PCR room, spatially divided from the location of the thermal cycling reactions and thus any possible PCR-product contamination eventualities. The experiments were performed on an ABI7500/7500®Fast instrument and results were analysed with the ABI7500® Software v2.0.6. For all analyses, the threshold was always set just above background in the exponential phase of the amplification curve, as recommended by Applied Biosystems® (manuals for ABI7500/7500®Fast System). The threshold determines the Ct value of each sample, i.e. the point when amplification shifts into the exponential phase.

Figure 12 shows the amplification curves for all V1 samples. All negative samples showed the same dynamics as the positive curve which suggest contamination. Furthermore, the detection of Target1 with HyBeacon1 failed completely.

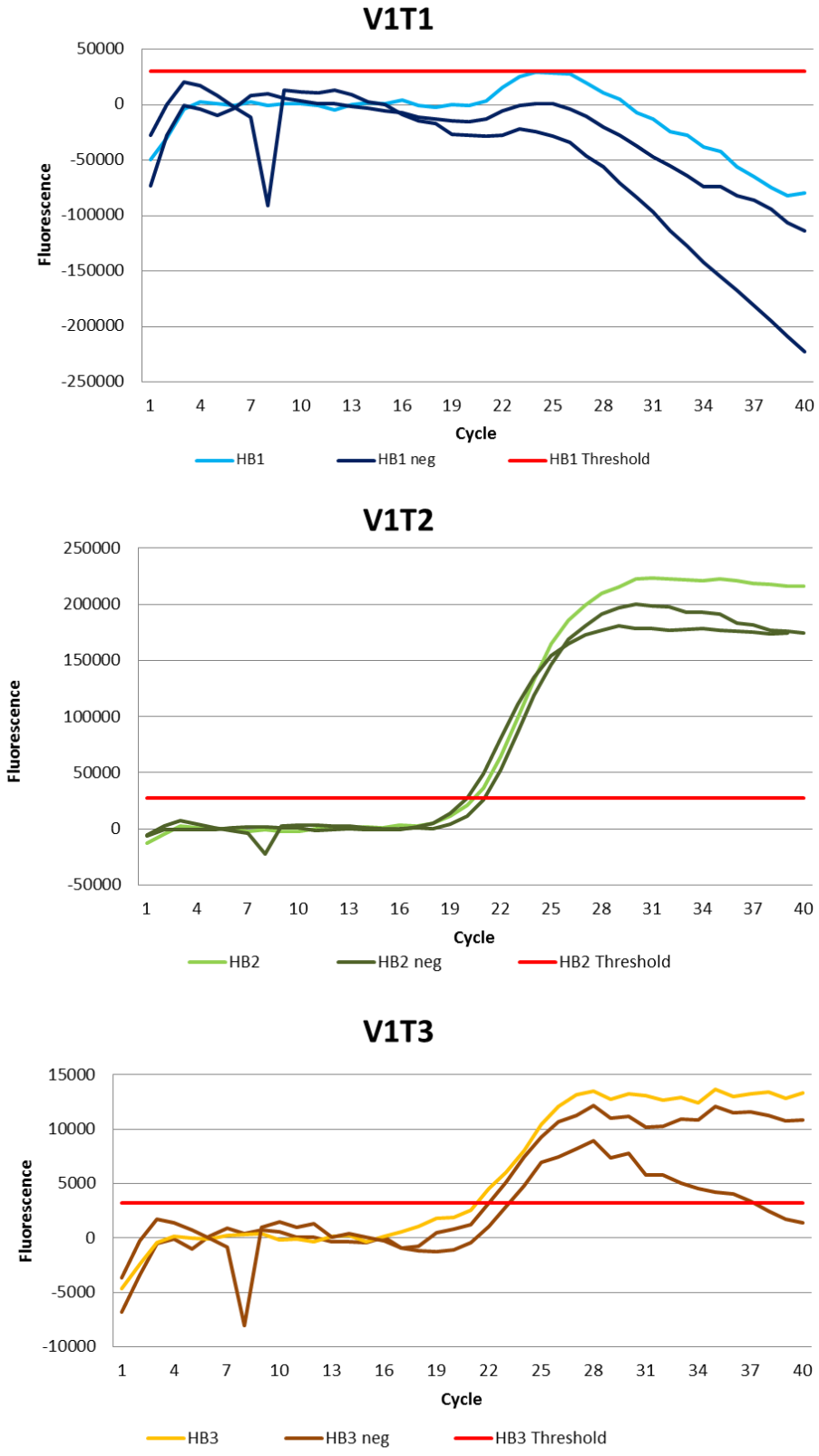


Figure 3.5: Amplification curves detected by HyBeacons® probes during qPCR
Reactions were set up in a pre-PCR room and run on an ABI7500®Fast instrument. The results shown are derived from the different HyBeacon®s of all three V1 constructs. The threshold was set just above the background fluorescence as is standard procedure.

New qPCRs were set up to compare the standard input of 2µl with a concentration of 100 molecules/µl and a 100-fold higher concentration (10,000 molecules/µl) (Fig. 13). Amplification plots showed that the T1 binding probe HB1 showed no sign of successful target detection. The best amplification and detection was observed in those amplicons containing Target2. i.e. VxT2 (HyBeacon2 binding site), with a distinctive difference between the two different input concentrations (Fig. 13b).

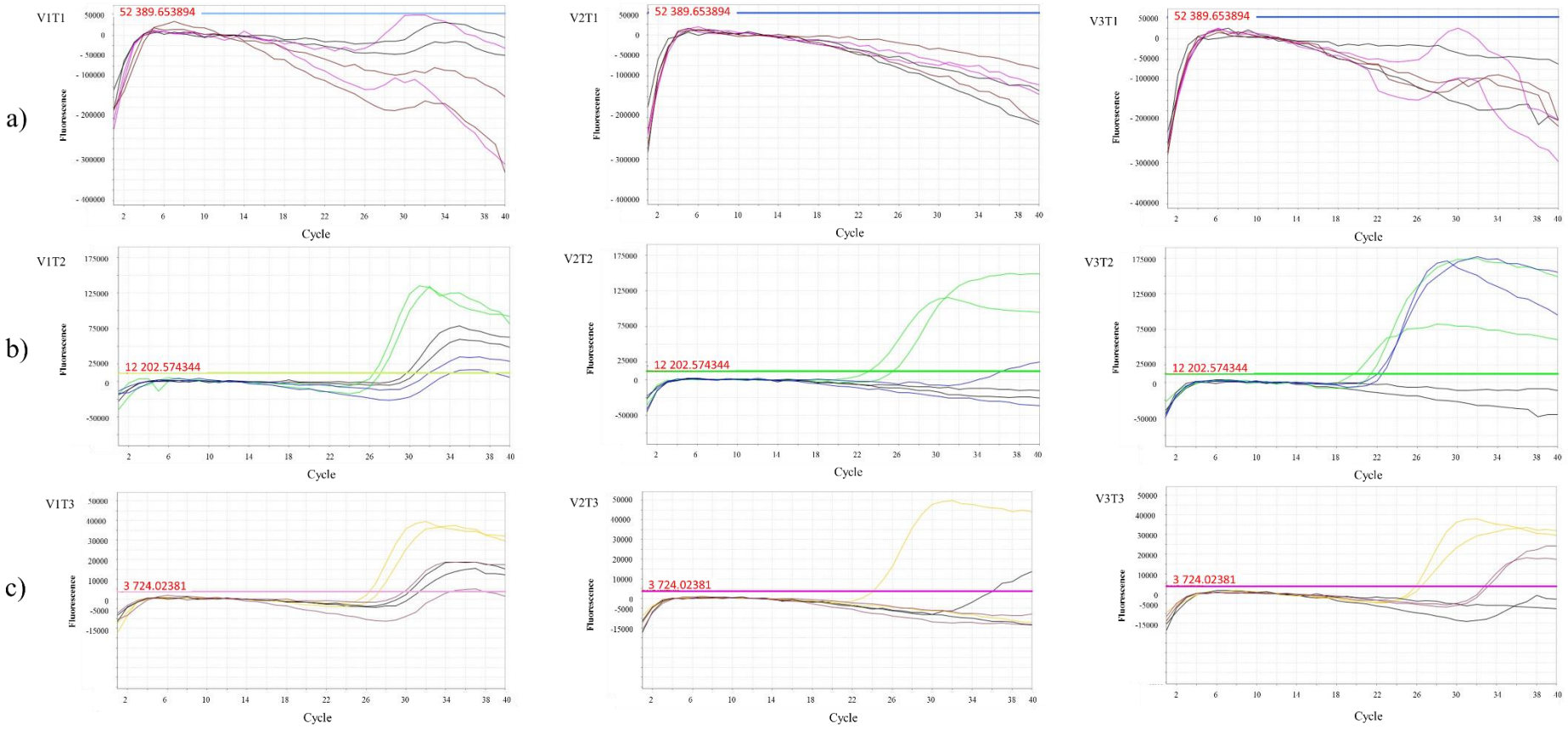


Figure 3.6: Comparison of template input
This qPCR experiment was performed to enable a direct comparison between an input of 200 and 20,000 molecules. Each reaction was set up in duplicate. The threshold was set directly above background noise and is constant within HyBeacon® amplification groups. a) Results shown for HB1 detection of T1, shown with amplification of oligonucleotides using all three primer sets. b) Only oligonucleotides containing T2 are shown, amplified with all three primer sets. c) Detection of T3 by HB3 in combination with all three primer pairs.

3.2.2 Asymmetric qPCR

A further modification intended to improve fluorescence output of the probes was asymmetric PCR (Poddar, 2000). Here, the primer generating the strand to which the HyBeacon® will bind is present at much higher concentration than the opposite primer. This means that the reaction will contain a higher number of single-stranded products to which the HyBeacon® probe can bind, thus resulting in a stronger fluorescent signal. In this case, it was always the reverse primer which was added in a 10-fold higher concentration than the forward primer. All reactions were performed in direct comparison to a regular symmetric PCR with equal primer concentrations. Figure 14a shows a representative amplification graph for each target detection, amplified with primer pair V1. Again, HyBeacon1 failed to produce any positive results, whereas for HyBeacon2 (T2) and HyBeacon3 (T3) an improvement in the fluorescence output was clear. The entire set of amplification plots can be found in the Appendix 5. Despite the difference in fluorescence at the end of amplification, this modification does not improve efficiency of amplification. This is demonstrated by the similar Ct values given by both symmetric and asymmetric reactions (Fig. 14b). As the amplification plot and the Ct value comparison showed, T1 detection with HB1 was still unsuccessful.

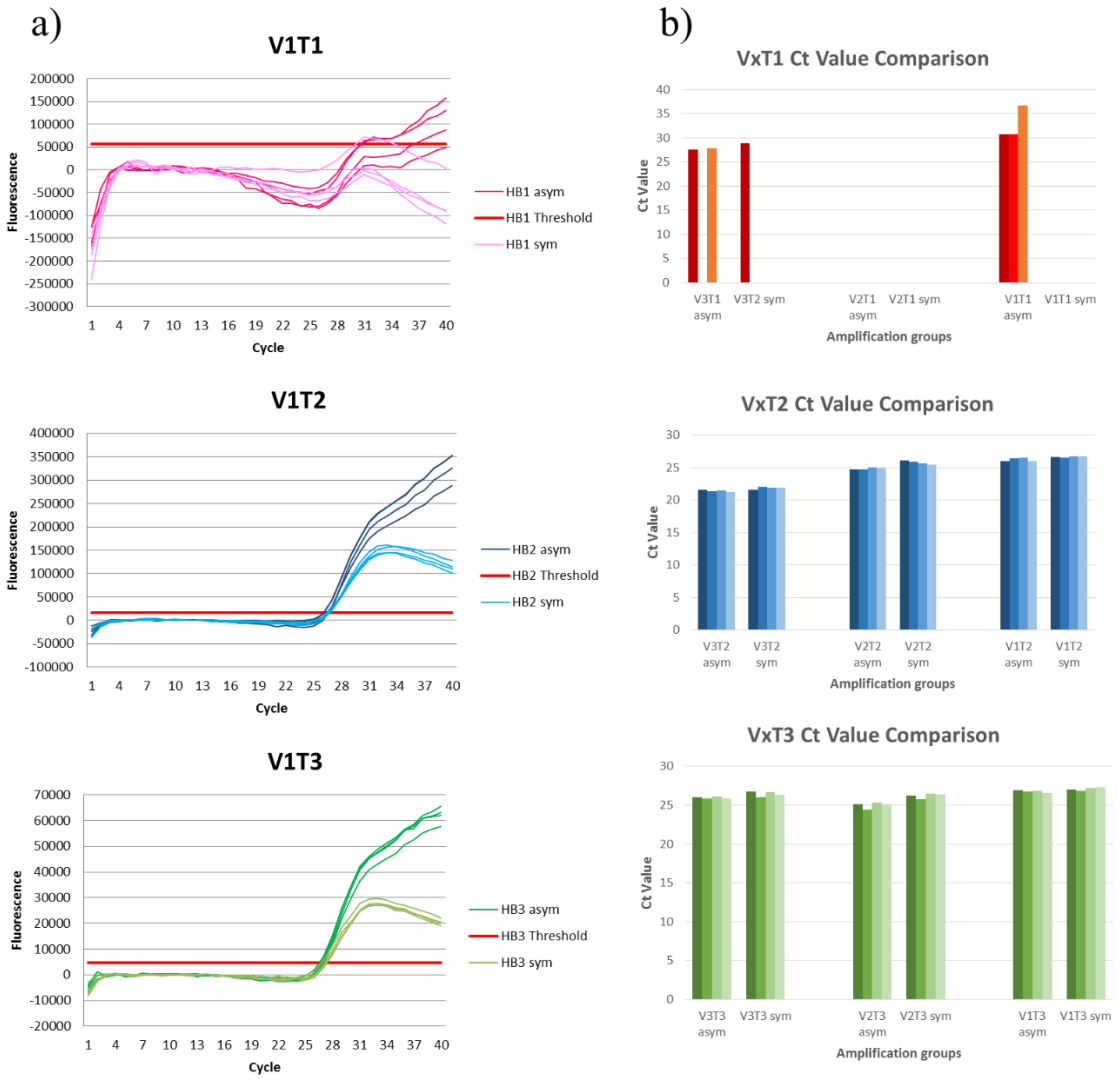


Figure 3.7: Asymmetric PCR

a) For direct comparison, templates were amplified with both primers at the same final concentration of 0.5 µM (symmetric PCR) and with the reverse primer in a 10-fold higher concentration (5 µM) (asymmetric PCR). 2 µl of the oligonucleotides, at 1×10^4 molecules/µl, were added to the reaction. The three amplification plots are representative for all nine oligonucleotides. b) The three graphs show the comparison of the Ct values obtained during amplification. The Ct value marks the cycle when the amplification surpasses the threshold and enters the exponential phase. It is dependent on the DNA concentration present in the reaction; i.e. a higher DNA concentration results in a lower Ct value and a lower DNA concentration increases the Ct value.

