Combination of fluorescence color and melting temperature as a two-dimensional label for homogeneous multiplex PCR detection

Yiqun Liao[1], Xiaobo Wang[2], Chao Sha[2], Zhongmin Xia[2], Qiuying Huang[2,*] and Qingge Li[2,*]

[1]Department of Translational Medicine, School of Pharmaceutical Sciences, Xiamen University, Xiamen, Fujian 361005, China and [2]Engineering Research Center of Molecular Diagnostics, Ministry of Education, Department of Biomedical Sciences, School of Life Sciences, Xiamen University, Xiamen, Fujian 361005, China

ABSTRACT

Multiplex analytical systems that allow detection of multiple nucleic acid targets in one assay can provide rapid characterization of a sample while still saving cost and resources. However, few systems have proven to offer a solution for mid-plex (e.g. 10- to 50-plex) analysis that is high throughput and cost effective. Here we describe the combined use of fluorescence color and melting temperature (T_m) as a virtual 2D label that enables homogenous detection of one order of magnitude more targets than current strategies on real-time polymerase chain reaction platform. The target was first hybridized with a pair of ligation oligonucleotides, one of which harbored an artificial sequence that had a unique T_m when hybridized with a reporter fluorogenic probe. The ligated products were then amplified by a universal primer pair and denatured by a melting curve analysis procedure. The targets were identified by their respective T_m values in the corresponding fluorescence detection channels. The proof-of-principle of this approach was validated by genotyping 15 high-risk human papillomaviruses and 48 human single-nucleotide polymorphisms. The robustness of this method was demonstrated by analyzing a large number of clinical samples in both cases. The combined merits of multiplexity, flexibility and simplicity should make this approach suitable for a variety of applications.

INTRODUCTION

Multiplex nucleic acid detection is a preferred approach in many applications such as screening for risk factors, typing of pathogen and detection of genetic disease predisposition. Multiplex essentially means the ability to simultaneously ask more than one question about a sample at the same time, or the ability to conduct more than one test on a sample in the same reaction. Therefore, multiplex assays not only facilitate timely and cost-effective detection, but they also offer more information in a sample than singleplex detections (1). Various multiplexing techniques have been described using measurement parameters detected on certain platforms. For example, multiplex polymerase chain reaction (PCR) exploits the length difference of amplicons to identify targets through gel electrophoresis. Similar strategy can be found in multiplex ligase-dependent probe amplification (MLPA), where up to 40 nucleic acid sequences could be detected in a single capillary electrophoresis (2). Array systems, such as planar array and a variety of particle arrays, have been developed for suggested use in high or medium density multiplexed assay (3,4). In addition, molecular weight has been used as tags for single-nucleotide polymorphism (SNP) genotyping or mutation detection on the mass spectroscopy platform (5,6). So far, few DNA testing platforms have proven to offer a solution for mid-plex analysis that is high throughput, ease-of-use and cost effective.

The emergence of real-time PCR has revolutionized nucleic acid detection in many respects owing to the reduced detection time and low risk of amplicon contamination. As a homogeneous detection by nature, real-time PCR distinguishes itself from the above-mentioned approaches in that the amplified products are detected in a closed-tube without post-PCR manipulations (7). Multiplex real-time PCR can be performed using a real-time thermocycler that has more than one detection channel, and thus allows different fluorophore-labeled probes to be simultaneously detected in one reaction. Because the number of detection channel, fluorophore and target are equivalent to each other in a classical

---

detection strategy, a real-time thermocycler equipped with four detection channels could accommodate a quadruplex detection. Higher multiplexed assays on a real-time PCR can be achieved using melting temperature (T_m) as a second dimension of multiplexing (8). For example, a recent study demonstrated that using four differently labeled probes, 27 different species of mycobacteria could be identified in one reaction (9). However, this T_m-based multiplexity was restricted to targets differed in one variant region encompassed by a single pair of primers. Alternatively, a multicolor combinatorial probe-coding method has been proposed to detect multiple targets in a single reaction (10). The limitation of this strategy is that it is difficult, if possible, to detect multiple co-existing targets in one reaction.

In the present study, we sought to develop a new multiplex approach to overcome some of the aforementioned difficulties while maintaining the homogeneous nature of real-time PCR. This approach is achieved by the combinatorial use of the fluorophore and T_m as 2D label, which enables an order of magnitude increase in multiplexity on the real-time PCR platform. As a proof of principle, the 2D label was first used to establish a 16-plex assay to genotype 15 high-risk human papillomaviruses (HPVs). This assay aimed to test whether the 2D label could be used to detect one or several among all possible targets in a single reaction. The robustness of this assay was validated by a comparison study with 517 clinical samples. In a second assay, the 2D label was used to establish a 96-plex assay for genotyping of 48 forensic SNPs. The purpose of this assay was to determine whether the 2D label could be used to detect all possible targets in a single reaction. The established assay was validated with 100 human DNA samples in a head-to-head comparison with a second commercially available method. Moreover, the utility of this assay was demonstrated in assessment of hematopoietic chimerism after bone marrow transplantation. The result from both assays demonstrated that the 2D label can be used as a universal label to detect the existence of one or a few among all possible targets or even simultaneous existence of all possible targets in one reaction.

MATERIALS AND METHODS

Construction of a library containing 50 2D labels

A library containing 50 2D labels was constructed by using five fluorophores (X = 5) and 10 T_m tags (Y = 10) (Supplementary Table S1). The five types of fluorophores were FAM, HEX, ROX, CAL Fluor Red 635 and Quasar 705. The sequences of the fluorophore-labeled probe were artificially generated and are not homologous to any known species. For each fluorophore-labeled probe, 10 T_m tags with predicated T_m values ranging from 40°C to 80°C with 1°C–5°C intervals were designed to hybridize with it. The T_m tag of the highest T_m is fully complementary to the probe. Other tags consist of different number of mismatched bases that are introduced to confer a gradual decrease in T_m values. All synthetic oligonucleotides were prepared at the polyacrylamide gel electrophoresis purity by Sangon, Inc. (Shanghai, China) and the fluorophore-labeled probes were from Biosearch Technologies, Inc (Novato, CA, USA).

The predicated T_m values were obtained by T_m Utility v1.3 (BioFire Diagnostics Inc., Salt Lake City, UT, USA) using the following conditions: 200 nM probe, 200 nM target, 3 mM $MgCl_2$ and 200 μM deoxyribonucleoside triphosphates (dNTP), where the probe was the fluorogenic probe and the target was the T_m tag. The actual T_m values were measured by melting curve analysis on a Rotor-Gene^{TM} 6000 real-time analyzer (Corbett Research, Mortlake, Australia) in a 25-μl solution of 10 mM Tris–HCl (pH 8.6) containing 50 mM KCl, 3 mM $MgCl_2$, 200 μM dNTP, 200 nM fluorogenic probe and 200 nM T_m tag. The melting curve analysis (MCA) procedure started from 95°C for 1 min, 35°C for 2 min followed by raising the temperature from 40°C to 85°C at 1°C/step with a 5 s stop between each step. Fluorescence intensity was recorded at each step in each of the five detection channels. The data obtained were plotted as fluorescence intensity versus temperature as well as the negative derivative of fluorescence intensity with respect to temperature. T_m values were identified from the peak position of the melting curve.

