Multiplex Detection and SNP Genotyping in a Single Fluorescence Channel

Guoliang Fu1,2*, Andrea Miles1, Luke Alphey1,2*

1 Oxitec Limited, Oxford, United Kingdom, 2 Department of Zoology, University of Oxford, Oxford, United Kingdom

Abstract

Probe-based PCR is widely used for SNP (single nucleotide polymorphism) genotyping and pathogen nucleic acid detection due to its simplicity, sensitivity and cost-effectiveness. However, the multiplex capability of hydrolysis probe-based PCR is normally limited to one target (pathogen or allele) per fluorescence channel. Current fluorescence PCR machines typically have 4–6 channels. We present a strategy permitting the multiplex detection of multiple targets in a single detection channel. The technique is named Multiplex Probe Amplification (MPA). Polymorphisms of the CYP2C9 gene (cytochrome P450, family 2, subfamily C, polypeptide 9, CYP2C9*2) and human papillomavirus sequences HPV16, 18, 31, 52 and 59 were chosen as model targets for testing MPA. The allele status of the CYP2C9*2 determined by MPA was entirely concordant with the reference TaqMan® SNP Genotyping Assays. The four HPV strain sequences could be independently detected in a single fluorescence detection channel. The results validate the multiplex capacity, the simplicity and accuracy of MPA for SNP genotyping and multiplex detection using different probes labeled with the same fluorophore. The technique offers a new way to multiplex in a single detection channel of a closed-tube PCR.

Introduction

Recently, real-time PCR, which is carried out in a closed-tube format, has become a very important tool for detection and quantification of specific sequences in a sample [1–4]. Multiplex PCR, which uses multiple pairs of primers to amplify multiple target sequences simultaneously in a single PCR reaction, is likely to be a more efficient approach than standard single primer-pair PCR when multiple targets need to be detected [5–8].

Multiplex real-time PCR offers numerous advantages, including time savings, elimination of contamination risk, reduced reagent costs, increased throughput, conservation of precious sample material and reliable results [9–12]. However, current probe-based methods allow detection of only one target sequence per fluorescence channel. The multiplex fluorescent PCR is therefore limited by the availability of fluorescence dyes and channels; up to five or six targets, depending on particular PCR machines, can be detected simultaneously in the current platforms [5,13].

We have developed a novel multiplex detection method (Multiplex Probe Amplification, MPA), which allows the detection of up to four targets in a single detection channel, using current standard instrumentation. As model targets against which to test this approach, we selected CYP2C9*2 SNP genotyping, and detecting and typing high-risk HPV sequences, each in a single closed-tube reaction. Variations in CYP2C9 gene may lead to differential responses and metabolism of numerous drugs, such as warfarin. Multiple-tube PCR, sequencing and microarray hybridisation techniques have been utilised for genotyping these SNPs [14,15]. Human papillomavirus (HPV) is one of the most common causes of sexually transmitted disease (STD) worldwide; high-risk types of HPV can cause cervical cancer. Accurate molecular diagnosis is needed to inform patient management and follow-up after treatment [16]. In this study, two alleles of one SNP or four strains of a pathogen sequence were successfully genotyped in a single detection channel. This opens up the prospect of multiplex detection of far more targets in a single closed-tube reaction than the 4–6 currently possible with conventional PCR technology.

Results

Principles and Melting Profile of Probes

The principles of MPA are as follows: Firstly, probes labeled with the same fluorescence dye can be distinguished if the probes themselves, independent of hybridising to target sequence, have different melting temperatures. Differential melting temperatures for free probes can be arbitrarily arranged by providing complementary oligonucleotide with sequences of varying length, base composition and/or complementarity to the target-hybridising oligonucleotide. Secondly, if a target is present, its corresponding probe is consumed during PCR amplification. Comparing melting profiles of the probes after the reaction reveals which probes have been consumed; this in turn indicates which targets are present in a sample.

