Increased single nucleotide discrimination in arrayed primer elongation by 4′*C*-modified primer probes†

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Received (in Cambridge, UK) 6th November 2006, Accepted 18th December 2006
First published as an Advance Article on the web 2nd January 2007
DOI: 10.1039/b616129d

Herein we describe the beneficial impact of immobilized 4′*C*-modified primer probes on detecting single nucleotide variations in arrayed primer extension by a DNA polymerase.

Analysis of the draft sequences of the human genome revealed considerable variability between individuals.[1] The most common variation in the genome can be accounted for by the presence of single nucleotide polymorphisms (SNPs) that are identified as single base alterations occurring in human DNA at a frequency of approximately 1 every 1000 bases.[2] As a consequence of a minor difference in DNA sequence, a major difference in the individual phenotype may result, so that many efforts in deciphering the human genome sequence were dedicated to the exploration of the genetic bases of complex inherited diseases or disease predispositions.[3] Methods that permit an efficient and immediate diagnosis of relevant single nucleotide variations will contribute to identifying functional allelic variations and should help to predict individual clinical disorders.[4]

Many approaches to the detection of nucleotide variations have been described.[5] The emerging development of DNA microarrays represents a highly parallel, addressable, and large-scale method for the multiplex detection of SNPs. The combination of enzymes and oligonucleotides on an array can be useful for direct analysis of sequence variations, in which enzymes enhance discrimination beyond what can be achieved by hybridisation alone.[6] Enzyme-based genotyping assays such as primer extension and ligation used on array surfaces achieve discrimination in genotyping captured PCR products.[7] In primer elongation processes, DNA polymerases are required to incorporate nucleotides successively only if they match the complement in the template; the terminal base of the primer must also match that of the template. There are several ways in which the reaction can be used to identify the sequence or a single base at a selected site in the template strand.[8] In single-base extension, also denoted as minisequencing or arrayed primer extension, a tethered oligonucleotide is used to capture the target sequence at a position next to a variable base. The identity of the base is determined from the labeled dideoxytriphosphate incorporated by a polymerase.[9] If fluorescence is used to tag the nucleotide precursors, this method can readily be adapted to multicolor detection. Allele-specific extension approaches are adapted from a refractory amplification on template DNA where allele-specific primers are being used to take advantage of the lowered intrinsic efficiency of DNA polymerases to extend mismatched primers compared to matched primers.[10] It is an advantage of enzyme-based methods that the label is incorporated during DNA polymerase catalyzed DNA synthesis, since through incorporation of several labels sensitivity might be increased. However, depending on the sequence context, tedious optimisations are often required to achieve satisfactory selectivity. Recently we have found that primer extension as well as single nucleotide discrimination in PCR can be achieved by employment of 4′*C*-modified primer probes in solution phase approaches.[11] In comparison to unmodified primer probes, a significantly higher amplification selectivity is observed with employment of 4′*C*-modified primer probes at their 3′ termini and *Vent* (exo-), a 3′-5′-exonuclease-deficient DNA polymerase variant from *Thermococcus litoralis*.

Here we report the transfer of the concept of allele-specific primer elongation to a microarray scale. An initial evaluation of several 4′*C*-modified primer probes in arrayed primer elongations was performed to assign the most appropriate modification in further multiplex primer extensions. For this 4′*C*-modified and unmodified primer probes in the Farber disease[12a] context were synthesised and covalently attached to 1,4-phenylene diisothiocyanate-activated glass slides *via* aminoalkyl linkage at their 5′ termini.[13] Oligonucleotides bearing 3′-terminal thymidines with unmodified and 4′*C* methyl, vinyl and methoxymethylene residues were arranged as six replicates in three individual grids (Fig. 1). The sensitivity and efficiency of this assay were determined in allele-specific primer extension reactions at varying DNA template concentrations and cycles, thereby Cy3-dCTP was used for incorporation and labelling along with all four dNTPs to enhance signal intensity (Fig. 2). Moreover, cyclic reaction steps of