Typing of 15 high-risk human papillomavirus genotypes

Step 1: Hybridization and ligation

The reaction was performed in a 10-μl solution containing 20 mM Tris–HCl (pH 7.6), 25 mM sodium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM NAD^+, 0.1% Triton X-100 containing 30 type-specific ligation oligonucleotides (500 pM each, Supplementary Table S2), two ligation oligonucleotides for the internal control (IC) (250 pM), 1 unit of Taq DNA ligase (New England Biolabs, Bejing, China), 5×10^2 copies *SUC2* gene (IC) and 5 μl of either artificial plasmid DNA templates extracted from *E. coli* DH5α by a plasmid mini kit (Omega Bio-Tek, Inc. Norcross, GA, USA) or DNA extracted from clinical specimens by a kit supplied with the HPV GenoArray test kit (Hybribio Ltd., Chaozhou, China). Clinical specimens were provided as on-shelf coded samples that had been collected previously for diagnosis purposes. The reaction was performed in a T3 thermocycler (Biometra, Göttingen, Germany) at the following temperatures: denaturation at 95°C for 5 min; 10 cycles of 95°C for 1 min, 70°C for 1 min, 68°C for 1 min, 66°C for 1 min and 64°C for 3 min.

Step 2: PCR/MCA

The reaction was performed in a 25-μl solution containing 10 mM Tris–HCl (pH 8.6), 50 mM KCl, 2.5 mM $MgCl_2$, 5% (V/V) glycerin, 1 U Taq DNA polymerase, 1 pmol limiting primer, 20 pmol excess primer, 5 pmol of fluorogenic probes for 2D label (Supplementary Table S2) and 2 μl reaction product from step 1. PCR/MCA was performed in the Rotor-Gene^{TM} 6000 at the following temperatures: denaturation at 95°C for 3 min, 42 cycles of 95°C for 10 s, 56°C for 10 s and 72°C for 20 s, followed by a temperature increase from 40°C to 85°C, 1°C/step, with a 5 s stop between each step. Fluorescence intensity was

measured in four detection channels: FAM (510 nm), HEX (555 nm), ROX (610 nm) and CAL Fluor Red 635 (660 nm).

Genotyping of 48 SNPs

Step 1: Hybridization and ligation
The reaction was performed in a 10-µl solution containing 20 mM Tris–HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 mM NAD^+, 0.1% Triton X-100, 144 SNP-specific ligation oligonucleotides (800–8000 pM each), 1 unit of Taq DNA ligase and 5 µl of human genomic DNA extracted from anonymous blood samples obtained from Xiamen Blood Center by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The reaction was performed in a T3 thermocycler at the following temperatures: denaturation at 95°C for 5 min; 10 cycles of 95°C for 2 min, 68°C for 2 min, 66°C for 2 min, 64°C for 2 min, 62°C for 2 min and 60°C for 2 min.

Step 2: PCR/MCA
The reaction was performed in a 25-µl solution containing 75 mM Tris–HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 50 mM KCl, 3 mM $MgCl_2$, 0.01% (V/V) Tween 20, 1 U hot start Taq DNA polymerase, 200 µM dNTP, 1 pmol forward primer, 20 pmol reverse primer, 5 pmol of fluorogenic probes for 2D labeling and 1 µl of reaction product from step 1. PCR/MCA was performed in a Rotor-Gene^{TM} 6000 at the following temperatures: denaturation at 95°C for 3 min, 50 cycles of 95°C for 5 s, 56°C for 10 s and 75°C for 15 s, followed by a temperature increase from 35°C to 90°C, 1°C/step, with a 5 s stop between each step, and fluorescence intensity was measured in four detection channels, as described above.
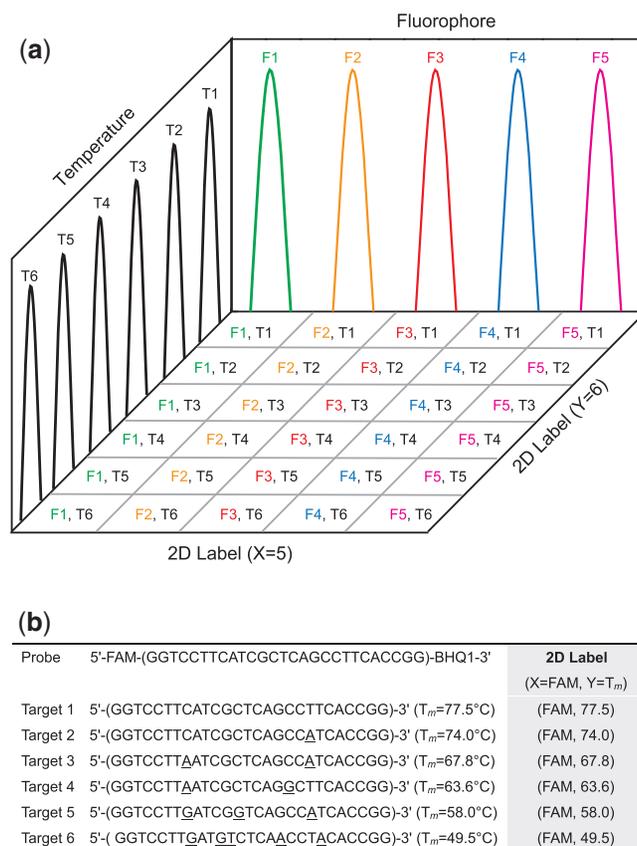
RESULTS

The working principle of 2D label

In 2D label, the type of different fluorophores (X) defines the first dimension and the value of resolvable T_m (Y) defines the second dimension. The total number of labels is equal to the product of X multiplied by Y. Figure 1a shows an example that combines five fluorophores (X = 5) and six T_ms (Y = 6), resulting in 30 different labels. To create a 2D library, a series of oligonucleotides, called Tm tags, are used to form hybrids with a fluorophore-labeled probe, resulting in different characteristic T_ms from this fluorophore. As shown in Figure 1b, six oligonucleotides and one FAM-labeled probe yield six 2D labels.

In a spectrofluorometric thermal cycler, the number of differently colored fluorophores that can be used is determined by the number of detection channels, which usually ranges from two to six, depending on the instrument. On the other hand, the number of T_ms that can be reliably distinguished from a melting curve varies from 6 to 10, depending on the temperature resolution of the instrument. Consequently, the maximal number of 2D labels can be detected is 12–60 with instruments currently available.

The design of 2D label assay

The design of 2D label assays is illustrated in Figure 2. Two ligation oligonucleotides are designed for one target. The left ligation oligonucleotide contains an upstream 22-nt universal priming sequence at the 5′-end, a 4-nt stuffer sequence, the T_m tag sequence and a 26–40-nt target-specific sequence at the 3′-end. The right ligation oligonucleotide contains a 30–50-nt target-specific sequence at the 5′ phosphorylated end and a downstream 21-nt universal priming sequence at the 3′-end (Figure 2a). The T_m tag in the left ligation oligonucleotide is fully or partially complementary to a particular fluorogenic probe and thus creates the specific X and Y values of a 2D label for the target. Melting temperatures of all probe target-specific sequences are 65°C–70°C under conditions of ligation. For the design of the hybridizing parts, sequence information available from the public databases (www.ncbi.nlm.nih.gov) was used.