For each target we designed a probe consisting of two oligonucleotides: a dual-labeled target-hybridising oligonucleotide (THO) and a partially complementary oligonucleotide (PCO).
THO is equivalent to the hydrolysis probe in the traditional TaqMan PCR method. THO and PCO are capable of hybridising to each other, forming a partially double-stranded probe (Figure 1). By design, each probe has unique melting properties, which are characterised by its melting temperature ($T_m$). THO, which is complementary to a target sequence, is labeled with a fluorophore at the 5’ end and a quencher at the 3’ end. PCO has its 3’ end blocked by modifying the 3’ end, for example, by attaching a label or a phosphate group, so that it cannot be extended. If the 3’ end of PCO comprises a moiety which is not a quencher (Figure 1A and 2A), fluorescence emission is at the highest amount when THO and PCO are hybridised. The fluorescence emission is

**Figure 1. Nucleic acid probe design and its melting profile.** (A) Probe consists of a target-hybridising oligonucleotide (THO) labeled with a Fluorophore at the 5’ end and a Quencher at the 3’ end, and a partially complementary oligonucleotide (PCO) without a label. The hybrid of THO:PCO is named plus probe. The melting curve shows decreased fluorescence emission when temperature rises (left panel); the negative derivative plot of the emission reading versus temperature reveals a positive value of the melting peak (right panel). (B) Minus probe consists of a THO labeled with a Fluorophore at the 5’ end and a Quencher at the 3’ end, and a PCO labeled with a Quencher at the 3’ end. The melting curve shows increased fluorescence emission when temperature rises (left panel); the negative derivative plot of the emission reading versus temperature reveals a negative value of the melting peak (right panel).

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The key feature of consumption of THO could be achieved by several methods. For example, THO might be incorporated into a PCR product, where THO acts as a primer. In this study, we designed the THO to work as a hydrolysis probe, which is hydrolysed during the amplification [17]. The degradation of THO, like ordinary probe-based real-time PCR, results in an increase in fluorescence emission. We genotyped 25 samples using this design. Figure 3 shows amplification plots (Figure 3A) and melting curves (Figure 3B, 3C and 3D). The melting curve of a homozygous sample of “G” allele along with no amplification controls is shown in Figure 3B. A heterozygous sample along with no amplification controls is shown in Figure 3C. A homozygous of “A” allele along with the no amplification controls is shown in Figure 3D. This result is 100% in concordance with TaqMan® SNP Genotyping Assays from Applied Biosystems.

Minus Probes Detecting Four HPV Sequences

To test the approach further, with minus probes, different target sequences and more independent targets per channel, we designed four minus probes labeled with FAM dye for HPV16, HPV31, HPV52 and HPV59 sequences. The probes’ THOs are complementary to the conserved L1 region of the HPV genome sequences. HPV59 probe has a TM of 35°C; HPV31 probe has a TM of 43°C; HPV16 probe has a TM of 51°C; and HPV52 probe has a TM of 57°C. After amplification, melting profiles were performed. Compared with the negative control, each individual HPV target sequences can be correctly distinguished in the FAM detection channel (Figure 4A–D).

Sensitivity of the assays on genomic DNA

The limit of detection of the HPV assays using plus and minus probes was evaluated in a real-time PCR format. HeLa cell line genomic DNA containing HPV16 and SiHa cell line genomic DNA containing HPV18 were serially diluted in 10-fold increments. Real-time PCR assays were performed on the dilution series and negative controls; all were performed in duplicate. The HPV negative K562 cell line gDNA control and water control were all negative. The HeLa cell genome used contains 10 to 50 copies of HPV18 DNA, whereas the SiHa cell genome contains a single integrated copy of HPV16 [18]. A sensitivity of 0.03ng of gDNA was determined for both plus and minus probe assays. As 0.03ng of human genomic DNA is about 10 genome equivalents, this indicates that the assays can detect at least 10 copies of HPV16 containing the target HPV sequences, and a no DNA negative control, were PCR amplified, and fluorescence emission was collected in the FAM channel. Typical real-time PCR amplification plots for each series of template dilutions are shown in Figure 2. Panel C – HPV16, panel E – HPV18, panel G – HPV16+ HPV18. Melting profiles were obtained after amplification. Compared with the negative control, when the target HPV16 is present, the melting peak at 46°C in the melting curve has disappeared (Figure 2D). When the target HPV16 is present, the melting peak at 37°C has disappeared (Figure 2F). When both targets HPV16 and HPV18 are present, the two melting peaks have both disappeared (Figure 2H). The results clearly demonstrate that HPV16 or HPV18 or both HPV16+HPV18 can be detected individually and distinguished, even in the same detection channel.