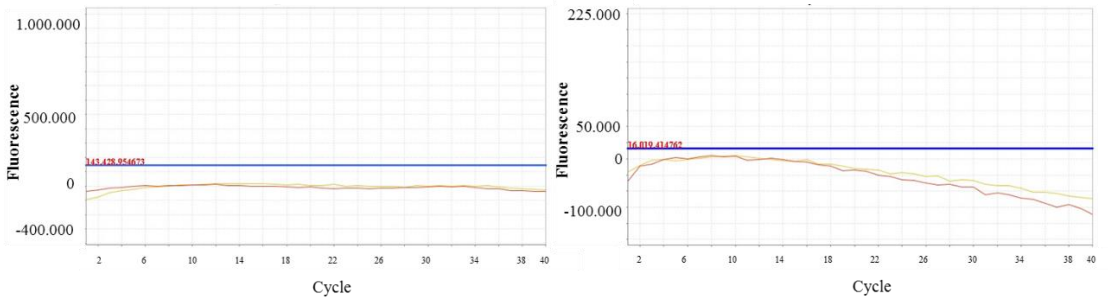
3.2.3 "Hotstart" enzymes

The results presented indicated that the detection of target T1 using HyBeacon1 was not successful. A possible explanation for the negative results using HB1 was failure of the qPCR instrument to detect the fluorophore FAM™, which is incorporated within HB1. To exclude instrument malfunction, a comparison with a second machine of the same model was performed. At the same time, this experiment was used to evaluate the effect of using different hotstart *Taq* DNA polymerase formulations. To decrease primer dimerisation and non-specific priming, it has been suggested hotstart polymerase enzymes can be effective (Birch & Laird, Walter J., Zoccoli, Michael A., 1997; Butler, 2005). Three hotstart enzymes, Qiagen HotStar*Taq*®, Kapa HotStart and Ampli*Taq* Gold®, were compared to the Kapa*Taq* Polymerase used in previous experiments. Figure 15a shows exemplary amplification plots of V2T1 with two different enzymes (Kapa HotStart and Ampli*Taq* Gold®), on the two machines. Repeatedly, HB1 failed to produce positive results. This led to the conclusion that the HyBeacon® design was not optimal. However, an important caveat is that for this experiment, a single high temperature activation time had to be set which was not optimal for all enzymes. Kapa HotStart needed only 5 minutes at 95°C for activation according to the manufacturer, whereas Ampli*Taq* Gold® needed 5-10 minutes and Qiagen HotStar*Taq*® needed 15 minutes at 95°C. The latter time (15 minutes) was eventually used as activation time for all reactions. Nevertheless, as shown by the exemplary plots in 15b, amplification was successful for the other oligonucleotides with the three hotstart enzymes, with similar results between the two different machines (all oligonucleotides PCRs resulted in similar efficient amplification, data only partly shown). It was thus presumed that despite the difference in optimal activation time, all hotstart enzymes functioned.

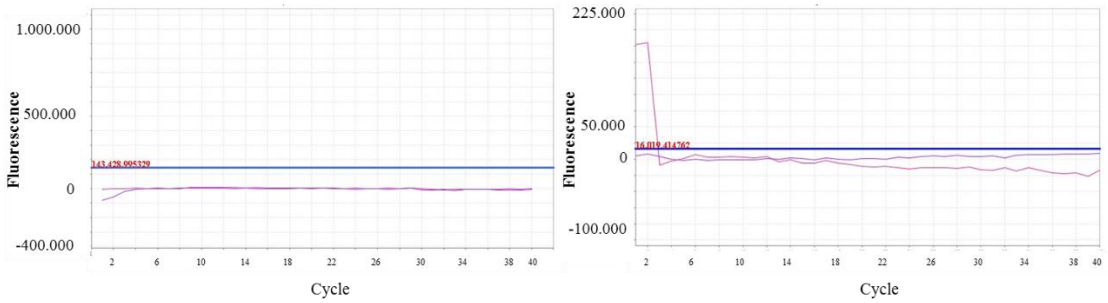
ABI® 7500/7500Fast-1 ABI® 7500/7500Fast-2

a) V2T1

Kapa HotStart

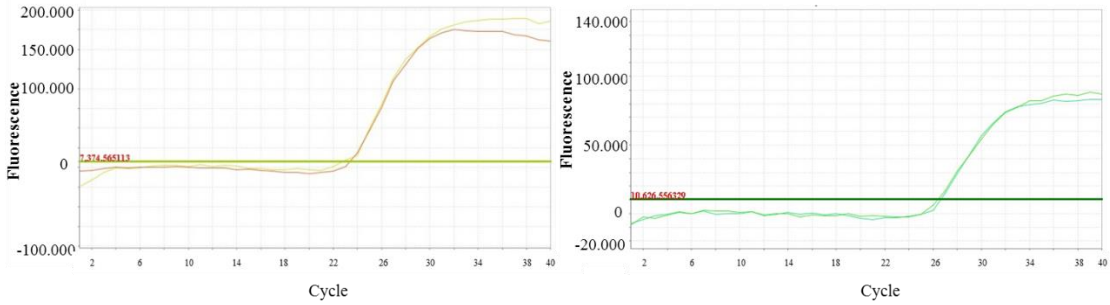


Ampli*Taq* Gold®



b) V3T2

Kapa HotStart



Ampli*Taq* Gold®

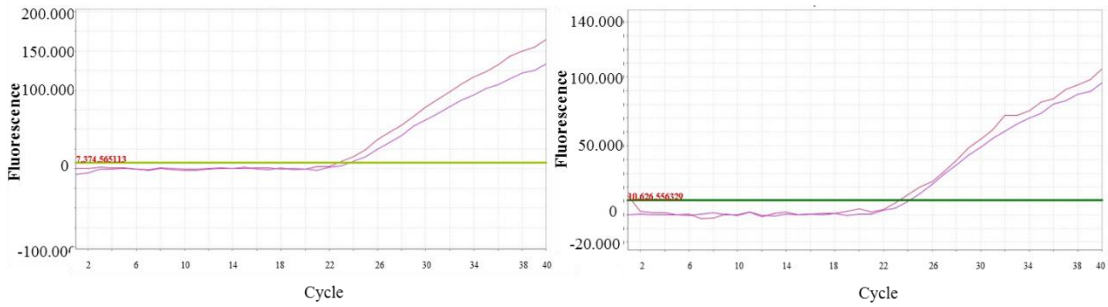


Figure 3.8: Hotstart enzyme test and FAM verification

Two experiments were set up identically and run in parallel on two different ABI7500® machines. Samples were added at a final concentration of 2×10^4 molecules/reaction. All samples were amplified using four different *Taq* polymerases, at a final concentration of 0.02 U/µl. a) A comparison of V2T1 amplification plots is shown, using Kapa HotStart DNA polymerase and Ampli*Taq* Gold®. b) The amplification plots of V3T2 show a successful identification of the target with all three hotstart *Taq* polymerase enzymes. These plots are shown as example for the amplification of all oligonucleotides detected with HyBeacon2 and HyBeacon3 in this experiment.

This experiment involved all nine oligonucleotides and resulted in successful and efficient amplification. All three tested hotstart enzymes seemed suitable for further experiments (Fig 8b). However, Ampli*Taq* Gold® is used as standard DNA polymerase in forensic laboratories worldwide (Hedman *et al.*, 2009). Given the nature of this project and the satisfactory results from the experiment, it was thus decided to continue all further experiments with Ampli*Taq* Gold®.

3.2.4 HBres 0170

Previous results showed that HBres0170 (HB1) did not perform as designed. Its sequence was thus analysed using the mfold web server (Zuker, 2003). This showed that the probe is self-annealing, forming a hairpin structure (Fig 16). The ΔG value represents the quantity of energy required to separate the secondary structure of DNA strand, i.e. the lower the ΔG value, the more energy is needed to break the bond of a hairpin or a primer dimer. Even though the value is not significantly low (values lower than -9 kcal/mol are commonly considered troublesome for primers in PCRs), it is enough to keep the hairpin structure stable, inhibiting the HB1 probe from binding to its target sequence. The self-annealing HyBeacon® will thus never efficiently hybridise to its target but instead will be in continuous state of emitting light. Even a small amount of amplification would not be detected due to the permanent light emission creating a high background noise of fluorescence. As a consequence, HB1 was excluded from further experiments.

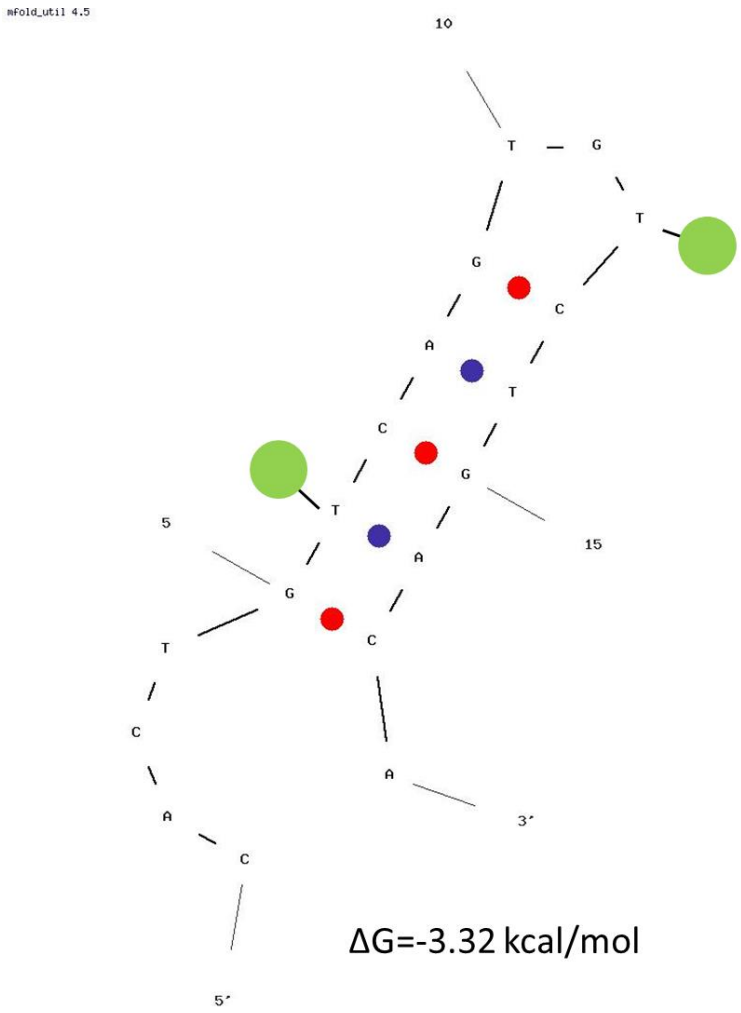


Figure 3.9: HyBeacon1 analysis
Using mfold (http://mfold.rna.albany.edu/?q=mfold) and the OligoAnalyzer® (https://www.idtdna.com/calc/analyzer) the self-annealing region of HB1 was identified and ΔG=-3.32 kcal/mol was calculated. Green circles indicate the fluorescent reporters. These reporters emit light once the HyBeacon® hybridises to its target sequences. The light is then measured by a qPCR machine and the amplification can thus be analysed. However, HyBeacon1 is self-annealing and is thus in continuous state of emitting light and gives inconclusive results. After analysing HyBeacon1's sequence, this probe was considered not suitable for this project.

3.2.5 Template mixtures and T2 and T3 interaction

A major aim of the project was to decode a mixture of several templates. Therefore, a mixture was created which contained templates for all three primer pairs. The mixture contained constructs V1T2, V1T3, V2T2, V2T3, V3T2 and V3T3 (Fig 17). Both targets (T2 and T3) were successfully detected in V1 primer pair amplification whereas T3 was not detected in either V2 or V3 primer pair amplifications (Fig 17b and 10c). Ampli*Taq* Gold® was used as the standard polymerase enzyme, with an activation time of 10 minutes at 95°C. As already mentioned in section 2.3, all hotstart enzymes had different activation times suggested by the manufacturer. All experiments which contained Ampli*Taq* Gold® subsequent to the comparison tests were performed using the optimal activation time for the enzyme, i.e. 10 minutes.