(a)

Fluorophore

Temperature

(b)

Probe	5'-FAM-(GGTCCTTCATCGCTCAGCCTTCACCGG)-BHQ1-3'	2D Label
		(X=FAM, Y=T_m)
Target 1	5'-(GGTCCTTCATCGCTCAGCCTTCACCGG)-3' (T_m=77.5°C)	(FAM, 77.5)
Target 2	5'-(GGTCCTTCATCGCTCAGCC<u>A</u>TCACCGG)-3' (T_m=74.0°C)	(FAM, 74.0)
Target 3	5'-(GGTCCTT<u>A</u>ATCGCTCAGCC<u>A</u>TCACCGG)-3' (T_m=67.8°C)	(FAM, 67.8)
Target 4	5'-(GGTCCTT<u>A</u>ATCGCTCAG<u>G</u>CCTTCACCGG)-3' (T_m=63.6°C)	(FAM, 63.6)
Target 5	5'-(GGTCCTT<u>G</u>ATCG<u>G</u>TCAGCC<u>A</u>TCACCGG)-3' (T_m=58.0°C)	(FAM, 58.0)
Target 6	5'-(GGTCCTT<u>G</u>AT<u>GT</u>CTCA<u>A</u>CCT<u>A</u>CACCGG)-3' (T_m=49.5°C)	(FAM, 49.5)

Figure 1. The concept of 2D label and its composition. (**a**) Combination of five fluorophores and six T_ms to create 30 2D labels. The five fluorophores (shown in color as fluorescence emission wavelengths) are those detectable in a common real-time PCR machine. The six T_ms (shown as six melting peaks) are obtained from melting curve analysis in one detection channel. (**b**) The labeling fluorophore of the probe, FAM (X = 1) and the six T_m tags (Y = 6) together form six 2D labels. Among the six T_m tags, the one with the highest T_m is fully complementary with the probe, all others possess base pair substitutions (underlined) that result in mismatched base pairs in the probe: tag hybrid. The T_m variations of the hybrid are determined by the number and position of mismatched base pairs present.
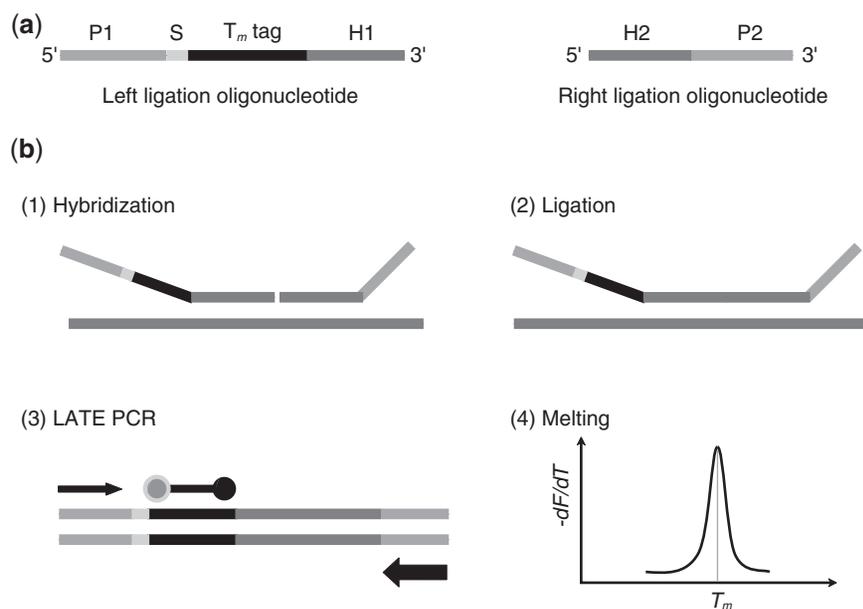
Figure 2. The design of 2D label assays. (**a**) Two unreacted ligation oligonucleotides in 2D label assays. The left ligation oligonucleotide consists of four segments: an upstream priming region (P1), a stuffer sequence (S), a T_m-tag sequence that serves as the identification label of the target and a hybridization region (H1) that is complementary to the target sequence in the genomic DNA. The right ligation oligonucleotide consists of two segments: a hybridization region (H2) that is complementary to the target sequence in the genomic DNA, and a downstream priming region (P2). (**b**) Flow chart of 2D label assays. (1) The hybridization regions of the two ligation oligonucleotides hybridize to the target sequence. (2) The two oligonucleotides are ligated to each other, producing a joined full-length sequence. (3) The ligated product is amplified with a universal pair of LATE-PCR primers. The LATE-PCR primer that is present in excess is shown as a thick arrow, and the LATE-PCR primer that is present in limited concentration is shown as a thin arrow. (4) Melting curve analysis in certain detection channel yields a T_m value identifying the target sequence.

During the hybridization-ligation process, the two oligonucleotides hybridize to the genomic DNA sample. Because hybridization occurs before any amplification steps, no amplification bias can be introduced into the assay. Following hybridization, a DNA ligase seals the nick between the left and the right ligation oligonucleotides to form PCR templates (11) that can be amplified with a universal PCR primer pair designed for asymmetric Linear-after-the-exponential (LATE) PCR (12). The LATE PCR is performed in the presence of a fluorogenic probe using a real-time PCR machine. After thermal cycling, a melting curve analysis allows readout of the unique T_m values displayed by the hybrids formed between T_m tags and their corresponding fluorescent probes in the respective fluorophore channels, and thereby producing the X and Y values for the targets that exist (Figure 2b).

Multiple targets can be detected using different target-specific pairs of ligation oligonucleotides. These targets are differentiated by their T_m tags. We have designed a library of 50 T_m-tag sequences, 10 of which serve as cassettes that can be detected by one fluorogenic probe. These pre-optimized T_m-tag sequences can be incorporated into any ligation oligonucleotides for multiplex assays of target DNA sequences of interest. These T_m-tag-probe combinations are compatible with all mainstream spectrofluorometric thermal cyclers (Supplementary Table S1).

Detection of 15 high-risk HPV types

We set out to validate the 2D label scheme by detecting part of all the possible targets in a multiplex assay, which was

exampled by detecting 15 high-risk HPV types in one reaction. Figure 3a illustrates the flow chart of the detection procedure. In this reaction, 16 target-specific ligation oligonucleotide pairs were present, and each pair possessed a unique T_m-tag sequence. Four differently colored probes were present, the FAM-labeled probe detected amplicons generated from an IC target sequence; the three other amplicon probes each detected the amplicons generated from five different HPV target sequences. The identity of each target sequence was revealed by the combination of probe color and probe-amplicon T_m. The entire procedure comprised a 16-plex ligation, a singleplex four-color PCR and an MCA. Using artificial plasmid DNA as HPV templates, the MCA results showed that each of the 15 HPV types was correctly identified by its 2D label (Figure 3b).