Plus Probes Genotyping SNP CYP2C9*2

To test if this method can be used for SNP genotyping, we designed probes and primers for genotyping SNP CYP2C9*2 in a single fluorescent detection channel in a real-time PCR machine. Two plus probes were designed, one corresponds to the A allele (c.430T) and one corresponds to the G allele (c.430C). “A” allele probe has a TM of 44°C; “G” allele probe has a TM of 39°C. If a particular SNP is present in a sample, its corresponding probe is hydrolysed by the 5’ nuclease activity of Taq polymerase during amplification, resulting in an increase in fluorescence emission. We genotyped 25 samples using this design. Figure 3 shows amplification plots (Figure 3A) and melting curves (Figure 3B, 3C and 3D). The melting curve of a homozygous sample of “G” allele along with no amplification controls is shown in Figure 3B. A heterozygous sample along with no amplification controls is shown in Figure 3C. A homozygous of “A” allele along with the no amplification controls is shown in Figure 3D. This result is 100% in concordance with TaqMan® SNP Genotyping Assays from Applied Biosystems.

Multiplex Detection and Genotyping

HPV target sequences can be correctly distinguished in the FAM channel. Typical real-time PCR amplification plots for each series of template dilutions are shown in Figure 2. Panel C – HPV16, panel E – HPV18, panel G – HPV16+ HPV18. Melting profiles were obtained after amplification. Compared with the negative control, when the target HPV16 is present, the melting peak at 46°C in the melting curve has disappeared (Figure 2D). When the target HPV16 is present, the melting peak at 37°C has disappeared (Figure 2F). When both targets HPV16 and HPV18 are present, the two melting peaks have both disappeared (Figure 2H). The results clearly demonstrate that HPV16 or HPV18 or both HPV16+HPV18 can be detected individually and distinguished, even in the same detection channel.
A

HPV16THO 5’ FAM-TTCAGGACCCACAGGACCC-BHQ1 3’
HPV16PCO 3’ Ph-AAGTTCTGAGTCTCGTTG 5’

HPV18THO 5’ FAM-AGCCCCAAATGAAATCCGGTGACC-BHQ1 3’
HPV18PCO 3’ Ph-TCGGAGTATTATTGAGGTCATGG 5’

B

Melting Curve

Fluorescence (R’(T))

Temperature (°C)

C

Amplification Plots

Fluorescence (dR)

Cycles

D

Melting Curve

Fluorescence (R’(T))

Temperature (°C)

E

Amplification Plots

Fluorescence (dR)

Cycles

F

Melting Curve

Fluorescence (R’(T))

Temperature (°C)

G

Amplification Plots

Fluorescence (dR)

Cycles

H

Melting Curve

Fluorescence (R’(T))

Temperature (°C)
DNA and 100-500 copies of HPV18 DNA in the background of human genomic DNA.

Discussion

Compared with traditional hydrolysis probe-based PCR, this MPA method comprises an extra oligonucleotide, which is capable of hybridising to the labeled oligonucleotide. Since the extra oligonucleotide hybridises to the labeled oligonucleotide at a temperature lower than the annealing temperature, it does not affect the PCR process. In other words, in terms of PCR amplification there is no difference between this multiplex method and the traditional hydrolysis probe PCR. Therefore, this new method inherits all the benefits of the traditional hydrolysis probe-based PCR, but with an extra benefit of being able to multiplex using probes labeled with the same fluorophore and detection in a single fluorescent channel.

To assess the scope and flexibility of MPA, we have also performed experiments using additional fluorophores rather than FAM, using plus and minus probes together, using minus probes and a single-stranded probe, and even detecting all 14 high-risk HPV strain sequences in a single-closed tube reaction. In one experiment, four minus probes labeled with HEX dye were designed, detecting HPV18, HPV39, HPV58 and HPV68 sequences (Figure SIA-D). In another experiment, a combination of plus and minus probes was tested for detecting HPV33, HPV45 and HPV35 sequences (Figure SIE-G). A combination of minus probe and single-stranded probe was also tested. Two minus probes labeled with Cy5 dye for HPV56 and HPV66 sequences, and one single-stranded probe labeled with the same Cy5 dye for HPV51 sequence were included in a PCR reaction (Figure SII-J). These experiments demonstrated that an individual HPV sequence can be distinguished by using a combination of multiple minus probes, plus probes and single-stranded probes.