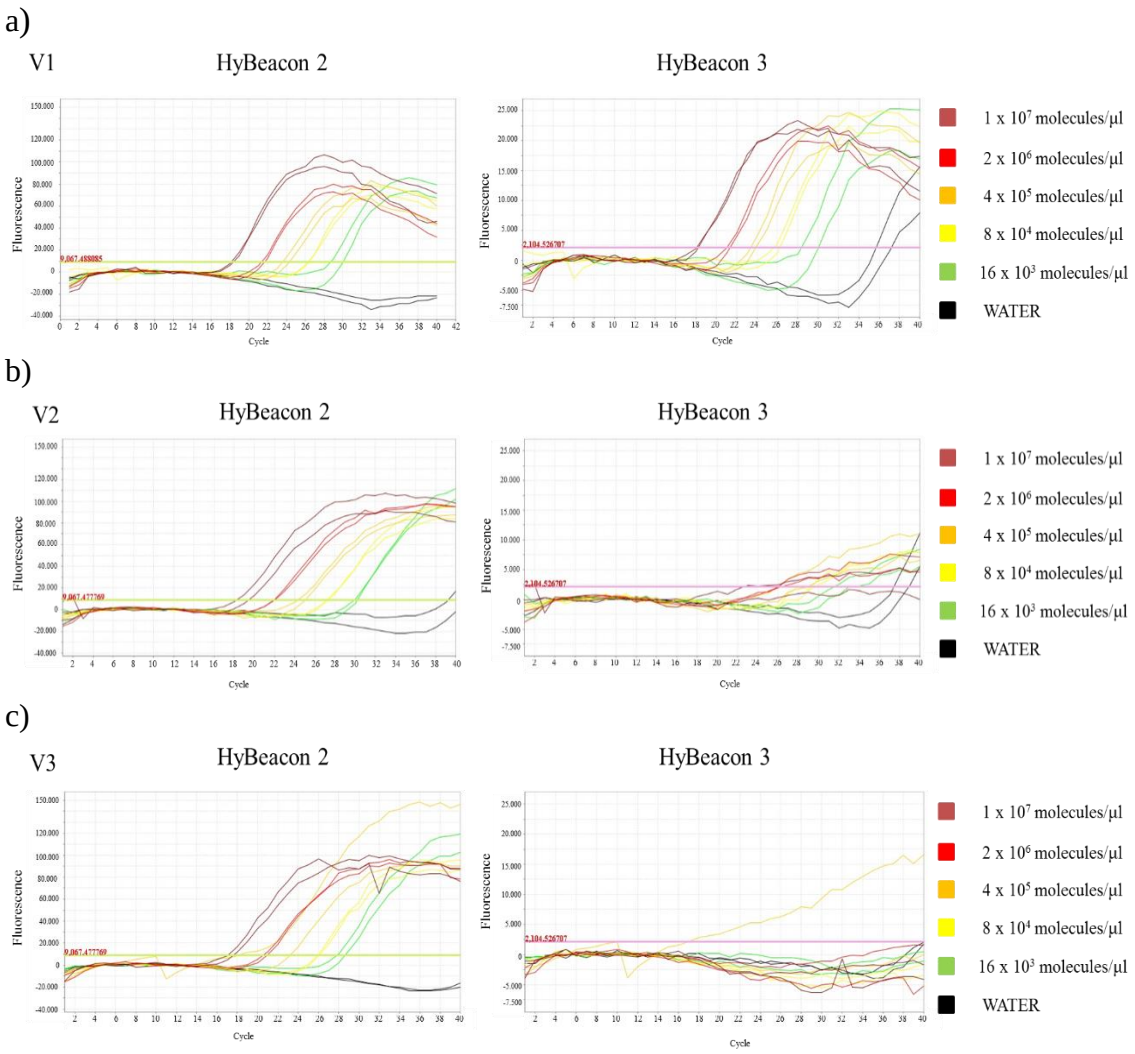


Figure 3.10: T2 and T3 mixtures
A dilution series for all samples was created, ranging from $1x10^7$ molecules/µl to $16x10^3$ molecules/µl. The mixtures contained equimolar amounts of each template at a final reaction volume of 20µl. The mixture was amplified using all three primer pairs, V1(a), V2(b) and V3(c).

It was known from previous experiments that T3 could be successfully detected when T3-containing constructs were amplified individually. However, when in mixture with T2 constructs, T3 failed to be detected indicating that the failures were most likely due to an interaction between T3 with T2 or V2. Table 6 shows the different combination in which all VxT3 samples were amplified and in which of those amplifications the T3 detection with HyBeacon3 was successful. The one oligonucleotide present in the unsuccessful detection experiment was V2T2. It is thus most likely that the failure of detection is due to the presence of V2T2 and could derive from an interaction of that oligonucleotide with HyBeacon3 or with V3T3.

Table 3.1: The different combinations present in V3T3 detection experiments.

Constructs present in amplification mixture	Successful detection of T3
V2T3 + V3T3	√
V2T2 + V2T3 + V3T2 + V3T3	X
V3T2 + V3T3	√

3.2.6 Conclusion

When summarising all these results, it became clear that HyBeacons® were not suitable for the successful completion of this project. The most problematic probe was HB1 (detects target T1) which was largely ineffective, most likely due to intramolecular interactions (see 2.4). The two remaining HyBeacons® were used to decode a mixture containing several templates but this also proved unsuccessful as a likely result of interactions between the target and primer sequences (see 2.5). Furthermore, HyBeacons® have been used most frequently for endpoint analyses, distinguishing between polymorphic target sequences via melt curve analysis (French *et al.*, 2001; French *et al.*, 2002; Dobson *et al.*, 2003). An endpoint analysis is not possible in this case because it would not allow the detection of all code components in a mixture, i.e. only target sequences of the different probes would be identifiable, not, however, the primer pair annealing sites, e.g. V3T2 and V1T2.

After the difficulties encountered, samples of oligonucleotides, primers and HyBeacon® probes were sent to David French to evaluate described issues. The report

showed a good efficiency of PCR primers and found similar problems with HyBeacon1 (evaluation report under disclosure). However, no imminent solution for the described problems was proposed and so all further experiments involved TaqMan® probes.

3.3 TaqMan®

On balance, previous experiments using HyBeacons® indicated they were not suitable for use with the designed combinatorial system. As a result TaqMan® probes were designed and used in all further experiments. These probes require specific criteria for successful use; e.g. the probe's T_m should be 5-10°C higher than the amplifying primers' T_m and cannot contain a 5′-G base. To meet all these requirements, the oligonucleotides designed for the HyBeacons® system (58-60 nt) were found to be too short and consequently, the original sequences were used as a basis for designing new 82 nucleotide long templates.

Preliminary experiments were carried out using only the V3T2 primer and target combination contained within 110 and 82 nt oligonucleotides. During the first experiments all tests were performed on 112 bp long PCR-built molecules (see Chapter 3.1) which is also the proposed length for the final product. The newly chosen oligonucleotide provider (Sigma-Aldrich), however, only produced oligonucleotides up to 110 nt which was eventually used as comparison to the original length of 112 bp. In order to have a further comparison to the much shorter oligonucleotides used for the HyBeacon® experiments, the original sequences were shortened as much as possible. Due to the length of one the TaqMan® probes and its position - close to the forward primer annealing site - the sample had a final length of 82 nucleotides (Fig 18a). For detecting T2, three different TaqMan® probes were designed, using FAM™ as fluorescent dye. Instead of using a fluorescent quencher, the first probe contained a minor groove binder-non-fluorescent quencher (MGB-NFQ) and was 15 nucleotides long. This TaqMan® probe was designed using Primer Express® Software v3.0.1. The additional two probes were 21 and 27 nucleotides long, respectively and both included a Black Hole Quencher (BHQ®) group with FAM™ as the fluorescent dye (Fig 18b). The latter probes were named MPTQ (27-mer) and NTTQ (21-mer) to distinguish between the probe designers. NTTQ was designed by the industrial sponsor's consultant, external to the current project.

a)

5′
ATCTTGTCGGTACGGAGGTGCGTTTACCTTGTGTCCAGAGACTAACGATACTACTCCCAGCTCTGACT
TCAACTGCTTGGACTGAGGTCTCTCAACGGAGTCCACGTAACCT3′
(112 nt)

5′
ATCTTGTCGGTACGGAGGTGCGTTT··CTTGTGTCCAGAGACTAACGATACTACTCCCAGCTCTGACT
TCAACTGCTTGGACTGAGGTCTCTCAACGGAGTCCACGTAACCT3′
(110 nt)

5′
ATCTTGTCGGTACGGAGGTG··························CTACTCCCAGCTCTGACT
TCAACTGCTTGGACTGAGGTCTCTCAACGGAGTCCACGTAACCT3′
(82 nt)

b)

MGB-NFQ

```
                                  5′FAM AAGCAGTTGAAGTCA MGB3′
                                        ||||||||||||||||
3′TCCAATGCACCTGAGGCAACTCTCTGGAGTCAGGTTCGTCAACTTCAGTCTCGACCCTCATCATAG
CAATCAGAGACCTGTGTTCCATTTGCGTGGAGGCATGGCTGTTCTA5′
```

T_m: 64°C 5′FAM AAGCAGTTGAAGTCA MGB3′

MPTQ (BHQ1®)

```
                        5‘FAM ACCTCAGTCCAAGCAGTTGAAGTCAGA BHQ13′
                              ||||||||||||||||||||||||||||
3′TCCAATGCACCTGAGGCAACTCTCTGGAGTCAGGTTCGTCAACTTCAGTCTCGACCCTCATCATAG
CAATCAGAGACCTGTGTTCCATTTGCGTGGAGGCATGGCTGTTCTA5′
```

T_m: 68.2°C 5‘FAM ACCTCAGTCCAAGCAGTTGAAGTCAGA BHQ13′

NTTQ (BHQ1®)

```
                                                                BHQ13′
                                          5‘FAM GAAGTCAGAGCTGGGAGTAG
                                                ||||||||||||||||||||
3′TCCAATGCACCTGAGGCAACTCTCTGGAGTCAGGTTCGTCAACTTCAGTCTCGACCCTCATCATAG
CAATCAGAGACCTGTGTTCCATTTGCGTGGAGGCATGGCTGTTCTA5′
```

T_m: 61.2°C 5‘FAM TGAAGTCAGAGCTGGGAGTAG BHQ13′

Figure 3.11: TaqMan® probe and oligonucleotide sequences
Sample V3T2 (shown in a)) was identified as a model oligonucleotide for the TaqMan® experiments and was shortened for further application. Furthermore, three different TaqMan® probes were designed to identify the most efficient quencher and probe design. The underlined sequences correspond to the V3 priming sites. The sequences in red designate the original HyBeacon® binding sites. a) All three sequences of V3T2 starting with original design at 112 nt, the second at 110 nt and the third at 82 nt. The middle dots indicate those nucleotides left out. The shortening was done to create a more stable oligonucleotide. b) Three TaqMan® probes were designed, two with a Black Hole Quencher (BHQ®) and one with a Minor Groove Binding Quencher (MGB-NFQ). All three quenchers are non-fluorescent and the probes were designed to meet recommended standards, e.g. avoiding triple-G or-C sequences, have a T_m 5-8°C higher than the primers' T_m and lack a 5′-G.

3.3.1 Testing TaqMan® probes of three different designs

Initial experiments involved only the MGB-NFQ TaqMan® probe with dilution series of the 82 and 110 bp oligonucleotides (Fig 19). An established TaqMan® assay acting on human DNA served as a positive control (assay LCT13, the kind gift of Dr Cristina Tufarelli, University of Nottingham). The positive control showed the expected increasing Ct values with decreasing sample concentration. The different concentrations were not as cleanly separated in V3T2 (110 nt) as they were in V3T2 (82 nt). Furthermore, the latter showed higher fluorescence output values. Consequently, the shorter oligonucleotides (82 nt) were used for all further experiments.