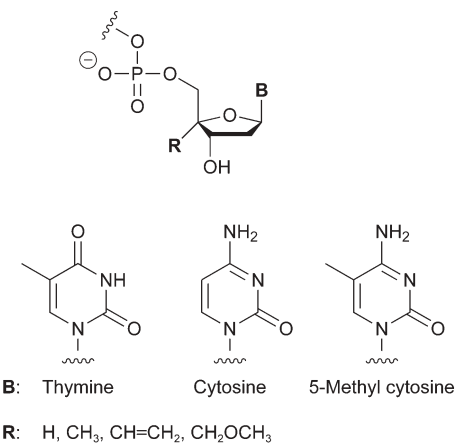

Fig. 1 Employed 4′*C* and nucleobase modifications as allele-specific primer probes. Thymidine derivatives: T^{H}, T^{Me}, T^{Vin}, T^{CH_2OMe}; 2′-deoxycytidine derivatives: C^{H}, $^{5Me}C^{CH_2OMe}$.

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† Electronic supplementary information (ESI) available: Experimental section. See DOI: 10.1039/b616129d

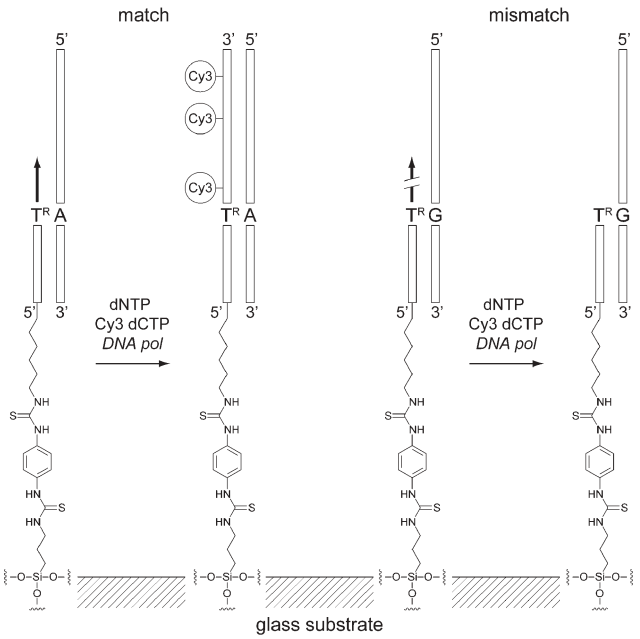

Fig. 2 Schematic illustration of arrayed 4′*C*-modified primer elongation. Covalently attached primer probes at their 3′-termini are complementary or non-complementary to single nucleotide variations in the template to be discriminated. In match extension, DNA polymerase *Vent* (exo-) generates a signal by incorporating fluorescent Cy3-modified 2′-deoxycytidines into the nascent allele-specific primer strand, whereby mismatch extension at 4′*C*-modified primer probes should be obviated.

denaturation, annealing and extension were applied to increase the yield of elongated labelled products at matched primer–template duplexes (for experimental details see Electronic Supplementary Information).[7b]

As can be seen in Fig. 3, amplification efficiency and the ability to discriminate between match and mismatch template varied according to the thymidine analogue employed. The use of native thymidines induced no significant discrimination between match and mismatch. Fluorescence data analysis emphasised the superior discrimination features of the 4′*C* methoxymethylene modification, resulting in an increased match to mismatch ratio after 20 cycles (Fig. 4). Thus, the 4′*C* methoxymethylene modification was used for further evaluation in subsequently conducted multiplex primer elongations.