In clinical samples, it is common for each HPV type to have various subtypes owing to the existence of polymorphic nucleotides. This added complexity was addressed by using a mixture of ligation oligonucleotides that cover all polymorphic sites, but harbor one common T_m tag, such that all the polymorphisms belonging to the same type were identified as a single HPV type (Figure 4). Co-infection by more than one HPV type also occurs clinically. Using dual infection of HPV-16 and HPV-18 as an example, we studied the ability to distinguish the two types when they are simultaneously present in the same reaction. By varying their relative ratios, the results showed that as low as 1% of one type in the presence of 99% of the other type could be detected (Figure 5).
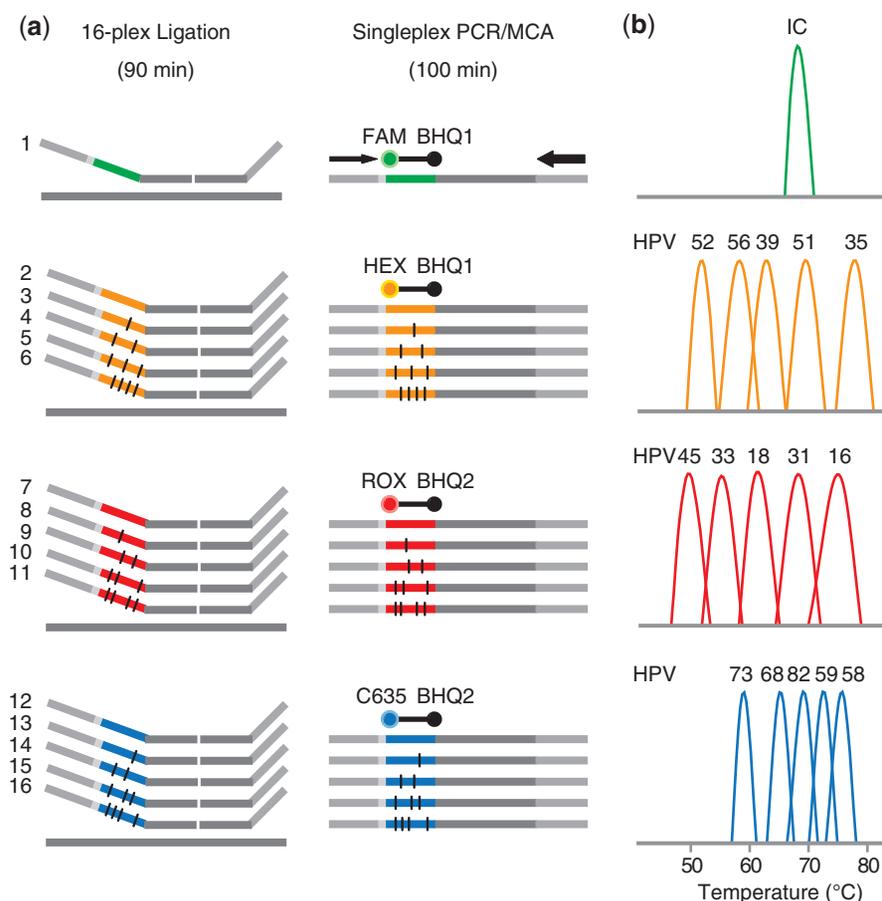
Figure 3. The application of 2D label to the typing of 15 HPV types in a single reaction. (**a**) Flow chart of the assay. In the ligation step, 16 pairs of ligation oligonucleotides were used to detect 15 HPV genotypes and one IC. In the PCR/MCA step, the ligated products were amplified by one pair of primers in the presence of four differently labeled fluorogenic probes. T_m tags are shown in different colors (corresponding with the labeling fluorophores in the fluorogenic probes), and the black bars within the T_m tags represent those substituted mismatched nucleotides. (**b**) Readout for HPV typing using the 2D label method. Each HPV type was identified by its unique 2D label, i.e. the combination of a fluorophore type and a T_m value. To visually illustrate the T_m values, the melting curves were normalized between 0 and 1, and the normalized value portions between 0.4 and 1 are shown. FAM: green line; HEX: orange line, ROX: red line and C635: blue line. The numbers above each melting peak identify the HPV genotype.

We then studied the concentration range of the assay using plasmid templates. The results showed that 5×10^2–5×10^7 copies plasmid DNA per reaction could be repeatedly detected regardless of the genotypes (Supplementary Figure S1).

The robustness of the 2D label-based assay was validated by comparing genotyping results of 517 cervical swab samples with a commercial chip-based system (GenoArray, Hybribio, Chaozhou, China) in a blind manner. The overall agreement between our method and the GenoArray assay was 96% (495/517), while the type agreement between the two methods was greater than 99% (Table 1). These results demonstrated the suitability of our 2D label-based strategy for clinical applications.

Genotyping of 48 forensic SNPs

We further tested the 2D label strategy by establishing a second assay to genotype 48 forensic SNPs that are randomly distributed among 23 human chromosomes.

Unlike the HPV typing assay in which only a portion of the 15 types were present in a sample, the genotyping of 48 SNPs could have 48–96 alleles simultaneously present in one reaction. To set up this assay, we designed two left ligation oligonucleotides harboring different T_m tags for each allele and one right ligation oligonucleotide for each locus. Thus, the ligation step of this assay was a 96-plex reaction containing 144 ligation oligonucleotides. To identify all the 96 alleles of 48 SNPs, 3 PCR reactions were used, each covering 32 alleles of 16 SNPs (Figure 6a). Thirty-two 2D labels were used for each reaction (Supplementary Table S1). After PCR/MCA, the melting curves (Figure 6b) obtained from the four channels were imported into the GeneMarker software (Softgenetics, State College, PA, USA) for data transformation. The function Melt Analysis, which provides data interpolation, baseline calibration, wavelet transformation, fast Fourier transformation and automatic peak detection with allele identification, was used to analyze the melting data and identify the allele of each
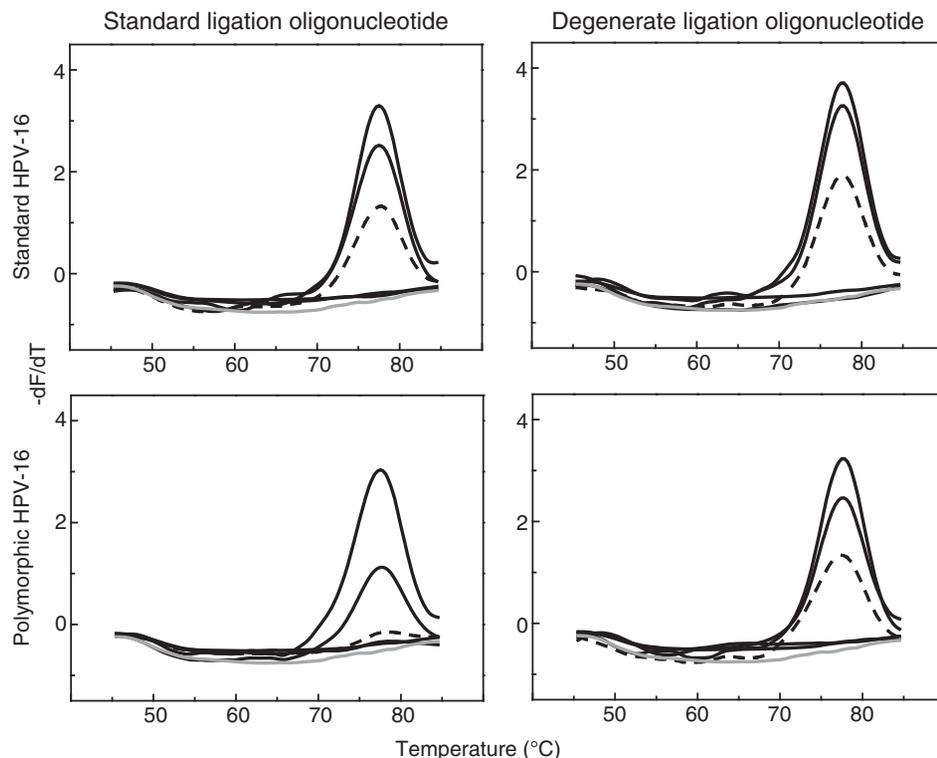
Figure 4. The effect of using degenerate ligation oligonucleotides on the performance of the 2D label assay. Both a standard ligation oligonucleotide and a degenerate ligation oligonucleotide mixture were used to detect a standard HPV-16 target and a polymorphic HPV-16 target, respectively. The polymorphic site is at the penultimate nucleotide at the 3′-terminus of the left ligation oligonucleotide sequence. The template DNAs used were artificial plasmids with concentrations of 1×10^4, 1×10^3, 1×10^2, 1×10^1 and 1×10^0 copies/μl, respectively. Note that the melting curve peak of the 1×10^2 copies/μl (dashed lines) was lowest when a standard ligation oligonucleotide was used to detect the polymorphic HPV-16. Non-template controls (NTCs) are shown as grey lines.