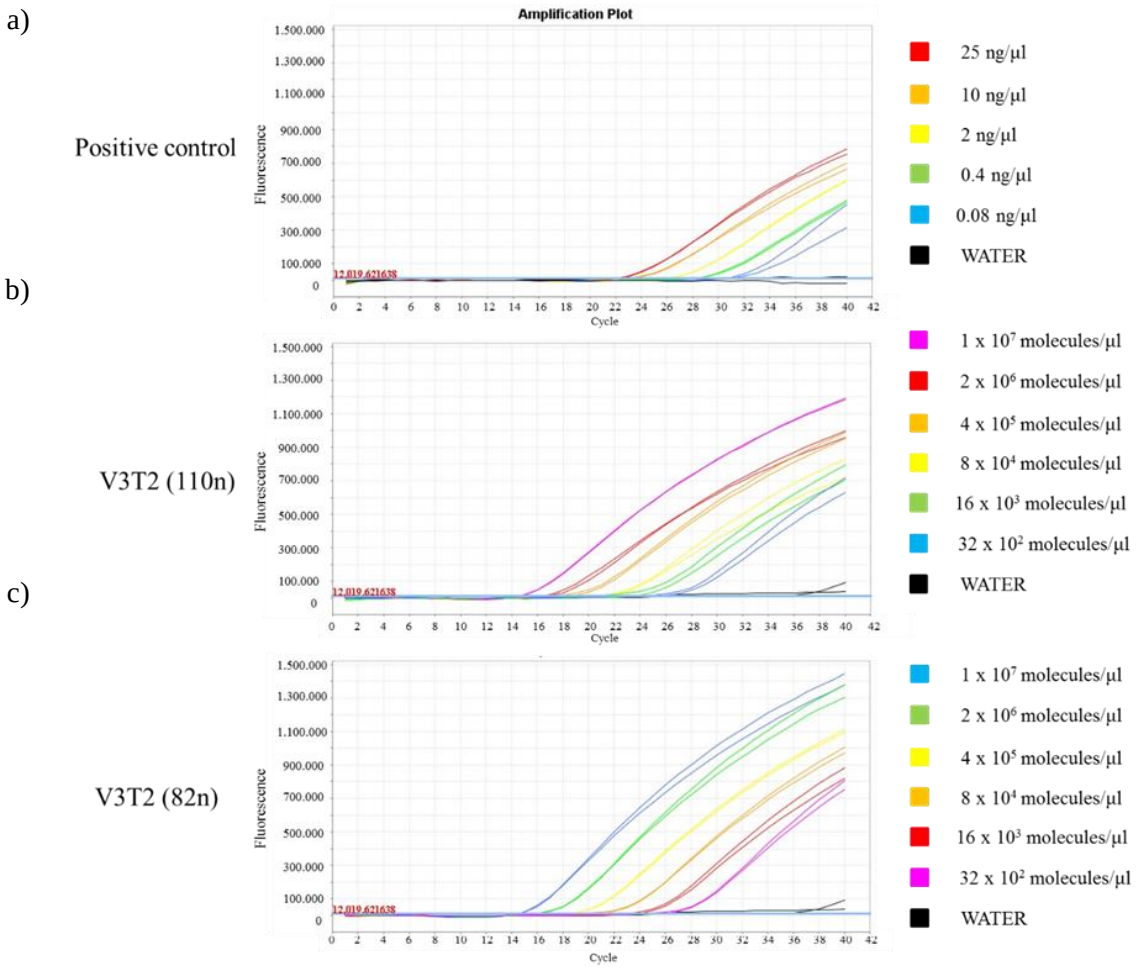


Figure 3.12: TaqMan® MGB-NFQ test
a) A positive control assay, using human DNA, in a dilution series ranging from 25 ng/µl to 0.08 ng/µl. The positive control assay utilised its own primer and TaqMan® probe mix, provided by Dr Cristina Tufarelli. b/c) V3T2 oligonucleotides (82 nt and 100 nt) were diluted and the dilution series amplified with V3 primers. The PCR reaction was performed using AmpliTaq Gold.

After establishing the experimental conditions in the preliminary experiments the three TaqMan® probes, MGB-NFQ, MPTQ and NTTQ (Fig 11b) were tested in comparison experiments using the V3T2 (82 nt) oligonucleotide as a template (Fig 20). All three probes showed a similar amplification dynamic, as shown in the first experiment using only the MGB-NFQ TaqMan® probe. As previously described, the Ct value increased with decreasing template concentration, i.e. the exponential amplification phase occurred later.

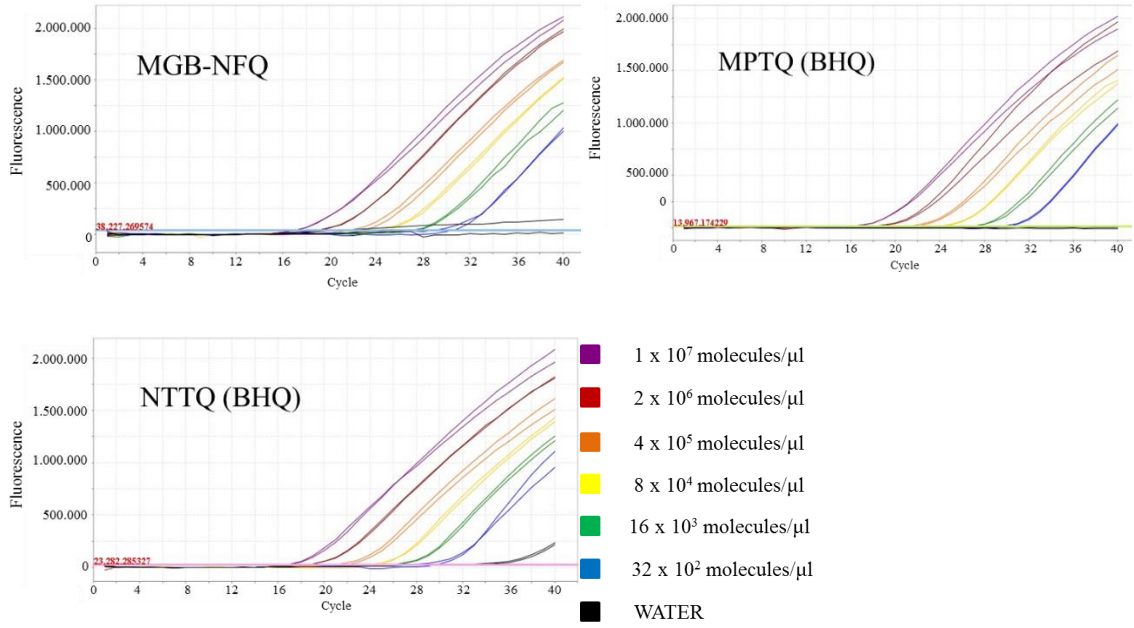


Figure 3.13: Comparison of three TaqMan® probes
Three mastermixes were prepared with each a different TaqMan® probe at a final concentration of 0.25 µM. A dilution series of V3T2 (82 nt) was used as input, and reactions were amplified in duplicates.The reactions were set up in the pre-PCR room.

The three different TaqMan® probes shown in Figure 13 did not differ in their efficiency. However, as already mentioned, the Applied Biosystems® owned MGB-NFQ is not suitable due to a lack of licensing agreement with Spyral. The quencher system selected for use was thus BQH1®. As the longer probe (MPTQ) was designed according to the requirements for TaqMan® probes, MPTQ was further used for all T2 detection experiments.

3.3.2 Testing of all nine oligonucleotides using new TaqMan® probes

After establishing that detection using TaqMan® probes was successful, two more probes using the BHQ1® were designed for T1 (labelled with JOE™) and T3 (labelled with TAMRA™). For all further experiments the formerly labelled MPTQ TaqMan® probe was used and all probes were referred to as TaqMan1, TaqMan2 (previously MPTQ) and TaqMan3, numbered respectively to their target annealing sites. All input oligonucleotides used in the following experiments were 82 nt long. Dilution series were created as previously described, from $1x10^7$ molecules/µl to $32x10^2$ molecules/µl. All nine oligonucleotides proved to have the same amplification dynamics as seen in Figure 13. Duplicates displayed the same trend during amplification and

fluorescence decreases with decreasing concentration of DNA input, as expected. All amplification plots can be found in Appendix 6. With a successful amplification rate and detection using the described TaqMan® probes, experiments were then continued utilising these probes along with the 82 nt long oligonucleotides.

3.3.3 Template mixtures

Due to previous experiences with HyBeacons®, the mixtures were created systematically. All possible combinations of the nine oligonucleotides were created and assigned systematic ID (numerical) as well as random IDs, to blind the decoding process.

The experiments started with a cohort of 27 mixtures with three oligonucleotides. Each mixture contained one construct for each primer pair annealing site, i.e. each mixture included one V1, one V2 and one V3 sample (Table 7). The complete cohort was successfully decoded. The first cohort was followed by a second group with 3 mixtures, each containing three oligonucleotides; one mixture being all samples for one specific primer pair (Table 8). Finally, a cohort of 27 mixtures with 6 templates was amplified (Table 9). These last 27 mixtures represented an inverse design of the first cohort. The first mixtures contained three out of the total 9 oligonucleotides whereas the last mixtures contained the remaining six oligonucleotides in the respective mixtures.

Table 3.2: First cohort of mixtures to be decoded

Sys_ID	Oligo_content	Rand_ID	Notes
C61	V1T1 V2T1 V3T1	9369	
C62	V1T1 V2T1 V3T2	2367	
C63	V1T1 V2T1 V3T3	8429	
C65	V1T1 V2T2 V3T1	8272	
C66	V1T1 V2T2 V3T2	6968	
C67	V1T1 V2T2 V3T3	6292	**False positive V2T1**
C68	V1T1 V2T3 V3T1	2093	
C69	V1T1 V2T3 V3T2	3799	
C70	V1T1 V2T3 V3T3	5303	
C82	V1T2 V2T1 V3T1	8968	
C83	V1T2 V2T1 V3T2	9814	
C84	V1T2 V2T1 V3T3	8984	
C86	V1T2 V2T2 V3T1	2285	
C87	V1T2 V2T2 V3T2	7819	
C88	V1T2 V2T2 V3T3	2150	**False positive V2T1**
C89	V1T2 V2T3 V3T1	6080	
C90	V1T2 V2T3 V3T2	4638	
C91	V1T2 V2T3 V3T3	7306	
C97	V1T3 V2T1 V3T1	8300	
C98	V1T3 V2T1 V3T2	2595	
C99	V1T3 V2T1 V3T3	8733	
C101	V1T3 V2T2 V3T1	1967	
C102	V1T3 V2T2 V3T2	1796	
C103	V1T3 V2T2 V3T3	4884	**False positive V2T1**
C104	V1T3 V2T3 V3T1	3338	
C105	V1T3 V2T3 V3T2	6075	
C106	V1T3 V2T3 V3T3	7334	

There were no instances where targets that were present in the mixture failed to be detected in the first cohort. However in subsequent experiments two templates (V2T3 and V3T2) stood out as often being undetected. Another feature of these experiments was systematic observation of a false positive V2T1 signal, which only appeared in mixtures containing both V2T2 and V3T3. This means that the oligonucleotide V2T1 was identified as present in the mixture, despite its absence. This false positive usually showed a slightly higher Ct value (24-30) compared to the more usual value of 16-20. This is most likely due to an overlap of the fluorophore spectra. The excitation and emission spectra of FAM™ and TAMRA™ (detecting T2 and T3 respectively) overlap which falls into the emission spectrum of JOE™ which detects T1. The overlap may thus create a false signal for JOE™, resulting in a false positive result for T1 oligonucleotides (this topic is explored in more detail in section 3, Discussion).

Table 3.3: Second Group of mixtures to be decoded
Green text indicates positive results and red text indicates a drop out (failed detection).

Sys_ID	Oligo_content	Rand_ID
C46	V1T1 V1T2 V1T3	6023
C110	V2T1 V2T2 V2T3	9572
C129	V3T1 V3T2 V3T3	1608

Table 3.4: Third cohort of mixtures containing 6 templates
Red text indicates drop out and green coloured font indicates templates that were successfully detected.

Sys_ID	Oligo_content	Rand_ID	Notes
C405	V1T1 V1T2 V2T1 V2T2 V3T1 V3T2	2092	
C406	V1T1 V1T2 V2T1 V2T2 V3T1 V3T3	5289	
C407	V1T1 V1T2 V2T1 V2T2 V3T2 V3T3	5602	
C408	V1T1 V1T2 V2T1 V2T3 V3T1 V3T2	680	
C409	V1T1 V1T2 V2T1 V2T3 V3T1 V3T3	9509	
C410	V1T1 V1T2 V2T1 V2T3 V3T2 V3T3	8366	
C412	V1T1 V1T2 V2T2 V2T3 V3T1 V3T2	6644	
C413	V1T1 V1T2 V2T2 V2T3 V3T1 V3T3	6730	**False positive V2T1**
C414	V1T1 V1T2 V2T2 V2T3 V3T2 V3T3	8846	**False positive V2T1**
C420	V1T1 V1T3 V2T1 V2T2 V3T1 V3T2	7225	
C421	V1T1 V1T3 V2T1 V2T2 V3T1 V3T3	8448	
C422	V1T1 V1T3 V2T1 V2T2 V3T2 V3T3	5023	
C423	V1T1 V1T3 V2T1 V2T3 V3T1 V3T2	6658	
C424	V1T1 V1T3 V2T1 V2T3 V3T1 V3T3	2006	
C425	V1T1 V1T3 V2T1 V2T3 V3T2 V3T3	9601	
C427	V1T1 V1T3 V2T2 V2T3 V3T1 V3T2	7464	
C428	V1T1 V1T3 V2T2 V2T3 V3T1 V3T3	9607	**False positive V2T1**
C429	V1T1 V1T3 V2T2 V2T3 V3T2 V3T3	5861	**False positive V2T1**
C441	V1T2 V1T3 V2T1 V2T2 V3T1 V3T2	312	
C442	V1T2 V1T3 V2T1 V2T2 V3T1 V3T3	3768	
C443	V1T2 V1T3 V2T1 V2T2 V3T2 V3T3	3109	
C444	V1T2 V1T3 V2T1 V2T3 V3T1 V3T2	5510	
C445	V1T2 V1T3 V2T1 V2T3 V3T1 V3T3	8837	
C446	V1T2 V1T3 V2T1 V2T3 V3T2 V3T3	9212	
C448	V1T2 V1T3 V2T2 V2T3 V3T1 V3T2	7584	
C449	V1T2 V1T3 V2T2 V2T3 V3T1 V3T3	2690	**False positive V2T1**
C450	V1T2 V1T3 V2T2 V2T3 V3T2 V3T3	8343	

Due to the known emission spectra of the chosen fluorophores, the false positive might have resulted from the overlapping spectra of FAM™ and TAMRA™. To test this

hypothesis, three mixtures from cohort one (C67, C88 and C103) were freshly made and amplified with three different TaqMan® concentrations: 0.25 μM, 0.125 μM and 0.0625 μM. These experiments also resulted in a successful detection of all three constructs with an expected decrease in the fluorescent signal according to a decreasing TaqMan® concentration. However, the false positive signal V2T1 was still present.

