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Mutant DNA Polymerase for Improved Detection of Single-Nucleotide Variations in Microarrayed Primer Extension

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The vast majority of genomic alteration events are based on single nucleotides.[1] Single nucleotide polymorphisms (SNPs) are changes in a single base at a specific position in the genome. These changes are differentiated from point mutations in their frequency in a population and occur on average approximately every 1000–2000 nucleotides in human DNA.[2] SNPs can lead to modified structures, activities and functions of expressed proteins if they are located in coding and regulating gene regions. Hence, these variations in our genetic make-up are closely associated with complex disorders, such as cancer, diabetes, vascular diseases, some forms of mental illness, and are known to be major players in an individual's predisposition to side effects of drugs.[3]

Many methods for the detection of nucleotide variations have been described.[4] Single nucleotide variations are commonly discriminated by sequence-specific hybridisation or dye-labelled nucleotide incorporation, ligation or invasive cleavage. These systems exploit small differences, for example, in thermodynamic stability and electrostatic interactions, as well as within enzyme recognition processes for obtaining allele-specific properties.[4] Enzymatic approaches can enhance the discrimination of sequence variants beyond what can be achieved by hybridisation approaches alone.[5] A spatially addressable allele-discriminating probe layout, for example, DNA microarrays, represent a promising method for multiplexed detection of SNPs. They are highly efficient, parallel and have the potential to be used in a high-throughput manner for medicinal diagnostics.

In previous publications, we have shown, that reliable and enhanced single-nucleotide discrimination by primer extension or PCR can be achieved and increased by chemical modification of the 3′-terminal nucleotide of the primer probe, which binds opposite the corresponding SNP site of a DNA template.[6] Recently, we have also described DNA polymerase mutants that exhibit significantly increased selectivity in extending a matched primer–template duplex in comparison with the mismatched counterpart.[7,8] Along these lines, one enzyme mutant derived from *Pyrococcus furiosus* (Pfu) DNA polymerase, which contains the amino-acid exchanges D541L/K593M (henceforth called M2), was designed by substituting two polar amino acid residues by hydrophobic amino acids with similar steric demands.[8] We demonstrated that M2 exhibits increased primer-extension selectivity in homogeneous assays, such as, allele-specific real-time PCR. Here, we transfer the concept of allele-specific primer extension from the solution phase to a solid support. We established a primer-extension reaction in microarray format, and used the selectivity-increased DNA polymerase mutant M2 as an allele-specific sensor.

Signal generation in microarray formats relies on the incorporation of, for example, a fluorophor. In order to validate whether the mutant DNA polymerase M2 is capable of incorporating a dye-labelled nucleotide, we first studied primer-extension reactions with a commercially available fluorophor-labelled dUTP analogue (F3-dUTP) as surrogate of the native dTTP. We found, that a ^{32}P-5′-end labelled primer could be elongated to yield the full-length product by the wild-type and M2 enzymes in the presence of all four natural dNTPs (Figure 1, lanes 2). The wild-type enzyme added an additional nucleotide in a nontemplated manner as has been observed for 3′→5′ exonuclease-deficient DNA polymerases.[9] Interestingly, M2 produced the blunt-ended 35 nt full-length product without significant formation of longer products. When the natural dTTP was substituted with F3-dUTP a reaction product was formed that migrated slower than the original band derived from experiments with dTTP (lanes 3) in denaturing polyacrylamide gel electrophoresis. The retardation can be explained by the additional size of the chromophore. Similar effects have been reported before.[10] It is worth noting that the respective bands were fluorescent when readout of the dried

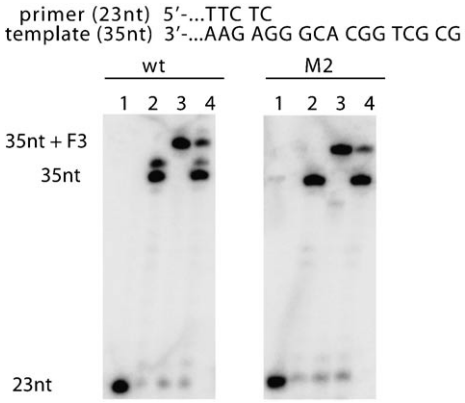

Figure 1. Radioactive primer-extension reactions to assess the incorporation of F3-dUTP by wild-type (wt; left panel) and M2 (right panel) DNA polymerase. A section of the primer template is also shown. Lanes 1: reactions without enzyme; lanes 2: reactions with all four native dNTPs; lanes 3: primer-extension reactions with dCTP, dATP, dGTP and F3-dUTP; lanes 4: primer-extension with all four dNTPs and 7.5% substitution of dTTP with F3-dUTP. All reactions were conducted under the same conditions.