SNP. A typical result from Reaction 1 was shown in Figure 6b.

We investigated the lowest amount of human genomic DNA required for these genotyping assays. The result showed that at least 20 ng DNA per assay was needed to achieve reproducible genotyping readout. Such starting genomic DNA amount required for our 2D label assays was similar to that required for MLPA (2).

The 48-SNP genotyping assay was used to analyze 100 human genomic DNA samples and the results were compared with a commercial singleplex real-time PCR-based genotyping assay (ForenSNPs, Zeesan Biotec, Xiamen, China). A 100% agreement was achieved between these two methods (Supplementary Table S3). As a clinical application, the 48-SNP genotyping assay was used to assess hematopoietic chimerism after bone marrow transplantation. A patient who had a sibling bone marrow donor possessed an identical human leucocyte antigen with the donor. However, SNP 14 was found to have a homozygotic disparity between the donor and the recipient and thus was chosen to track the level of donor granulocyte engraftment. The results showed that donor monocytes began to appear 10 days after transplantation and became dominant 20 days after transplantation (Figure 7). This result indicated that a semi-quantitative clinical analysis could be carried out by using 2D labels.

DISCUSSION

The 2D label is representative of two measurable parameters, i.e. fluorescence and T_m, which can be detected in a homogeneous way on real-time PCR machines. It was realized by using artificial T_m tags and corresponding fluorogenic probes. In this study, we used ligation reaction as the labeling procedure, which allowed the use of a single universal primer pair to amplify all ligated products. Such a labeling strategy enabled the transformation of the multiplex assays into a singleplex PCR detection. The total number of the targets that could be detected in a 2D labeled assay is equal to the number of readily resolvable T_m values multiplied by the number of fluorescence detection channels of a real-time PCR machine. For a standard 4-color real-time PCR machine that has a T_m resolution of 5°C in the range of 45°C–85°C, the 2D label strategy could accommodate a 36-plex detection. Higher plex levels could be achieved if the machine has more detection channels and/or a better temperature resolution.

The multiplexing capability of 2D label-based assays fills the gap between multiplex real-time PCR and array-based assays. Such an intermediate complexity is especially useful in molecular assays of routine use. For example, diagnosis of sepsis (13), gastrointestinal infections (14) and respiratory diseases (15) often involve dozens of pathogens of different types including viruses,
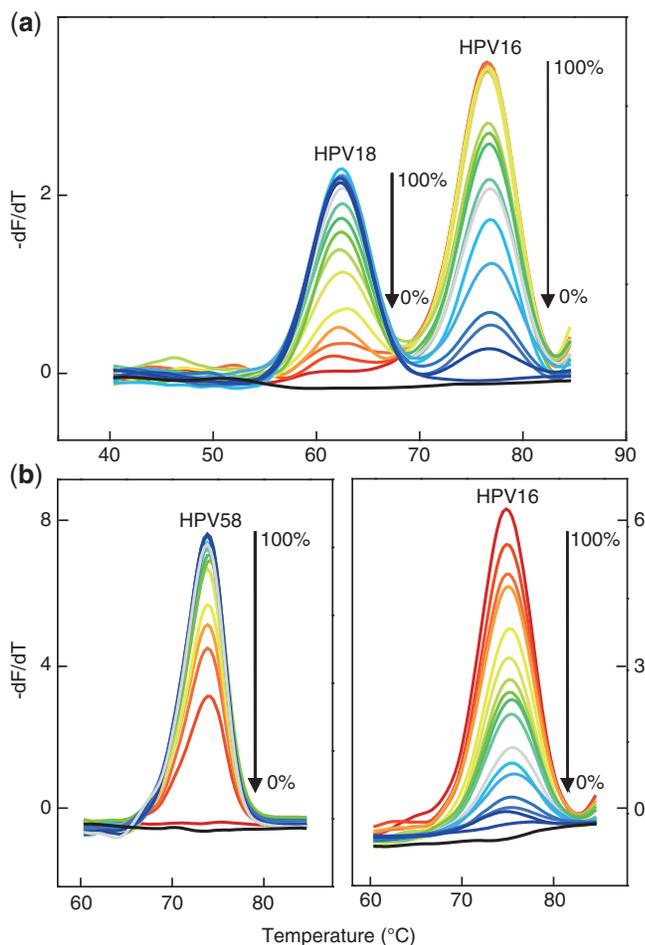
Figure 5. Simultaneous detection of multiple HPV genotypes in mixed infections. (**a**) Two co-existing genotypes were detected in the same channel. (**b**) Two co-existing genotypes were detected in two different channels. Melting curves of artificial plasmid templates containing two HPV genotypes of various percentage ratios (0:100, 1:99, 3:97, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 95:5, 97:3, 99:1 to 100:0) were tested. The overall template concentration was 10^6 copies per reaction. NTCs are shown as black lines.

bacteria and parasites. Similarly, inherited diseases and tumors are often caused by a variety of mutations found in different exons or genes (16,17). To detect these multiple targets or variants, current real-time PCR-based assays have to use multiple reactions, and other methods with higher order multiplexity require complicated post-PCR manipulations or expensive detection platforms.

Using this new 2D label concept, we have successfully established two multiplex assays and obtained comparable results with those well-established methods. The HPV typing assay involved simultaneous detection of 15 high-risk HPV types responsible for cervical cancer and one IC from a single sample in a single reaction. The method could detect intratype variations and multiple types in mixed infections, had a wide detection range and was highly concordant with a commercial array-based system when tested with 517 cervical swab samples. Most importantly, this assay could analyze 96 samples in 190 min without any post-PCR manipulations and the consumable cost per assay was close to a singleplex 4-color real-time PCR. By contrast, the comparison method could only process up to 30 samples in a working day (18). There are reported assays for 14 HPV types in a single reaction that could be finished within a shorter time. However, such assays could not give a full genotyping result (19,20). The SNPs genotyping assay was able to genotype 48 forensic SNPs randomly distributed among the 23 pairs of human chromosomes from a single sample in a single reaction. This is a sharp contrast with a reported melting curve analysis method that could only genotype six forensic SNPs in one reaction on a six-channel real-time PCR machine (21). The accuracy of the assay was validated by analyzing 100 human genomic samples with a complete concordance with the comparison singleplex real-time PCR method. In addition, the robustness of this assay was demonstrated in the semi-quantitative detection of the level of donor granulocyte engraftment after bone marrow transplantation.