During this experiment, a repetition of cohort 2 (Table8) was performed as well which resulted in an exact replicate of the previous results; a failure to detect V3T2 in mixture C129.

3.3.4 Conclusion

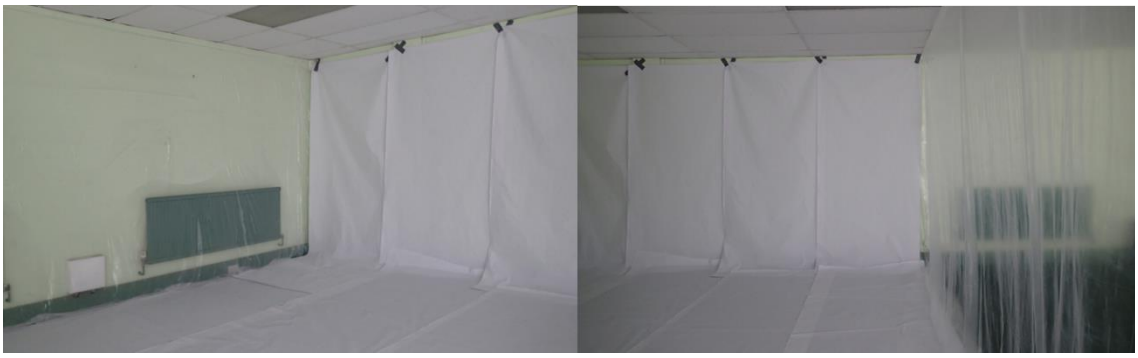
Through the use of qPCR it was established that TaqMan® probes were suitable and highly effective reporters for the objective of this project. The comparison of the initial TaqMan® probes designed for Target2 indicated that there was no difference between different quenchers (MGB and BHQ1®) and the experiments were continued using only BHQ1® following commercial considerations. All subsequent experiments to identify constructs individually were equally successful. Furthermore, but most importantly, the TaqMan® system proved to be able to detect several constructs within one mixture. Despite the advantages shown for using TaqMan® probes, addressing the issues of false positive results and even only one unidentified component (see section 3.3), is critical for the continuation of this project. It is crucial to be able to detect all components without error and to be sure that no false-positives can occur. Even if occurring at a small scale, these issues must not be ignored and will require further optimisation of the system.

3.4 DNA Deployment tests

An essential part of this project was to test deployment of the coded DNA and subsequent recovery and analysis in the laboratory. For this reason a prototype device was constructed by an independent company. DNA diluted in 75% (v/v) EtOH and 10 mM Tris, pH 8.5 was used for these deployment tests. This DNA solution was deployed in a clean room that had not been previously been used for dispersal tests. A room was hired and the walls and floor were covered with polythene film and disposable white paper (Fig 21a). The distance between the deployment unit and the target varied between 3 and 4 m. The device was comprised of a battery and a bracket for the cylinder containing the DNA solution (Fig 21b). It was part of a larger framework which enabled a flexible position for the deployment (Fig 21c). The framework with the deployment device was positioned on top of a table and the nozzle from which the DNA solution was sprayed was at a height of approximately 180 cm. The cylinder held up to 20 ml of DNA containing solution which was fully deployed in four sprayings.

During the procedure of deployment, crime-scene suits were worn. These were used to prevent contamination of the scene with DNA from the lab as well as preventing deployed DNA being brought back into the laboratory unintentionally. A clean suit was used for each experiment and the suits were disposed of at the conclusion of the test at the test facility.

a)



b) c)

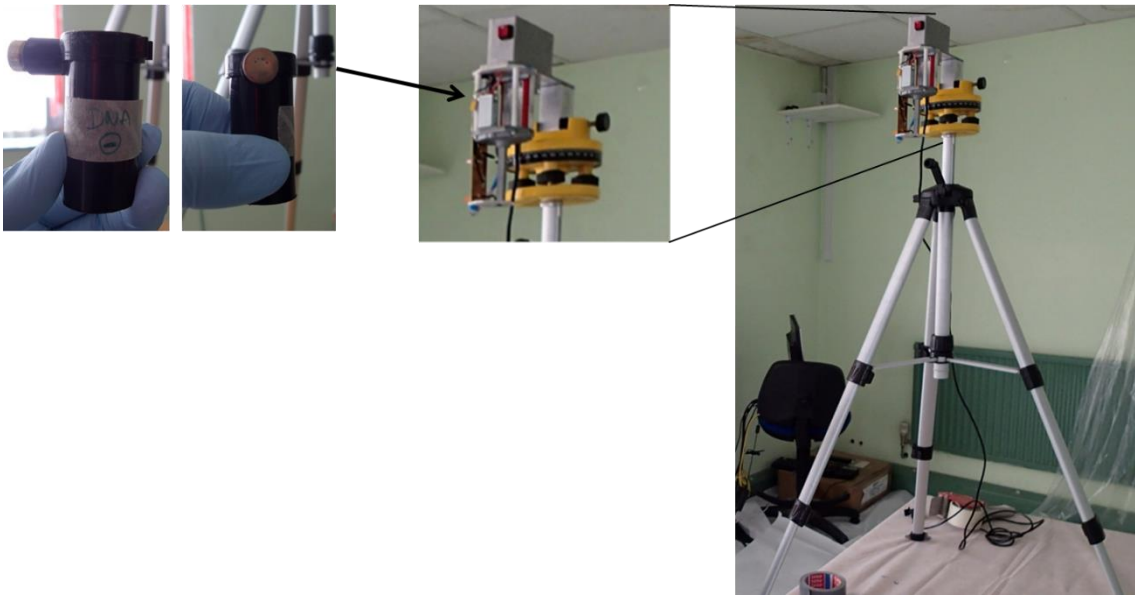


Figure 3.14: Deployment location and device
The location where analyses of initial experiments took place had to be spatially separated from the location of the first deployment tests to avoid the possibility of contamination. a) The area used was laid out with polythene film and disposable white paper. If solution was deployed onto the floor or walls, the paper was replaced by clean white paper to avoid any contamination. b) The container could hold 20 ml of solution and had a nozzle with 4 holes through which the solution was deployed by pressure, generated by a mechanical piston. The battery-powered device and could be attached to a larger framework which allowed alteration of the height and angle of deployment (c).

The target comprised an A4 blotting paper sheet on which small paper discs, with a diameter of 6 mm, were evenly spread in a grid (Fig 22). Those paper discs which were hit by the DNA solution were then collected individually into 1.5 ml tubes for further analysis in the laboratory. A small amount of bromophenol blue was added to the solution to enable the identification of target discs that had been hit.

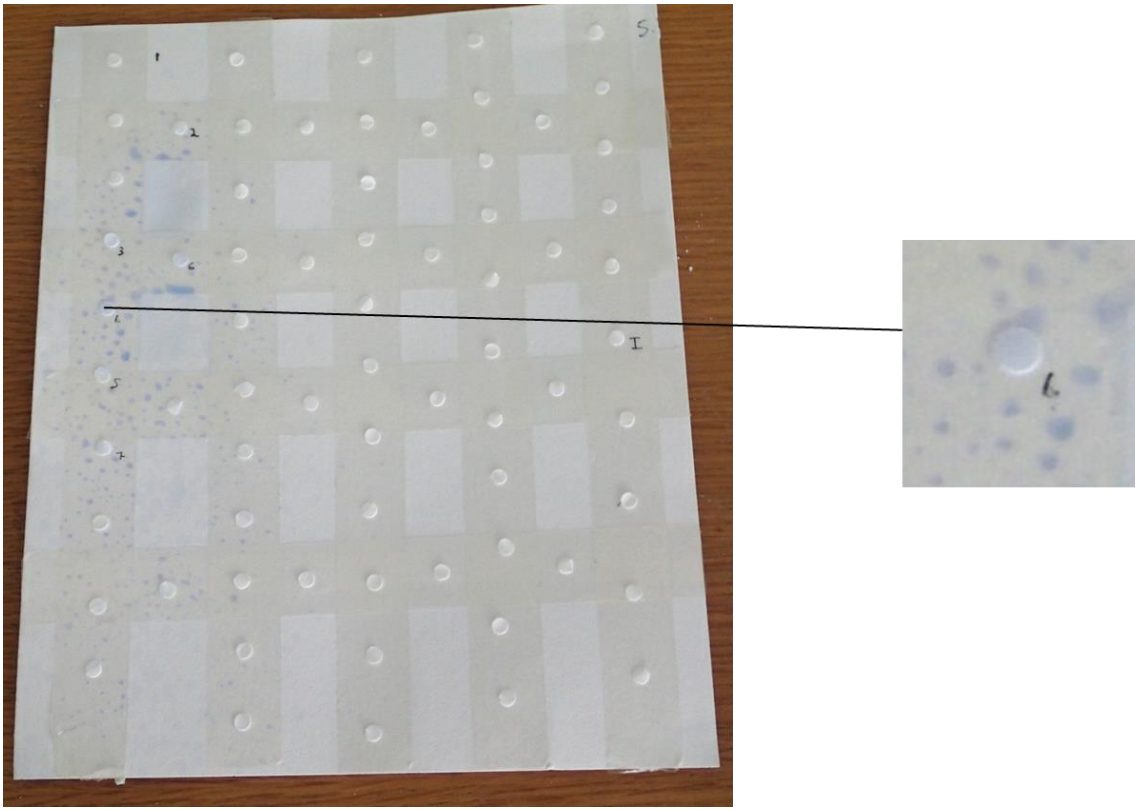


Figure 3.15: Target discs for deployment and subsequent analyses
In order to be able to analyse the spatter resulting from the deployment, 6 mm discs of 3MM chromatography paper were created. These discs were fixed onto an A4 blotting paper sheet in a pattern as is shown in the figure. At least two solutions were prepared for each deployment test; one without DNA and one containing DNA. Discs which were hit by the solution were collected in 1.5 ml tubes, labelled and subsequently analysed in the laboratory. For every test, a negative disc was collected, i.e. a disc that was not visibly struck by the solution.

For the first deployment tests, ΦX174/*Hae*III DNA marker was used as DNA input to avoid possible contamination of the deployment scene. Two solutions were prepared, one with and one without ΦX174/*Hae*III DNA; the latter was the negative control and deployed first. The DNA concentration of the positive control solution was approximately 5 ng/µl. Subsequently, the discs were first analysed using the QIAcube® system to purify DNA. This fully automated process eluted the DNA in a final volume of 200 µl. The protocol used for this procedure was "Blood and body fluid spin protocol" with the QIAamp® DNA Mini Kit, and can be found on the Qiagen website. QIAcube® protocols are generally used to purify DNA/RNA from tissue, blood or other body fluids (http://www.qiagen.com/gb/products/catalog/automated-solutions/sample-prep/qiacube/). In principle, purification may not have been needed as pure DNA was deployed, but automated DNA extraction systems are commonly employed in forensic analysis and provide a standardised procedure for sample handling. To test the effect of

DNA extraction on the recovery of the deployed DNA a group of discs hit by the DNA solution were chosen and incubated in 200 µl dH_2O for one hour at room temperature. The discs were then removed and the remaining liquid used as PCR input (1 µl per reaction). Subsequently, a PCR was performed for both groups using primers which amplified a fragment very similar in size to the 118 bp band of ΦX174 ladder (Fig 23).

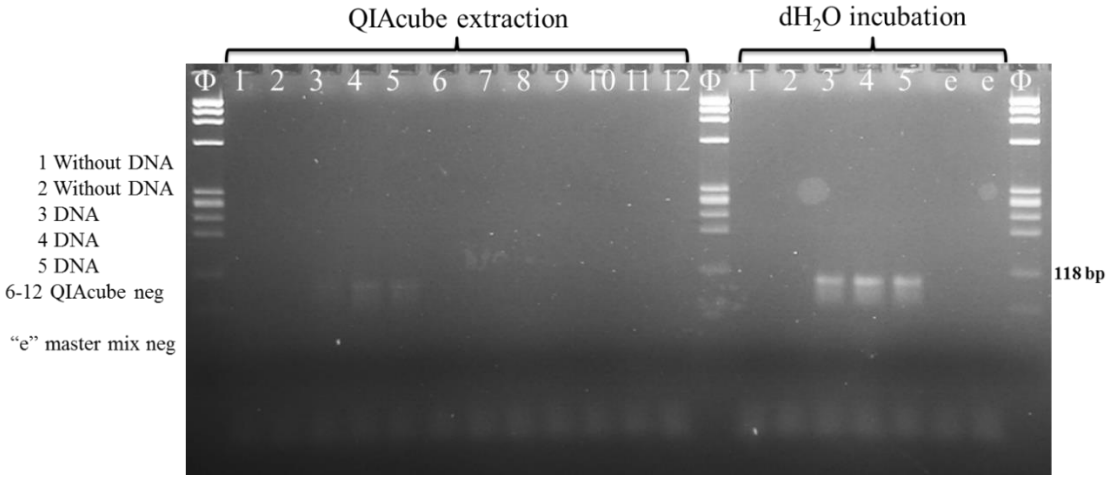


Figure 3.16: Amplification of recovered discs containing DNA solution
Paper discs that were collected during the deployment tests were either incubated in 200 µl dH_2O for one hour at room temperature to release the DNA or run through the QIAcube® system. The latter is commonly used in forensic laboratories for DNA extraction from body fluids. The process of extraction is hereby fully automated and eventually DNA is eluted in 200 µl AE Buffer (from QIAamp® DNA Mini Kit). 1 µl of both groups of extracted DNA were used as PCR input using primers which generated a fragment close to the 118 bp size. In both extraction groups, two discs with a no-DNA solution were present, along with three discs which were exposed to DNA solution. The QIAcube® system is designed for a total of 12 samples and has to be fully loaded. To exclude the possibility of cross-contamination during the automated process, all samples which did not include any sample, were also included in the PCR and run out on a 3% (w/v) LE agarose gel.