When analysing the melting profiles, we have noticed that peaks adjacent to the ‘true’ peak may also show a modest reduction in peak height in minus probe assays for some HPV targets (Figure 4C, SIA-B). This “shrunk neighbouring peaks phenomenon” could potentially lead to the erroneous conclusion that two or more targets were present in the original template. In follow-up experiments, using different PCR conditions and master mix, we found this phenomenon to be assay-specific but highly reproducible. Though the origin of this effect remains unclear, it does not affect the diagnostic specificity and accuracy of the assay – the signature melting profile for single HPV target is clearly different from the melting profiles for double or triple HPV targets. One always can judge whether it is single, double or triple HPV infection by comparing with reference melting profiles generated from single and double target standards.

Recently, a novel method named Multicolor Combinatorial Probe Coding (MCPC) was described for increasing the target numbers of real-time PCR detection in one reaction [19]. This strategy uses differently coloured fluorophores in various combinations to label individual probes. It successfully detected up to 15 targets in a single reaction. However, one of the major limitations of MCPC is that it is not suitable for simultaneous detection of multiple targets in one reaction, as the colour overlaps between singly labelled, doubly labelled, and multiply labelled probes complicate the MCPC profiles. Fortunately MPA, with its different and much simpler labelling strategy, does not suffer from this limitation, indeed in our HPV assay we routinely detected multiple targets in one reaction (Figure 2 and data not shown).

Our results demonstrate that it is possible to overcome the current one-channel-one-target limitation. By this method a greater number of target sequences can be analysed in a single closed tube by designing sets of probes that hybridise to different target sequences and have different melting temperatures. If a target sequence is present, its corresponding probe is consumed. The target can then be determined based on the comparison of the melting profiles of the probes before and after the reactions. Groups of probes in a set can be attached with the same label, allowing for independent monitoring of multiple targets at a single emission wavelength. Our method provides a significant improvement over the current closed-tube multiplex PCR technology, allowing for a 2–4 fold increase in the number of targets being analysed in the current instruments.

Materials and Methods

Cell line genomic DNA and plasmid DNA

The HPV18 positive cervical HeLa cell DNA was purchased from New England Biolabs; the HPV16 positive cervical SiHa cell line was purchased from Abgent; the HPV negative K562 cell line DNA was purchased from Promega. Genomic DNA was extracted using GeneJET™ Genomic DNA Purification Kit from Fermentas according to manufacturer’s instruction. Plasmid DNAs containing HPV sequences were synthesised by GeneArt. Serial 10-fold dilutions were undertaken to produce a titration series representing from 30pg to 0.039ng/μL for genomic DNA, and 106 to 1012 copies/μL for plasmid DNA.

Probe Design and Oligonucleotide Synthesis

Two plus probes were designed, targeting the E6/E7 region of the HPV genome sequence. The target-hybridising oligonucleotides (THOs) were synthesised with a FAM label at the 5‘ end and a BHQ1 at the 3‘ end. The nucleotide sequence for HPV16 THO is: 5’ TTCAGGACCCACAGGAGCGACC 3‘. The nucleotide sequence for HPV18 THO is: 5’ AGGCCAAAATGAAATTCCGGTTGACC 3‘. Partially complementary oligonucleotides (PCOs) were synthesised with the same length as the THOs and were attached with a phosphate group at the 3‘ end. The nucleotide sequence for HPV16 PCO is: 5’ GGGT-TGCTTTGCTGAGTCCGGAAC 3‘. The nucleotide sequence for HPV18 PCO is: 5’ GGTTAAGAGGTTATATTAGGAGGC 3‘. A THO and PCO were combined at a ratio of 1:2 to form a partially double-stranded nucleic acid probe. Forward and reverse primers were designed to be upstream and downstream of the probe-binding region. The sequences for HPV16 forward and reverse primers are 5’ AGACATTTTATG-CACGAAAAGAGAAC 3‘ and 5’ TCTGTGCAATACTGAC-
TGGTAACCTTTCTG 3', respectively. The sequences for HPV18 forward and reverse primers are 5' GTATGCATGGACCATAGGCAC- TAAAGCAAC 3' and 5' TCGCTTAATGCTCGTAGACATGA 3', respectively. Oligonucleotides were synthesized by Eurogentec.