**Table 1.** Comparison of the HPV genotyping results of 517 specimens between two assays

HPV type	Number of samples (2D label/GenoArray)				Number of discrepant results	Agreement (%)	Kappa (95% CI)
	+/+	+/−	−/+	−/−			
16	49	2	0	466	2	99.6	0.978 (0.962–0.994)
18	8	2	0	507	2	99.6	0.887 (0.808–0.966)
31	17	1	0	499	1	99.8	0.970 (0.940–1.000)
33	12	1	0	504	1	99.8	0.959 (0.918–1.000)
35	3	0	0	514	0	100	1.000 (1.000–1.000)
39	11	3	0	503	3	99.4	0.877 (0.807–0.947)
45	2	0	2	513	2	99.6	0.665 (0.442–0.888)
51	5	2	2	508	4	99.2	0.710 (0.572–0.848)
52	37	3	2	475	5	99.0	0.931 (0.901–0.961)
56	6	2	0	509	2	99.6	0.855 (0.754–0.956)
58	34	1	0	482	1	99.8	0.984 (0.968–1.000)
59	8	0	0	509	0	100	1.000 (1.000–1.000)
68	14	1	0	502	1	99.8	0.965 (0.930–1.000)
73[a]	1	0	0	516	0	100	1.000 (1.000–1.000)
82[a]	3	0	0	514	0	100	1.000 (1.000–1.000)

[a]HPV-73 and HPV-82 were not included in the GenoArray technique. Their detection results obtained from the 2D assay were compared with the sequencing method.
CI = confidence interval.
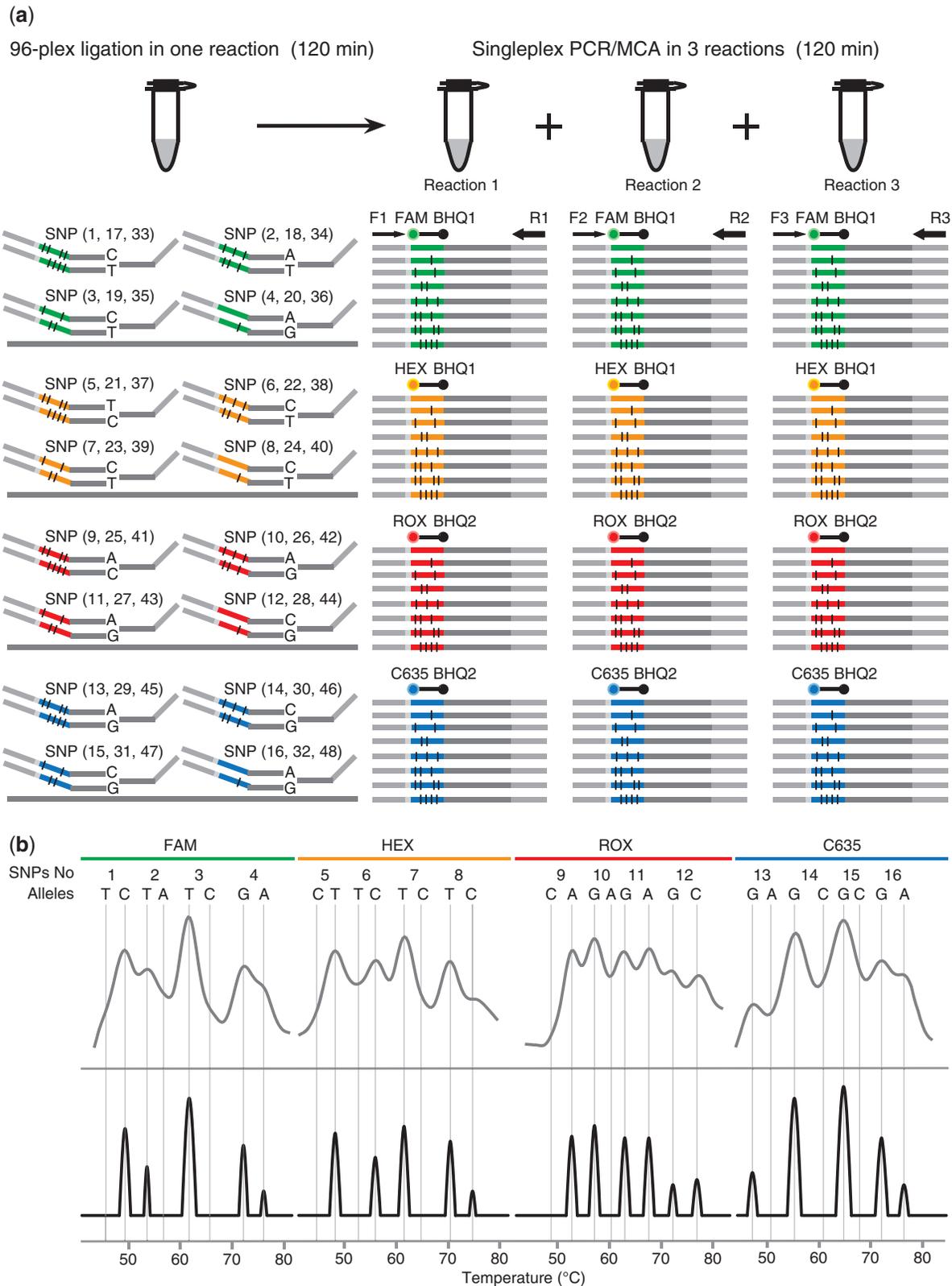
Figure 6. The application of 2D label in genotyping of 48 SNPs. (**a**) Flow chart of the assay. Forty-eight groups of ligation oligonucleotides (containing two left ligation oligonucleotides and one right ligation oligonucleotide for each SNP) were used to target 96 alleles of the 48 SNPs. The allele types of SNPs 1–16 were given at the 3′-terminus of the left ligation oligonucleotides. T_m tags are shown in different colors (corresponding with the labeling fluorophores in the fluorogenic probes), and the black bars within the T_m tags represent those substituted mismatched nucleotides. In the PCR/MCA step, the ligation products were amplified by three independent pairs of LATE-PCR primers (F1/R1, F2/R2 and F3/R3) in the presence of the four fluorogenic probes. (**b**) Typical readout of 16 SNPs in reaction 1 from one human genomic DNA sample. Upper panel shows the melting curves and the lower panel shows the exported results from GeneMarker software. The allele types of the 16 SNPs were identified according to their detection channels. SNPs 1–4: FAM; SNPs 5–8: HEX; SNPs 9–12: ROX; SNPs 13–16: C635.
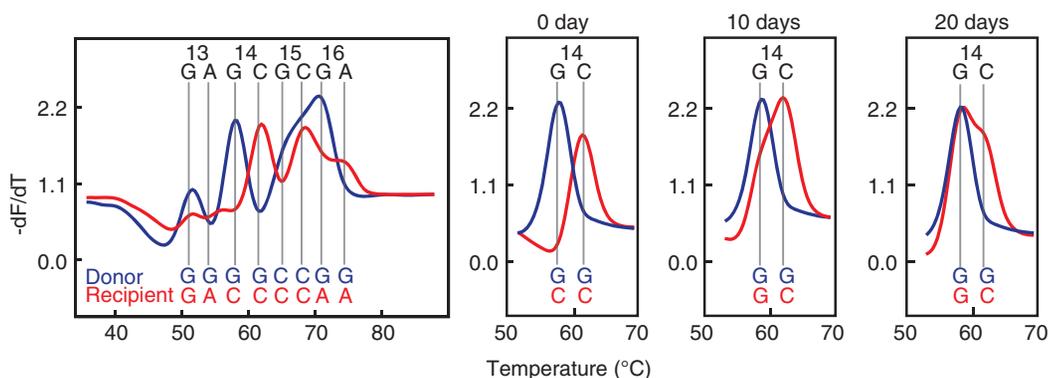
Figure 7. SNP genotyping for assessment of hematopoietic chimerism after bone marrow transplantation. The leftmost panel shows that SNP 14 has a homozygotic disparity between the donor (blue line) and the recipient (red line). The other three panels display the time-dependent change from allele G to allele C of SNP 14 in the recipient.