For most efficient results the QIAcube® machine is ideally fully loaded, thus six empty tubes were included during the purification process. To ensure that no cross-contamination had occurred, these "QIAcube neg" samples were included in the detection PCR. Samples entitled "without DNA" refer to the control solution which was deployed without any DNA, only containing 75% (v/v) EtOH, 10mM Tris pH 8.5 and bromphenol blue. As expected, no amplification occurred in either the "without DNA" samples or the QIAcube® negative samples. Samples numbered 3-5 each contained one disc with DNA solution on it and were either purified via QIAcube® or dH_2O incubation. In all six cases, bands were detected in amplification processes however the gel electrophoresis showed that the simple incubation in dH_2O resulted in a higher efficiency, shown by the brighter

bands. As a result, during all further deployment experiments, DNA was recovered by dH_2O incubation at room temperature for one hour.

The subsequent deployment experiments involved a DNA solution with one of the oligonucleotides itself and was detected via qPCR. V3T2 was used for this experiment because it had previously been chosen as a model oligonucleotide for the TaqMan® experiments. This further granted the opportunity to detect the oligonucleotide with three different TaqMan® probes (see section 3.1), in the event of a failure of detection. The construct was added with the aim of creating a final DNA concentration in the solution of approximately 1.5×10^7 molecules/µl (equal to 0.65 pg/µl). This was a considerably smaller amount of DNA, but as previously shown (section 3.1), DNA inputs of as little as 1×10^2 molecules/µl could be detected by the TaqMan® system.

A total of 26 discs with DNA solution and six discs with a solution which did not contain the oligonucleotide were analysed (Fig 24). For each group, a disc was included which was not hit by the solution. This was done to exclude any possible cross-contamination during the deployment itself. Additionally, two negative controls, i.e. only dH_2O was added to the PCR reactions, were included in the analyses. Oligonucleotide templates were detected using TaqMan® probe MGB-NFQ. Figure 19 depicts all described reactions and it can be observed that all samples which included the oligonucleotide showed amplification with Ct values between 23 and 32. All negative samples which include negative controls and discs with solution but no DNA, are below the threshold and show close to no fluorescence.

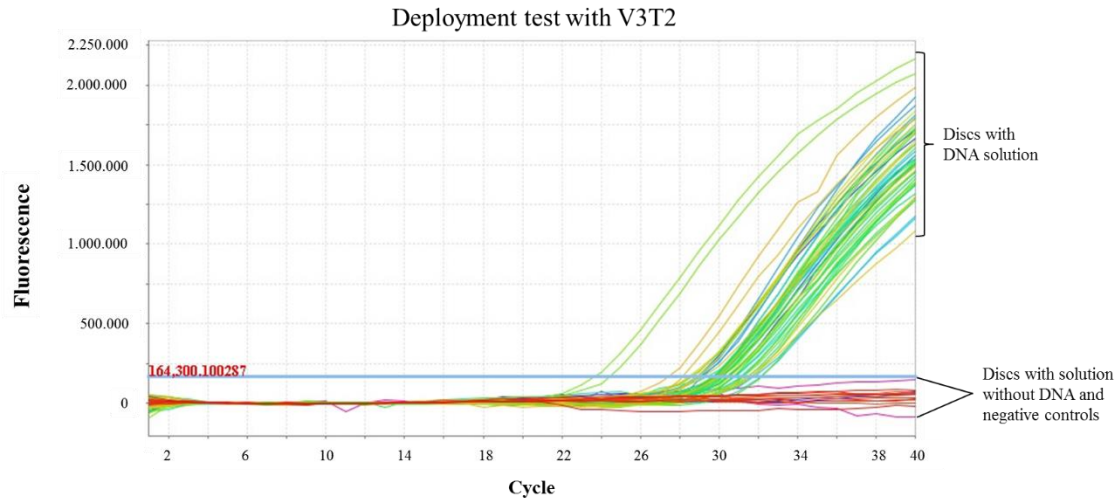


Figure 3.17: qPCR analysis of deployment test samples
After establishing the feasibility of DNA recovery and subsequent identification, a DNA solution was created containing the construct V3T2. Discs were collected, incubated in 200 µl dH_2O at room temperature and a qPCR was performed. The probe for detection was the MGB-quencher carrying probe from Applied Biosystems®.

3.4.1 Conclusion

The deployment experiments performed showed that recovery from the designed target discs was successful. The first experiments used PCR and gel electrophoresis for detection and latter experiments showed equally successful detection via qPCR. The decoding of a mixture deployed with the spray system could not be established due to the limited timespan of the project.

Chapter 4: Discussion

This project involved the development of a security device using synthetic single-stranded DNA, and creating a unique code that could be decoded after deployment using fluorescent probes. After verifying that the initial design for the decoding approach could be achievable, the experiments were developed to use qPCR and fluorescent probes to first detect fragments of the oligonucleotides amplified, and subsequently decode mixtures of such oligonucleotides. It will be considered how successful the different experiments were and what is further needed to accomplish the aim of creating the envisaged security device.

4.1 PCR-built construct validation

The first six months of this project mainly focussed on the verification of the existing constructs, the optimisation of the PCR assays, the introduction of synthetic oligonucleotide targets and development of qPCR assays. Initially, oligonucleotides were only tested in separate reactions; however, the final design involves a mixture of oligonucleotides. With not only several primer annealing sites but also different targets in one reaction, the PCR needs optimised primer pairs, which can be achieved by testing them in singular reactions, minimising any interactions. Hence, these experiments and their success are crucial for all proceeding experiments. The assay containing multiple oligonucleotides is designed to involve a sample with an unknown code that were amplified with all possible single primer pairs in separate reactions to identify the variable regions present in the sample. Those reactions may contain only one of up to 12 different primer pairs.

It was shown that all pGEMT-easy stored constructs were successfully extracted via PCR and further amplifiable. Despite initial problems, the optimisation of the assay resulted in a stable system. This is important for the project's further course. These experiments have shown that the amplification and thus identification of the variable sequences is successful. To further demonstrate the ability to identify the different targets,

PCR products were hybridised via ASO hybridisation. As shown in the results (Section 1.2 primer and target identification), the target-specific hybridisation was successful. However radioactive oligonucleotide hybridisation is not a commercially viable detection system, so further development was necessary.

4.2 HyBeacons®

Initial qPCR experiments with HyBeacons® were not successful. Several difficulties were encountered. Amplification plots did not show high amplification efficiency which might be due to a low detection efficiency of the HyBeacons® in this particular experimental design. Furthermore, data suggested contamination in the negative controls, which was confirmed by gel electrophoresis. The failure of the experiment, i.e. the failure of detection, was most likely caused by the incorrectly designed HyBeacons®. The probes' designs were previous experiments involved in this project, and were provided by LGC.

The detection of possible contamination found in all negative samples during qPCR and after gel electrophoresis might be explained by initial mishandling of the original oligonucleotides. These synthetic DNA strands are produced in extremely high concentration and cross-contamination might have occurred during preparing dilutions series or the actual PCR preparation. This supports the approach of using qPCR and not only relying on endpoint analysis, but being able to analyse the dynamics of amplification.

The failure to detect different oligonucleotides in subsequent experiments led to the suspicion that the low amplification efficiency derived from too small an input. The experiments were repeated using two different concentrations per oligonucleotide with the result that the lower concentration of the DNA samples was most likely miscalculated and a much smaller copy number was present in those dilutions. Throughout all experiments, the fluorescent signals were not as strong as expected which is probably due to low efficiency of HyBeacons® in this particular experimental set-up, rather than insufficient amplification.

The detectable concentration of deployed target DNA is a significant aspect in this project because it has to be known what concentration of oligonucleotides should be

added to the device for the system to function. Too low concentrations would mean that the marker might not be detectable and the device will fail to tag the intruder as intended. Too high a concentration will be a waste of material and thus increase the costs. Another potential issue would be that if an unnecessarily high concentration were used, cross contamination during preparation of the device is a real possibility, leading to incorrect decoding. No matter whether the container with DNA solution will be manufactured automatically or by hand, when the oligonucleotide concentration is too high, an error cannot be excluded. It is thus advisable to keep any possibilities for cross-contamination as small as possible. This possibility can be mitigated by observing the expected differences in amplification dynamics between subsequent deployments, another advantage of qPCR versus endpoint analysis.

HyBeacons® were originally designed to perform fast primary molecular analyses, such as DNA quantification, SNP detection and allele discrimination directly from body fluid samples (Liu, 2014; French *et al.*, 2001; French *et al.*, 2002). They are currently successfully used in the ParaDNA® system which tests crime scene evidence samples directly at the scene to determine quantity and quality of DNA and sex of the donor in the sample prior to further processing (Henco *et al.*, 1999; Wittwer *et al.*, 2001). These analyses are based on melting curves of the amplified DNA, i.e. it is an end point analysis. Despite much effort, the HyBeacon® probes were determined to be unsuitable for this project. The design of the constructs requires primer and probe annealing site detection in one single experiment run and it was shown that this can be achieved via qPCR amplification curves. However, the HyBeacon® probes not only failed in the determination of components in a mixture, but one of the previously designed probes (HyBeacon1) failed entirely due to self-annealing (see Results, section 2.4). An evaluation was performed by David French (LGC, inventor of HyBeacons®) to verify the findings presented and possibly find a solution for the encountered difficulties. All primer pairs were tested for their efficiency with satisfactory results. However, the experiments performed to resolve the problems with HyBeacon1 and the inability to distinguish between constructs in a mixture did not fulfil the requirements of the proposed system design. The experiments were focussed on end point analyses and mixtures were created that did not contain the problematic T3 and T2 combinations. As shown in the results, section 2.5, T3 could not be detected within a mixture of oligonucleotides under certain circumstances. The nature of this project makes a faultless identification of each

component in a mixture vital. The evaluation report, despite providing important and helpful information concerning primers, did not fully satisfy the criteria of decoding; neither did it provide a solution for the presented problem. A lot of time was invested in the optimisation for the use of HyBeacons® but our experiments showed that this system does not represent the ideal design, and so other probes were considered.

4.3 TaqMan®

The experiments using TaqMan® probes for detection showed that they are more efficient and likely to be successful for the final realisation of the product. It was shown that 3200 molecules of original input which was the smallest amount used in this study, could be successfully detected. It was important to determine how much of the oligonucleotides could be detected, to minimise the amount of oligonucleotides in the final container, which will be part of the security device. One container can hold enough liquid for approximately four deployments and it has to be assumed that the deployments take place over long intervals, i.e. several months. As the aim is to create not only a fast detection system but also an affordable one, it is necessary to avoid adding a large excess of oligonucleotides, which would inevitably increase the cost.

Moreover, the identification of different components in several mixtures, using TaqMan® probes, was more successful in comparison to the HyBeacon® experiments. Throughout the process of testing various mixtures, however, an issue surfaced when all three targets were present (see Results, section 3.3). When looking at the results, a false positive V2T1 appeared throughout. This could be due to overlapping excitation and emission spectra from the other two fluorescent dyes (Fig. 25).

a) b)

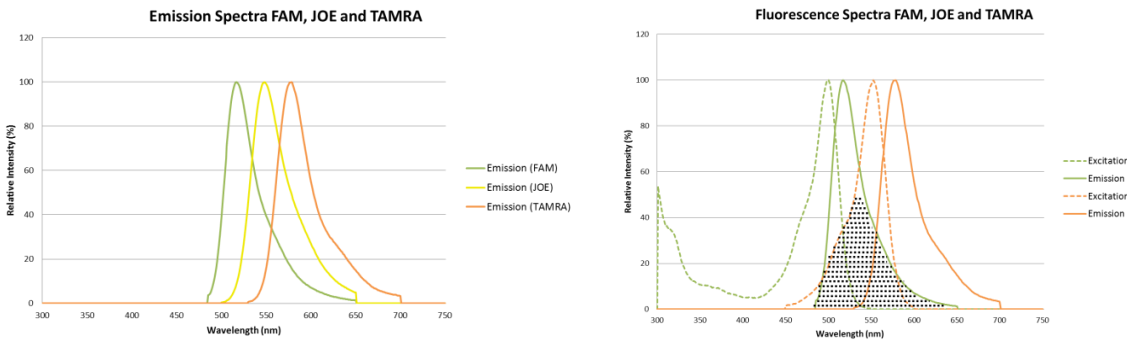


Figure 4.1: Emission spectra of reporter fluorophores
Data for three fluorophores have been taken from Life Technologies' SpectraViewer (http://www.lifetechnologies.com/uk/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html) a) Emission spectra of all three fluorophores used for the detection experiments. The spectral peak at 518 nm, 548 nm and 580 nm for FAM™, JOE™ and TAMRA™ respectively. b) The excitation and emission spectra for FAM™ and TAMRA®, which are incorporated in the probes detecting T2 and T3 respectively, show that there is an overlap in the spectra, falling into the emission value of JOE™ which detects T1. The maximum absorption for FAM™ and TAMRA™ are 494 nm and 555 nm. Values are taken from the atdBio website http://www.atdbio.com/content/33/FAM-fluorescein-HEX-JOE-ROX-TAMRA-TET-Texas-Red-and-others).