Two plus probes were designed, targeting the SNP CYP2C9*2. The target-hybridising oligonucleotides (THOs) were synthesised with FAM at the 5' end and BHQ1 at the 3' end. The nucleotide sequence for the "G" allele probe is: 5' CTTGAACACGGTCCTCAATGC 3'. The nucleotide sequence for the "A" allele probe is: 5' TCTTGAACACGGTCCTCAATGC 3'. The partially complementary oligonucleotide (PCO) was the same for both allele probes and was attached with a phosphate group at the 3' end. The nucleotide sequence of the PCO is: 5' GCATTAAGGACTGTGTCGAAGA 3'. Forward and reverse primers were designed to be upstream and downstream of the probe-binding region. The sequences for forward and reverse primers are 5' TAAGGTCAATGGATA TGGAGTAGG 3' and 5' GAATTGTTTT- CAGCAATGGAAAGAA 3', respectively.

Figure 3. Plus probes genotyping SNP CYP2C9*2. (A) is the graphic presentation of the amplification plot of PCR for genotyping SNP CYP2C9*2 on three genomic DNA samples and three negative controls. (B) is melting curves showing one sample is homozygous for G allele. (C) is melting curves showing one sample is heterozygous. (D) is melting curves showing one sample is homozygous for A allele.
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Consensus primers BSGP5+/BSGP6+ targeting the highly conserved L1 region that have the potential to detect all mucosal HPV types were used in PCR detection with minus probes. PCR amplification by BSGP5+/BSGP6+ has been described [20]. Four minus probes labeled with FAM dye were designed, targeting the conserved L1 region of the HPV genome sequences of HPV16, HPV31, HPV52 and HPV59. The target-hybridising oligonucleotides (THOs) were synthesized with FAM at the 5' end and BHQ1 at the 3' end. The nucleotide sequence for HPV16 probe is: 5’ CTGCCATATCTACTTCAGAAACTACATATAA 3’. The nucleotide sequence for HPV31 probe is: 5’ CTGCCATATCTACTTCAGAAACTACATATAA 3’. The nucleotide sequence for HPV52 probe is: 5’ CTGCCATATCTACTTCAGAAACTACATATAA 3’. The nucleotide sequence for HPV59 probe is: 5’ CTGCCATATCTACTTCAGAAACTACATATAA 3’. The partially complementary oligonucleotide (PCO) was the same for HPV16 and HPV31 probes and was attached with a Dabcyl group at the 3’ end, having a sequence 5’ GTATATTTTGACGGAGACTCTGTAGAAAG 3’.

Real-time PCR and Melting Profile Analysis

PCR reactions in a final volume of 25 μl consist of two equal amounts of mix: 12.5 μl of 2 x FastStart Universal Probe master (Rox) (Roche Diagnostics GmbH, Mannheim Germany) and 12.5 μl primer/probe mix. Primer/probe mix was created as follows: the primers and probes were mixed to a final concentration of 0.4 μM of probes and 0.6 μM of primers, and 1 μl of various amount of target templates were added. Amplification reactions and melting profiles were performed in a Stratagene real-time PCR MX3005P system. The thermal profile was: 95°C for 9 min 30 sec; 40 cycles of 95°C for 20 sec and 60°C for 60 sec. Fluorescence measurements were recorded during the read steps at 60°C. Post-amplification melting profile had the following conditions: after the last cycle of PCR, heat at 95°C for 10 sec, cool to 30°C and hold for 30 sec, then slowly increase the temperature to 80°C. The fluorescence emission data is continually collected during the rising temperatures. The negative

![Figure 4. Melting curve analysis of the amplification reactions of various HPV targets.](https://www.plosone.org/article/funding figure)
derivative of the emission reading, with respect to temperature, is plotted against the temperature to form melting curves, and the peak of the curve corresponds to the $T_{m}$ of the probe.

**Supporting Information**

Figure S1 Melting curve analysis of the amplification reactions of various HPV targets.

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**Author Contributions**

Conceived and designed the experiments: GF LA. Performed the experiments: GF AM. Analyzed the data: GF AM LA. Contributed reagents/materials/analysis tools: GF. Wrote the paper: GF AM LA.