Melting temperature was proposed as a second layer of multiplexing more than a decade ago to serve to augment multiplexing capability (8). However, high order multiplexing remains to be achieved by existing strategy in which the T_m is obtained from the probe-target hybrid. This strategy has been widely used for detection of mutations in close proximity or in one amplicon (22–25). However, if the variants are in different amplicons, such a strategy will encounter difficulty in dealing with the multiple primer pairs and adjusting the T_m values to have balanced amplifications and discriminable T_m values. Another problem comes from the unexpected mutations (26) and polymorphism (27) that might occur in the probe-binding region of the targets. Such mutations and/or polymorphisms might give similar T_m value with the variant to be detected and cause false positive results. One mutation/polymorphic site may be easy to deal with but if multiple mutations/polymorphic sites are present, discrimination of variants by one probe becomes a challenging task. By comparison, the 2D label strategy uses a multiplex ligation reaction followed by a universal PCR, a well-established procedure that can detect dozens (2) and thousands (28) of loci from different amplicons. The presence of polymorphism in the target would exert no impact on the T_m predefined for the target because the T_m value was obtained from the T_m tag-probe hybrid rather than from the target-probe hybrid. Problems might be encountered if the polymorphic nucleotides occur and prevent ligation reaction. Such problems can be resolved by using degenerate nucleotide or universal binding base at the polymorphic sites or, if possible, changing the ligation region. Moreover, the use of T_m tag could obviate the possible abnormal melting curve caused by secondary structure in the target, which otherwise might cause unexpected result in probe-target hybrid-based melting curve analysis.

Compared with other multiplexing techniques, the 2D label approach has advantages in many respects. First, as a homogeneous assay by nature, its amplification and detection are carried out within a consecutive procedure and in a closed-tube format. Like in real-time PCR, this feature significantly simplifies the manipulations, reduces the risk for PCR product contamination and increases the

assay throughput. Second, by using a common 2D label library, the assay is easy to be standardized in set-up, detection and readout. Such standardization helps save the overall, and especially labor, cost. In the two exampled assays, the amount of ligation oligonucleotides used in one reaction was 5 fmol, and one standard commercial preparation for the ligation oligonucleotide (100 nmol) can therefore be used for 20 million reactions! Therefore, once the assay was established, the ligation oligonucleotides cost was negligible. The only costly reagents are fluorogenic probes. However these universal probes can be prepared in bulk because the same fluorogenic probes can be used for different assays (e.g. for both the HPV and SNP assays). In this study, one fluorogenic probe was designed to detect all the T_m tags in one channel. It might be possible to use more than one fluorogenic probe in some applications, but the number of fluorogenic probes is still drastically smaller than the targets to be detected. Consequently, the material cost of the 2D label-based assay for a double-digit number of targets is close to multiplex real-time PCR assays for a single-digit number of targets. Finally, the 2D label-based assay can be performed with almost any real-time PCR machines, making it an open system readily adoptable for different applications.

The proposed 2D label strategy for multiplex detection has several limitations worth further improvement. First, there is an extra ligation step before PCR in the 2D label assays when compared with the real-time PCR detection. This extra pre-PCR step increased the manipulations and lowered the throughput; however, it causes no risk of PCR amplicon contamination frequently encountered in strategies involving post-PCR manipulations. It is possible for this ligation reaction to be combined with PCR as reported previously (29), and therefore the additional step might be eliminated. Second, non-specific amplification might derive from the non-ligated oligonucleotides, producing false positive signals and lowering the overall analytical sensitivity. Although the non-specific signal can be largely reduced or eliminated by changing the ligation oligonucleotide design, one useful way is to include an IC in the system as exampled in the HPV assay. The amplification of the IC consumed the excess primer

and reduced opportunity for non-specific amplification in the absence of target. Another way to eliminate non-specific amplification is to add certain exonucleases to digest unligated oligonucleotides before amplification (30). It should be noted, however, that the concentration of the ligation oligonucleotides is extremely low compared with the universal primers, and the non-specific signals could be largely eliminated through design of the ligation oligonucleotide without digestion. Third, as an end-point detection, the 2D label-based assays are essentially non-quantitative and therefore restricting their use in screening rather than quantitative detection. However, as shown in the example of monitoring donor monocytes after bone marrow transplantation, a semi-quantitative detection could be achieved. Further research is now being undertaken to assess whether a combination of real-time PCR detection and melting curve analysis can improve the quantification ability. Finally, the analytical sensitivity of this method was relatively low as observed in the SNP genotyping assay, which might preclude its use in some forensic cases when extremely low amount of residue DNA was available. However, this barrier might be eliminated by the use of some pre-amplification procedure such as whole genome amplification (31).

In summary, we have developed a universal labeling system that can significantly increase the multiplexing capability for homogeneous detection. In addition, we have shown, using two examples, that the accuracy and robustness of the 2D label-based assays are comparable with those of well-established low-multiplex and low-throughput methods. Regarding the ease-of-use, low cost, inherent standardization and time and labor effectiveness of this approach, this system is expected to be used in screening pathogens responsible for many complex diseases and become a method of choice for high-throughput and multiplex mutation/SNPs genotyping assays. Prospectively, the strategy will have the potential to upgrade current mid-plex assays from various complex solid-phase systems to a universal homogeneous platform that can be performed on the widely available real-time PCR machines.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3 and Supplementary Figure 1.

Conflict of interest statement. None declared.