The two graphs in Figure 25 show the emission spectra of all three fluorescent reporters (a) and the combined emission and excitation spectra of FAM™ (HB2) and TAMRA™ (HB3) (b). It can be observed that the spectra are strongly overlapping creating a large area in a similar region of the spectrum as JOE™ (HB1). This could have led to a false positive signal, which was read by the instrument as an actual fluorescent signal. In order to get rid of these false signals, a repetition of the experiments with these false positive results was performed with different concentrations of the TaqMan® probes. By decreasing the probe concentration the strength of overlapping fluorescent signal was predicted to become lower. However, the repetition still showed the false positive signal in all concentrations. Evidently, these experiments will have to be repeated with more replicates to obtain a firm conclusion. One possibility is that the misreading was most likely due to a calibration error in the machine. Despite the significant overlap of the fluorescent spectra, the laser should still be able to distinguish between the different fluorophores. A reason for misreading could be the lack of a passive reference dye, usually ROX™, in the experiments. The passive reference dye is used to normalise for non-amplification related fluorescence signal variation, as recommended by the instrument manufacturer, but was not used in the reported experiments.

An unsolved problem is the failure of detection of oligonucleotide V3T2 and V2T3 in several of the mixtures. These drop outs seem random because in the experiments done to date, no pattern was detected which might predict the detection of either oligonucleotide. However, a similar drop out was observed with HyBeacons® and represents a major difficulty in the successful execution of the final product. This issue requires further experiments to understand and eliminate the inhibiting factor.

TaqMan® assays have been long established in molecular biology laboratories and are widely used. The nature of this project is such that the final product has to be considered even in this early stage of development, i.e. its judicial relevance. In the unlikely case that a conviction of an offender is doubted, the identification method must stand on strong scientific ground. The results obtained using TaqMan® probes showed that this identification of both components in the oligonucleotides was successful and stable even when using very small amounts of input. The obstacles encountered, i.e. a false positive and an oligonucleotide specific drop out, could potentially be solved by further optimisation of the qPCR. Conclusively, it can be said the HyBeacon® system was ineffective and using these probes is to be avoided, particularly as an efficient alternative was developed, and tested successfully.

4.4 Deployment tests

An essential part of this project was to show that the encoded DNA solution could be deployed, recovered and subsequently decoded. The final design for the product involves a wireless connection to the nearest police station that will ensure a quick arrival of police force. This rapid response will allow little chance for the intruder to dispose of the targeted, and thus evidentiary, clothing. The timeframe during the experiments from deployment to actual experiment, i.e. the extraction of DNA and subsequent qPCR, averaged between 1 to 3 hours, which is a large enough timeframe for the police to act.

It was shown that not only were the deployed DNAs effectively recovered but the initial experiments showed that they could be specifically identified. The initial experiments were performed using ΦX174/*Hae*III DNA marker instead of oligonucleotides. This was to protect the testing site from possible contamination before demonstrating that the deployment and subsequent recovery was actually successful. During the final steps, one oligonucleotide was deployed at a similar concentration to the

previously used ΦX174/*Hae*III DNA marker. The extreme sensitivity of qPCR could pick up signals in all targets that were hit by the solution, showing the system is fit for purpose, at least in principle.

Due to time restriction, no further deployment tests could be performed. Nevertheless, further experiments were planned, especially the deployment of mixtures with their subsequent decoding. As a selection of mixtures had already been successfully decoded, these would have made an ideal starting point for deployment tests, as it is already known that the decoding could be done. Final deployment tests, once the decoding has been successfully established, might involve a more rigorous experimental approach, e.g. changing the distance of the deployment apparatus, changing the angle, changing the spray dispersion pattern etc. The outcome of such experiments can help developing a guide for the final user, including advice where to best install the device (distance of deployment, angle, etc).

4.5 Technical considerations and limitations

The initial idea for this project involved a close collaboration together with LGC and it was therefore desirable to use HyBeacons® as fluorescent reporters in qPCR experiments. These probes are already widely in use for genotyping, foremost in forensic science in the ParaDNA® system (French *et al.*, 2002; Liu, 2014). However, HyBeacons® work best for endpoint analyses, i.e. melt curve analysis which, as shown, is not suitable for the prospect of this project. Despite efforts to optimise the system to fit the probes' needs, it was decided to move away from HyBeacons® and instead use TaqMan® probes as fluorescent reporters. The immediate success supported this decision. With fast progress, mixtures of oligonucleotides were successfully being decoded. However, also this system needs further optimisation to eliminate the encountered difficulties, such as the failure to detect a component in the mixture or false positive results. A main limitation for this project was time. Further experiments are needed to stabilise the decoding system and, moreover, to optimise and refine the deployment of the DNA solution.

This spraying device, as devices produced by SmartWater® and SelectaDNA®, will first and foremost act as a deterrent. Criminals are more likely to avoid premises marked as containing such spraying devices (Soper, 2010). Nevertheless, the system has to have the capacity of standing in court which means the successful recovery and a faultless decoding of a mixture is absolutely crucial. Furthermore, once the system is fully functional, i.e. neither oligonucleotide drop outs nor false positive signals are encountered, all experiments should be repeated in an ISO/IEC17025:2005 accredited forensic laboratory. All forensic research facilities and companies have to be ISO/IEC17025:2005 accredited by UKAS (United Kingdom Accreditation Service) to ensure technical competence of laboratory and staff for the appropriate research performed. Only when all the criteria are met, should the device be brought to market.

Appendices

Appendix 1

Sequences of all 36 shortened oligonucleotides for the original HyBeacon® approach.

Name	Sequence	Length	Tm
V1T1	CGTACGCGCCTTAAGTTTTCCACTGTCAGTGTCTGACATGCTATAGGGGTCGGTAACG	58	74.5
V1T2	CGTACGCGCCTTAAGTTTTCTGAAGTCAGAGCTGGGAGTTGCTATAGGGGTCGGTAACG	59	75
V1T3	CGTACGCGCCTTAAGTTTTCAGGAGTGTAGCCTACCAGCATGCTATAGGGGTCGGTAACG	60	75.5
V2T1	GTGGACCGACTAGGGACAAACACTGTCAGTGTCTGACAAAACGTGGGCGACCTATTTA	58	73.8
V2T2	GTGGACCGACTAGGGACAAATGAAGTCAGAGCTGGGAGTAAACGTGGGCGACCTATTTA	59	74.3
V2T3	GTGGACCGACTAGGGACAAAAGGAGTGTAGCCTACCAGCAAAACGTGGGCGACCTATTTA	60	74.8
V3T1	AGGTTACGTGGACTCCGTTGCACTGTCAGTGTCTGACACACCTCCGTACCGACAAGAT	58	75.2
V3T2	AGGTTACGTGGACTCCGTTGTGAAGTCAGAGCTGGGAGTCACCTCCGTACCGACAAGAT	59	75.7
V3T3	AGGTTACGTGGACTCCGTTGAGGAGTGTAGCCTACCAGCACACCTCCGTACCGACAAGAT	60	76.2
V4T1	GAATCCTAGGTCGACGCAACCACTGTCAGTGTCTGACACAATCGAGCGGACCTAAGAG	58	75.2
V4T2	GAATCCTAGGTCGACGCAATGAAGTCAGAGCTGGGAGTCAATCGAGCGGACCTAAGAG	59	75.2
V4T3	GAATCCTAGGTCGACGCAAAGGAGTGTAGCCTACCAGCACAATCGAGCGGACCTAAGAG	60	75.7
V5T1	CAGGTCGCACGTAGTATCCACACTGTCAGTGTCTGACATGAAGTCCTAACGTCCGGAG	58	75.2
V5T2	CAGGTCGCACGTAGTATCCATGAAGTCAGAGCTGGGAGTTGAAGTCCTAACGTCCGGAG	59	75.7
V5T3	CAGGTCGCACGTAGTATCCAAGGAGTGTAGCCTACCAGCATGAAGTCCTAACGTCCGGAG	60	76.2
V6T1	CACCGCCGATACTTAGTCGTCACTGTCAGTGTCTGACAGCTACCTACGCCCGATGTTA	58	75.2
V6T2	CACCGCCGATACTTAGTCGTTGAAGTCAGAGCTGGGAGTGCTACCTACGCCCGATGTTA	59	75.7
V6T3	CACCGCCGATACTTAGTCGTAGGAGTGTAGCCTACCAGCAGCTACCTACGCCCGATGTTA	60	76.2
V7T1	TCACCGAGTTAGACCGACCTCACTGTCAGTGTCTGACAATACTAATCCGACGGGTCCC	58	75.2

V7T2	TCACCGAGTTAGACCGACCTTGAAGTCAGAGCTGGGAGTATACTAATCCGAC GGGTCCC	59	75.7
V7T3	TCACCGAGTTAGACCGACCTAGGAGTGTAGCCTACCAGCAATACTAATCCGAC GGGTCCC	60	76.2
V8T1	GTCCGCCCGAGTCATAATAACACTGTCAGTGTCTGACAATTCCTACTAACGGC CCGAT	58	73.8
V8T2	GTCCGCCCGAGTCATAATAATGAAGTCAGAGCTGGGAGTATTCCTACTAACG GCCCGAT	59	74.4
V8T3	GTCCGCCCGAGTCATAATAAAGGAGTGTAGCCTACCAGCAATTCCTACTAACG GCCCGAT	60	74.9
V9T1	TATAAGCGGCGTGGGATTAGCACTGTCAGTGTCTGACAGGACTCACGTAGAG CGAAGG	58	75.2
V9T2	TATAAGCGGCGTGGGATTAGTGAAGTCAGAGCTGGGAGTGGACTCACGTAG AGCGAAGG	59	75.7
V9T3	TATAAGCGGCGTGGGATTAGAGGAGTGTAGCCTACCAGCAGGACTCACGTA GAGCGAAGG	60	76.2
V10T1	TTCGGACCTAGGCGTAGAAACACTGTCAGTGTCTGACAAATACGTTAGCGGG ACCCTT	58	73.8
V10T2	TTCGGACCTAGGCGTAGAAATGAAGTCAGAGCTGGGAGTAATACGTTAGCG GGACCCTT	59	74.4
V10T3	TTCGGACCTAGGCGTAGAAAAGGAGTGTAGCCTACCAGCAAATACGTTAGCG GGACCCTT	60	74.9
V11T1	CCCGATAGCACGTAGTCGATCACTGTCAGTGTCTGACAAAGTTTAGTAACGCG CACGG	58	74.5
V11T2	CCCGATAGCACGTAGTCGATTGAAGTCAGAGCTGGGAGTAAGTTTAGTAACG CGCACGG	59	75
V11T3	CCCGATAGCACGTAGTCGATAGGAGTGTAGCCTACCAGCAAAGTTTAGTAAC GCGCACGG	60	75.6
V12T1	TTAGCCTAAGCGGTCGAAAACACTGTCAGTGTCTGACAGATTGTTACGACGG TCCTGG	58	73.8
V12T2	TTAGCCTAAGCGGTCGAAAATGAAGTCAGAGCTGGGAGTGATTGTTACGACG GTCCTGG	59	74.4
V12T3	TTAGCCTAAGCGGTCGAAAAAGGAGTGTAGCCTACCAGCAGATTGTTACGAC GGTCCTGG	60	74.9

Appendix 2

Oligonucleotides (lab-built constructs):

SYNTH NAME	SEQUENCE	Length	Tm °C
V1T1	CGTACGCGCCTTAAGTTTTCGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGT CTGACA**CACACAAAATGCACGACACACATAACCTGCTATAGGGGTCGGTAACG	112	78.7
V1T2	CGTACGCGCCTTAAGTTTTCAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGG AGT**AGTATCGTTAGTCTCTGGACACAAGGTAAACGTGCTATAGGGGTCGGTAACG	112	79.4
V1T3	CGTACGCGCCTTAAGTTTTCTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCA GCA**GCACTATATGCTTAAGGACAGGCATTTGGCTTGCTATAGGGGTCGGTAACG	112	79.8
V2T1	GTGGACCGACTAGGGACAAAGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGT GTCTGACA**CACACAAAATGCACGACACACATAACCAAACGTGGGCGACCTATTTA	112	78.3

V2T2	GTGGACCGACTAGGGACAAAAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGG GAGT**AGTATCGTTAGTCTCTGGACACAAGGTAAACGAAACGTGGGCGACCTATTTA	112	79
V2T3	GTGGACCGACTAGGGACAAATCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACC AGCA**GCACTATATGCTTAAGGACAGGCATTTGGCTAAACGTGGGCGACCTATTTA	112	79.4
V3T1	AGGTTACGTGGACTCCGTTGGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTG TCTGACA**CACACAAAATGCACGACACACATAACCCACCTCCGTACCGACAAGAT	112	79
V3T2	AGGTTACGTGGACTCCGTTGAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGG GAGT**AGTATCGTTAGTCTCTGGACACAAGGTAAACGCACCTCCGTACCGACAAGAT	112	79.8
V3T3	AGGTTACGTGGACTCCGTTGTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACC AGCA**GCACTATATGCTTAAGGACAGGCATTTGGCTCACCTCCGTACCGACAAGAT	112	80.1

Appendix 3

112bp constructs

V1T1

CGTACGCGCCTTAAGTTTTCGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACAC
AAAATGCACGACACACATAACCTGCTATAGGGGTCGGTAACG