To perform genotyping experiments we synthesized native and 4′*C* methoxymethylene-modified allele-specific primer probes of

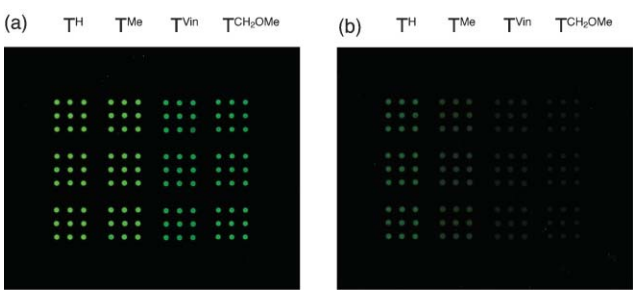

Fig. 3 Fluorescence images of arrayed primer extension. (Cy3, 532 nm). (a) Matched arrayed primer elongation after 20 cycles employing Farber A template. (b) Mismatched arrayed primer elongation after 20 cycles employing Farber G template.

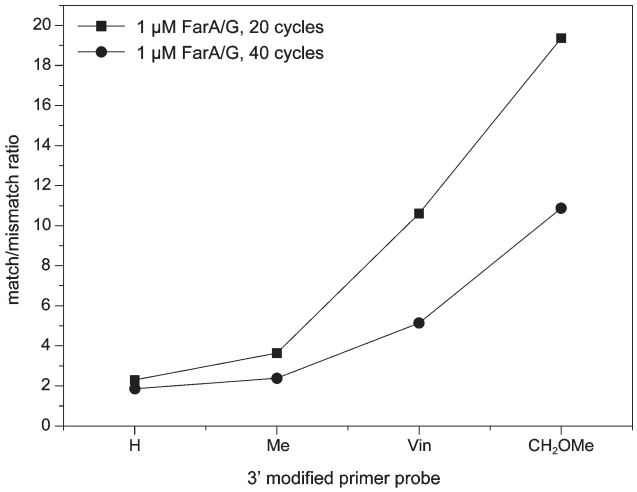

Fig. 4 Match-to-mismatch ratios of normalized fluorescence data from arrayed primer elongations.

several clinically relevant sequences in genetic deficiency contexts, *i.e.* human *N*-acylsphingosine amidohydrolase 1 (Farber), coagulation factor V (Leiden),[12b,c] and dihydropyrimidine dehydrogenase (DPyD).[12d] Among thymidine primer probes, the respective 2′-deoxycytidines were synthesized, derived from the respective thymidine analogue as described.[11e] 5′-terminal aminohexyl-modified oligonucleotides were spotted as six replicates arranged in pairs of 3′-terminal variants of native and modified T/C primer probes (Fig. 5). The signal generated by primer extension on the microarray surface detects the respective SNP. Primer elongations with templates of Farber, Leiden, and DPyD were conducted.

Single nucleotide discrimination by extension of arrayed unmodified *versus* 4′*C*-modified primer probes was facilitated simultaneously and in parallel fashion. Afterwards the slides were scanned to obtain fluorescence data with 532 nm excitation as described above.

In all multiplex experiments, unmodified primer probes revealed poor ability to discriminate between single nucleotide mismatches in this genotyping assay (Fig. 5), resulting in small match-to-mismatch ratios (see Electronic Supplementary Information). In contrast, 4′*C* methoxymethylene-modified primer probes were however suited for detection of sequence variations employing the three different DNA target sequences. We noticed that in all sequence contexts, analysis of normalized fluorescence data affirmed significant discrimination abilities of canonical *versus* non-canonical elongation of the respective A- or G-allele at substantially increased match-to-mismatch ratios.

In this study we have described the use of 4′*C*-modified oligonucleotides as a tool in arrayed primer elongation to increase the selectivity for single nucleotide polymorphism diagnostics. Primer probes bearing a 4′*C* methoxymethylene modification were able to significantly increase single nucleotide discrimination by *Vent* (exo-) DNA polymerase in comparison to their non-modified counterparts. Thus, we were able to interrogate single nucleotide variations in DNA targets to provide a simultaneous correlation to respective primers on multiplexed microarrays.