REFERENCES

1. Deshpandde,A. and White,P.S. (2012) Multiplexed nucleic acid-based assays for molecular diagnostics of human disease. *Expert Rev. Mol. Diagn.*, **12**, 645–659.
2. Schouten,J.P., McElgunn,C.J., Waaijer,R., Zwijnenburg,D., Diepvens,F. and Pals,G. (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res.*, **30**, e57.
3. Hacia,J.G. (1999) Resequencing and mutational analysis using oligonucleotide microarrays. *Nat. Genet.*, **21**, 42–47.
4. Oliphant,A., Barker,D.L., Stuelpnagel,J.R. and Chee,M.S. (2002) BeadArray technology: enabling an accurate, cost-effective approach to high-throughput genotyping. *Biotechniques*, **32**, 56–58.
5. Ross,P., Hall,L., Smirnov,I. and Haff,L. (1998) High level multiplex genotyping by MALDI-TOF mass spectrometry. *Nat. Biotechnol.*, **16**, 1347–1351.
6. Bray,M.S., Boerwinkle,E. and Doris,P.A. (2001) High-throughput multiplex SNP genotyping with MALDI-TOF mass spectrometry: practice, problems and promise. *Hum. Mutat.*, **17**, 296–304.
7. Heid,C.A., Stevens,J., Livak,K.J. and Williams,P.M. (1996) Real time quantitative PCR. *Genome Res.*, **6**, 986–994.
8. Wittwer,C.T., Herrmann,M.G., Gundry,C.N. and Elenitoba-Johnson,K.S.J. (2001) Real-time multiplex PCR assays. *Methods*, **25**, 430–442.
9. El-Hajj,H.H., Marras,S.A., Tyagi,S., Shashkina,E., Kamboj,M., Kiehn,T.E., Glickman,M.S., Kramer,F.R. and Alland,D. (2009) Use of sloppy molecular beacon probes for identification of mycobacterial species. *J. Clin. Microbiol.*, **4**, 1190–1198.
10. Huang,Q., Zheng,L., Zhu,Y., Zhang,J., Wen,H., Huang,J., Niu,J., Zhao,X. and Li,Q. (2011) Multicolor combinatorial probe coding for real-time PCR. *PLoS One*, **6**, e16033.
11. Hsuih,T., Park,Y.N., Zaretsky,C., Wu,F., Tyagi,S., Kramer,F.R., Sperling,R. and Zhang,D.Y. (1996) Novel, ligation-dependent PCR assay for detection of hepatitis C in serum. *J. Clin. Microbiol.*, **34**, 501–507.
12. Sanchez,J.A., Pierce,K.E., Rice,J.E. and Wangh,L.J. (2004) Linear-after-the- exponential (LATE)-PCR: an advanced method of asymmetric PCR and its uses in quantitative real-time analysis. *Proc. Natl Acad. Sci. USA*, **101**, 1933–1938.
13. Tsalik,E.L., Jones,D., Nicholson,B., Waring,L., Liesenfeld,O., Park,L.P., Glickman,S.W., Caram,L.B., Langley,R.J. and Van Velkinburgh,J.C. (2010) Multiplex PCR to diagnose bloodstream infections in patients admitted from the emergency department with sepsis. *J. Clin. Microbiol.*, **48**, 26–33.
14. Amar,C.F.L., East,C., Gray,J., Iturriza-Gomara,M., Maclure,E. and McLauchlin,J. (2007) Detection by PCR of eight groups of enteric pathogens in 4627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993–1996). *Eur. J. Clin. Microbiol.*, **26**, 311–323.
15. Endimiani,A., Hujer,K.M., Hujer,A.M., Kurz,S., Jacobs,M.R., Perlin,D.S. and Bonomo,R.A. (2011) Are we ready for novel detection methods to treat respiratory pathogens in hospital-acquired pneumonia? *Clin. Infect. Dis.*, **52**, S373.
16. Shi,M.M. (2001) Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. *Clin. Chem.*, **47**, 164–172.
17. Thomas,R.K., Baker,A.C., DeBiasi,R.M., Winckler,W., LaFramboise,T., Lin,W.M., Wang,M., Feng,W., Zander,T. and MacConaill,L.E. (2007) High-throughput oncogene mutation profiling in human cancer. *Nat. Genet.*, **39**, 347–351.
18. Liu,S.S., Leung,R.C.Y., Chan,K.K.L., Cheung,A.N.Y. and Ngan,H.Y.S. (2010) Evaluation of a newly developed GenoArray human papillomavirus (HPV) genotyping assay and comparison

with the Roche Linear Array HPV genotyping assay. *J. Clin. Microbiol.*, **48**, 758–764.

19. Heideman,D.A., Hesselink,A.T., Berkhof,J., van Kemenade,F., Melchers,W.J., Daalmeijer,N.F., Verkuijten,M., Meijer,C.J. and Snijders,P.J. (2011) Clinical validation of the cobas 4800 HPV test for cervical screening purposes. *J. Clin. Microbiol.*, **49**, 3983–3985.

20. Cuzick,J., Ambroisine,L., Cadman,L., Austin,J., Ho,L., Terry,G., Liddle,S., Dina,R., McCarthy,J., Buckley,H. *et al.* (2010) Performance of the Abbott realtime high-risk HPV test in women with abnormal cervical cytology smears. *J. Med. Virol.*, **82**, 1186–1191.

21. Nicklas,J.A. and Buel,E. (2008) A real-time multiplex SNP melting assay to discriminate individuals. *J. Forensic Sci.*, **53**, 1316–1324.

22. Bernard,P.S. and Wittwer,C.T. (2000) Homogeneous amplification and variant detection by fluorescent hybridization probes. *Clin. Chem.*, **46**, 147–148.

23. Elenitoba-Johnson,K.S.J., Bohling,S.D., Wittwer,C.T. and King,T.C. (2001) Multiplex PCR by multicolor fluorimetry and fluorescence melting curve analysis. *Nat. Med.*, **7**, 249–253.

24. Huang,Q., Liu,Z., Liao,Y., Chen,X., Zhang,Y. and Li,Q. (2011) Multiplex fluorescence melting curve analysis for mutation detection with dual-labeled, self-quenched probes. *PLoS One*, **6**, e19206.

25. Pont-Kingdon,G. and Lyon,E. (2005) Direct molecular haplotyping by melting curve analysis of hybridization probes: beta 2-adrenergic receptor haplotypes as an example. *Nucleic Acids Res.*, **33**, e89.

26. Mahadevan,M.S. and Benson,P.V. (2005) Factor V null mutation affecting the Roche LightCycler factor V Leiden assay. *Clin. Chem.*, **51**, 1533–1535.

27. Jeffrey,G.P., Chakrabarti,S., Hegele,R.A. and Adams,P.C. (1999) Polymorphism in intron 4 of HFE may cause overestimation of C282Y homozygote prevalence in haemochromatosis. *Nat. Genet.*, **22**, 325–326.

28. Shen,R., Fan,J.B., Campbell,D., Chang,W., Chen,J., Doucet,D., Yeakley,J., Bibikova,M., Wickham Garcia,E. and McBride,C. (2005) High-throughput SNP genotyping on universal bead arrays. *Mutat. Res.*, **573**, 70–82.

29. Eggerding,F.A. (1995) A one-step coupled amplification and oligonucleotide ligation procedure for multiplex genetic typing. *Genome Res.*, **4**, 337–345.

30. Battistella,S., Damin,F., Chiari,M., Delgrosso,K., Surrey,S., Fortina,P., Ferrari,M. and Cremonesi,L. (2008) Genotyping β-globin gene mutations on copolymer-coated glass slides with the ligation detection reaction. *Clin. Chem.*, **54**, 1657–1663.

31. Cheung,V.G. and Nelson,S.F. (1996) Whole genome amplification using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than one nanogram of genomic DNA. *Proc. Natl Acad. Sci. USA*, **93**, 14676–14679.