V1T2

CGTACGCGCCTTAAGTTTTCAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCGT
TAGTCTCTGGACACAAGGTAAACGTGCTATAGGGGTCGGTAACG

V1T3

CGTACGCGCCTTAAGTTTTCTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTATA
TGCTTAAGGACAGGCATTTGGCTTGCTATAGGGGTCGGTAACG

V2T1

GTGGACCGACTAGGGACAAAGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CAC
ACAAAATGCACGACACACATAACCAAACGTGGGCGACCTATTTA

V2T2

GTGGACCGACTAGGGACAAAAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGAAACGTGGGCGACCTATTTA

V2T3

GTGGACCGACTAGGGACAAATCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTA
TATGCTTAAGGACAGGCATTTGGCTAAACGTGGGCGACCTATTTA

V3T1

AGGTTACGTGGACTCCGTTGGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCCACCTCCGTACCGACAAGAT

V3T2

AGGTTACGTGGACTCCGTTGAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGCACCTCCGTACCGACAAGAT

V3T3

AGGTTACGTGGACTCCGTTGTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTCACCTCCGTACCGACAAGAT

V4T1

GAATCCTAGGTCGACGCAACGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCCAATCGAGCGGACCTAAGAG

V4T2

GAATCCTAGGTCGACGCAAAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCGT
TAGTCTCTGGACACAAGGTAAACGCAATCGAGCGGACCTAAGAG

V4T3

GAATCCTAGGTCGACGCAATCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTATA
TGCTTAAGGACAGGCATTTGGCTCAATCGAGCGGACCTAAGAG

V5T1

CAGGTCGCACGTAGTATCCAGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCTGAAGTCCTAACGTCCGGAG

V5T2

CAGGTCGCACGTAGTATCCAAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGTGAAGTCCTAACGTCCGGAG

V5T3

CAGGTCGCACGTAGTATCCATCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTTGAAGTCCTAACGTCCGGAG

V6T1

CACCGCCGATACTTAGTCGTGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCGCTACCTACGCCCGATGTTA

V6T2

CACCGCCGATACTTAGTCGTAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCGT
TAGTCTCTGGACACAAGGTAAACGGCTACCTACGCCCGATGTTA

V6T3

CACCGCCGATACTTAGTCGTTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTGCTACCTACGCCCGATGTTA

V7T1

TCACCGAGTTAGACCGACCTGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCATACTAATCCGACGGGTCCC

V7T2

TCACCGAGTTAGACCGACCTAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCGT
TAGTCTCTGGACACAAGGTAAACGATACTAATCCGACGGGTCCC

V7T3

TCACCGAGTTAGACCGACCTTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTATACTAATCCGACGGGTCCC

V8T1

GTCCGCCCGAGTCATAATAAGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCATTCCTACTAACGGCCCGAT

V8T2

GTCCGCCCGAGTCATAATAAAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGATTCCTACTAACGGCCCGAT

V8T3

GTCCGCCCGAGTCATAATAATCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTATTCCTACTAACGGCCCGAT

V9T1

TATAAGCGGCGTGGGATTAGGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CAC
ACAAAATGCACGACACACATAACCGGACTCACGTAGAGCGAAGG

V9T2

TATAAGCGGCGTGGGATTAGAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGGGACTCACGTAGAGCGAAGG

V9T3

TATAAGCGGCGTGGGATTAGTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTGGACTCACGTAGAGCGAAGG

V10T1

TTCGGACCTAGGCGTAGAAAGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCAATACGTTAGCGGGACCCTT

V10T2

TTCGGACCTAGGCGTAGAAAAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGAATACGTTAGCGGGACCCTT

V10T3

TTCGGACCTAGGCGTAGAAATCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTAATACGTTAGCGGGACCCTT

V11T1

CCCGATAGCACGTAGTCGATGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCAAGTTTAGTAACGCGCACGG

V11T2

CCCGATAGCACGTAGTCGATAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGAAGTTTAGTAACGCGCACGG

V11T3

CCCGATAGCACGTAGTCGATTCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTAAGTTTAGTAACGCGCACGG

V12T1

TTAGCCTAAGCGGTCGAAAAGCATTCGAGCATCCTACCACAAAATCT**CACTGTCAGTGTCTGACA**CACA
CAAAATGCACGACACACATAACCGATTGTTACGACGGTCCTGG

V12T2

TTAGCCTAAGCGGTCGAAAAAGAGACCTCAGTCCAAGCAGT**TGAAGTCAGAGCTGGGAGT**AGTATCG
TTAGTCTCTGGACACAAGGTAAACGGATTGTTACGACGGTCCTGG

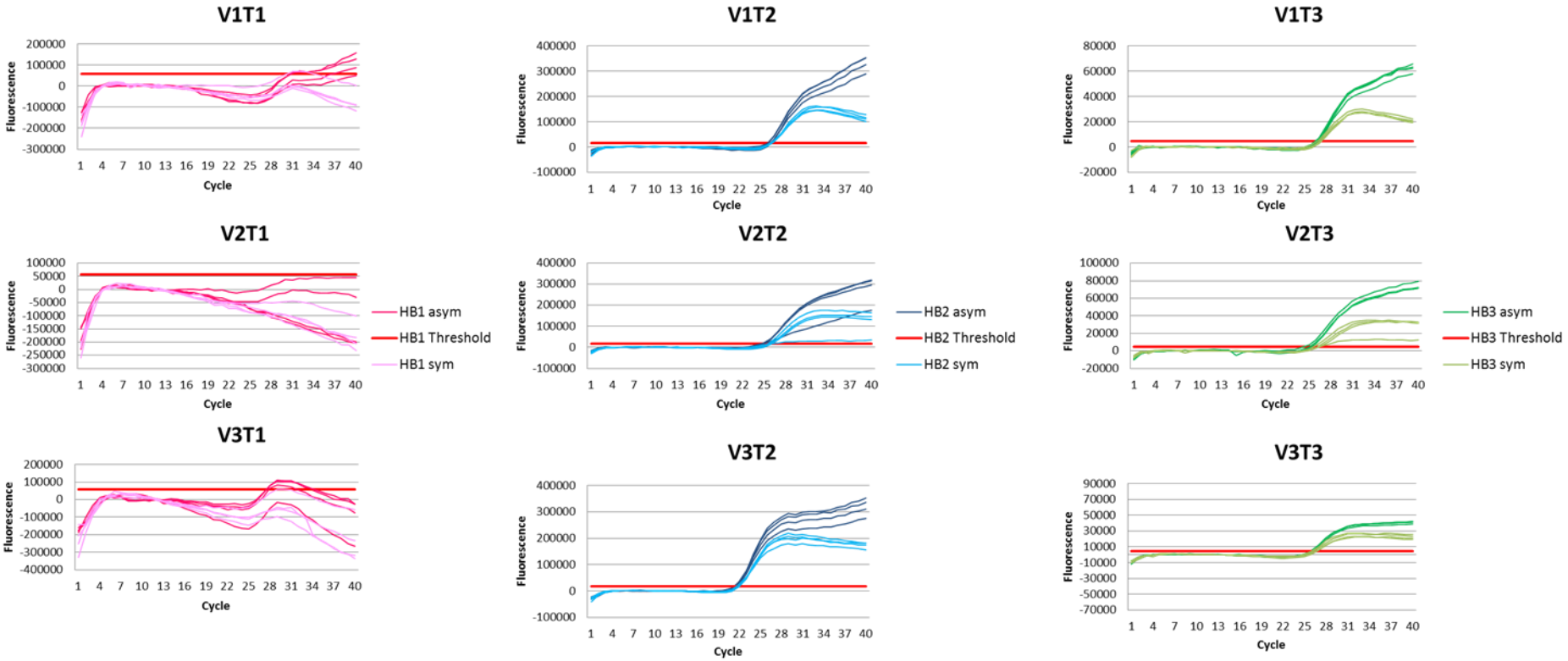
V12T3

TTAGCCTAAGCGGTCGAAAATCTTGGCTGATACCCGACATG**AGGAGTGTAGCCTACCAGCA**GCACTAT
ATGCTTAAGGACAGGCATTTGGCTGATTGTTACGACGGTCCTGG

.

Appendix 4

Asymmetric PCR plots



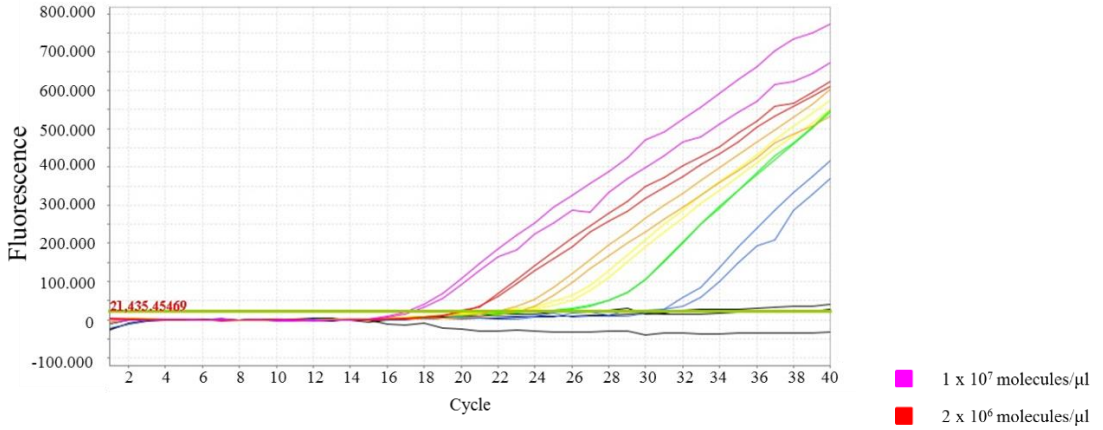
Asymmetric PCR

In a direct comparison, templates were amplified with both primers at the same final concentration of 0.5 µM (symmetric PCR) and with the reverse primer in a 10-fold higher final concentration (5 µM) (asymmetric PCR). 2 µl of the constructs, at $1x10^4$ molecules/µl, were added to the reaction.

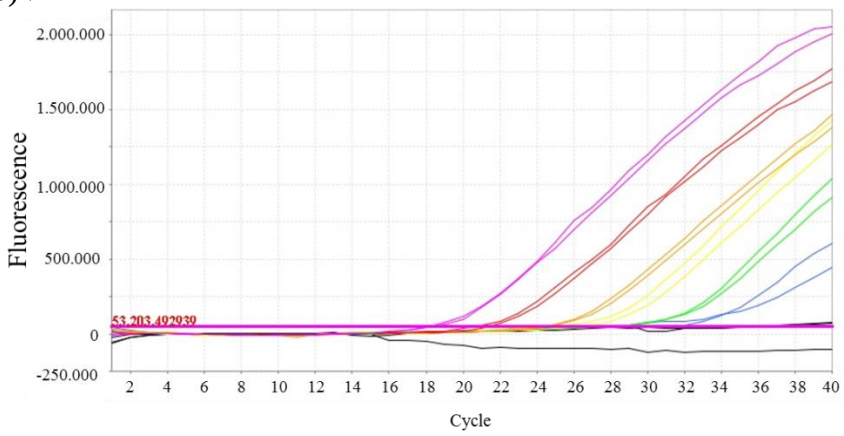
Appendix 5

Amplification of all V1Tx oligonucleotides and detection with all three TaqMan® probes

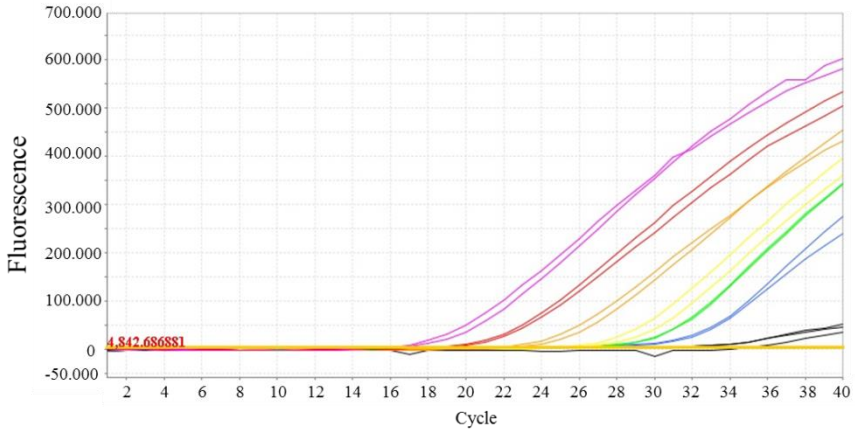
a)V1T1



b)V1T2



c)V1T3



Three TaqMan® probes for identification of all 82 nt long constructs

All nine oligonucleotides (82 nt) were amplified individually with the three different TaqMan® probes using qPCR. All three probes were present in each primer pair mastermix at a final concentration of 0.25 µM. A dilution series of each template was used as input, and reactions were amplified in duplicates. a) An example for all T1 containing oligonucleotides, detected with TaqMan1 which was labelled with JOE™. All other T1 oligonucleotides showed similar amplification dynamics. b) In order to create a complete comparison, all T2 oligonucleotides were also amplified and showed the same pattern as previously observed with the three initial TaqMan® probes. c) All oligonucleotides containing target3 were identified using TaqMan3

which had TAMRA™ incorporated as fluorescent reporter. As a model for all identified T3 samples, V1T3 is shown with an expected pattern in correlation with the initial DNA concentration. All T3 oligonucleotides (data not shown) displayed an almost identical diagram pattern.

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