Although this study is a first approach to increasing the selectivity of a microarray-based DNA detection system employing primer extension, in this phase, the primary basics for the

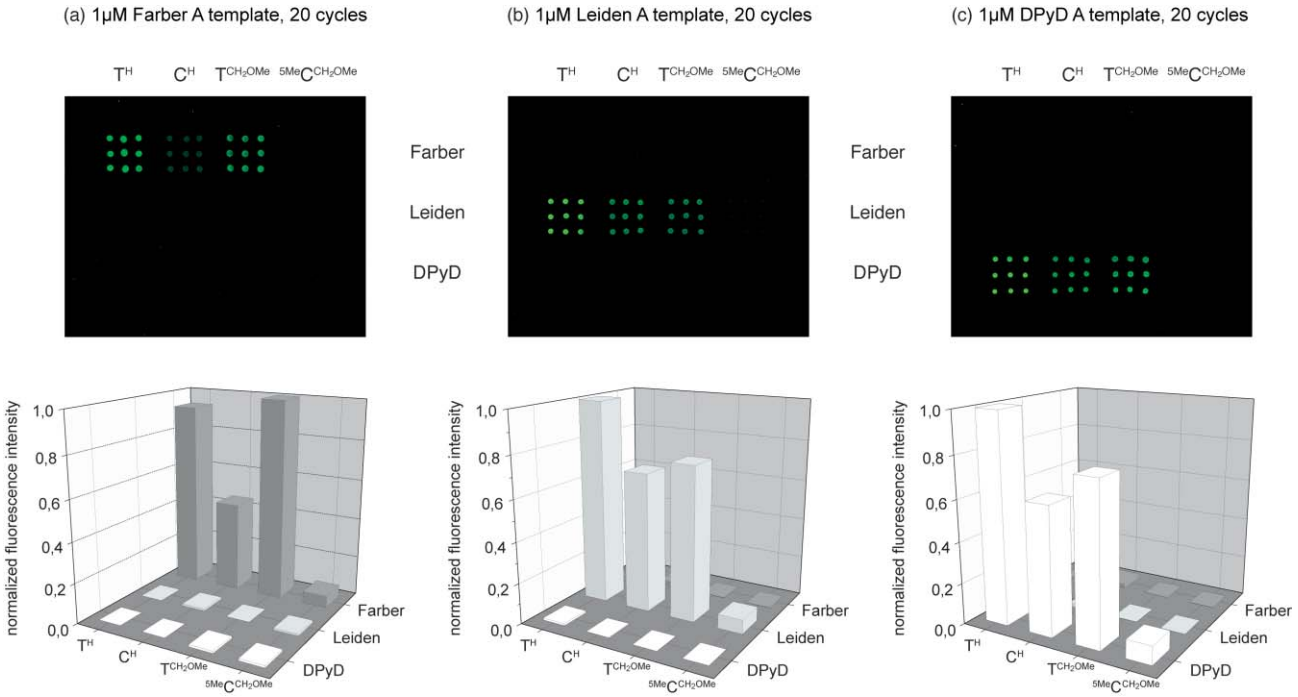

Fig. 5 Fluorescence images (Cy3, 532 nm) and charts of normalized fluorescence data acquired after 20 cycles of arrayed multiplex primer elongation reactions supplemented with respective templates at 1 μM concentration. (a) Arrayed primer elongation after 20 cycles employing 1 μM Farber A template. (b) Arrayed primer elongation after 20 cycles employing 1 μM Leiden A template. (c) Arrayed primer extension after 20 cycles employing 1 μM DPyD A template. Each bar in the chart renders the position of spotted replicates as a single grid on a glass slide.

development of future genotyping assays like accuracy, simplicity, and versatility were demonstrated. The results are promising for further improvements for establishing microarray-based allele-specific PCR assays.

We acknowledge the DFG for funding and wish to thank Prof. Dr Axel Meyer and Dr Walter Salzburger for their support in the use of the GenePix microarray scanner equipment.

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