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gel was performed with a fluorescence imager (data not shown). Furthermore, we could show that F3-dUTP is able to compete with dTTP incorporation since even with 7.5% F3-dUTP in the presence of dTTP the slowest migrating band could still be detected (Figure 1, lane 4).

In the next step, we transferred the concept of fluorophor-incorporation based primer extension to a solid support. We immobilised primer probes on 1,4-phenylene diisothiocyanate (PDITC)-activated glass slides by using an aminoalkyl linkage at their 5′ termini,[6f] and performed comparative primer-extension reactions with wild-type Pfu DNA polymerase as well as M2 (Scheme 1). Primer probes were found to be covalently

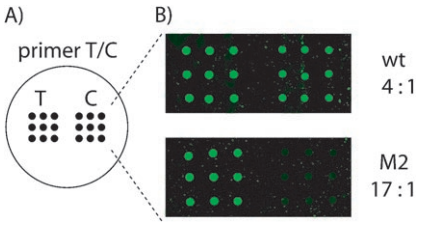
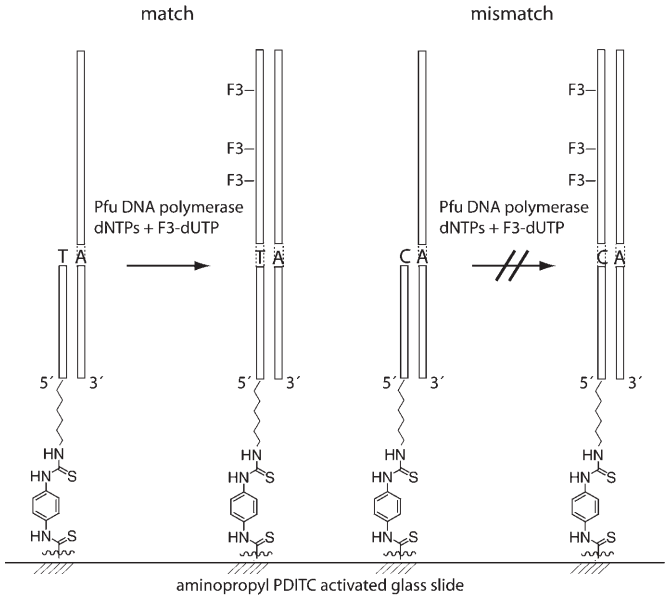


Figure 2. Allele-specific primer extension in microarray format with Farber A template. A) Microarray spotting design, Farber primer probes with 3′-terminal T or C were spotted in 3×3 blocks directly next to each other. B) Primer-extension reactions were carried out with wild-type and M2 Pfu DNA polymerase. All reactions were conducted under the same conditions, on the same slide, with identical amounts of enzyme, template and dNTPs.



Scheme 1. Schematic illustration of allele-specific primer-extension reactions.

attached to aminopropyl PDITC activated glass substrate. In the matched case primer extension by DNA polymerase was expected to proceed, whereas in the mismatched case the reaction should be prevented. A fluorescence signal is generated by partial incorporation of F3-dUTP substituted for the unmodified dTTP.

At first we conducted our experiment in the sequence context of human acid ceramidase comprising the transition mutation A107G, which is involved in the onset of Farber disease.[11] For immobilisation, we spotted the relevant 5′-terminal aminohexyl-modified oligonucleotide (20 nt) as nine replicate spots on the PDITC-activated glass slides. To study the match to mismatch ratio we spotted the primer probe blocks with the 3′-terminal nucleobase thymine (T) or cytosine (C) directly next to each other (Figure 2).

The ratio between the two fluorescence intensities (primer probe T:primer probe C) was derived after the primer-extension reactions in the presence of F3-dUTP, and readout was defined as the discrimination ratio and should be directly dependent on the degree of allele-specific discrimination. Both enzymes were able to show sufficient primer extension and incorporation of the fluorescent F3-dUTP on the slide surface. The primer-elongation reaction with wild-type Pfu DNA polymerase resulted only in poor discrimination properties with low match (T probe) to mismatch (C probe) ratios (Figure 2B). However, using M2 the discrimination ratio increased significantly (Figure 2B).

Next, we investigated whether the ability of the microarray system to discriminate between single-nucleotide variations could be applied to other sequence contexts in a selective and multiplexed manner. Therefore, we investigated two further single-nucleotide variations that are of considerable medicinal interest in addition to the Farber sequence context: the factor V Leiden G1691A mutation is believed to be responsible for a predisposition to thrombosis;[12] and mutation G735A in the human dihydropyrimidine dehydrogenase (DPyD) gene leads to reduced activity of this enzyme, and treatment with the anticancer drug 5-fluorouracil (5-FU) results in fatal haematopoietic, neurological and gastrointestinal toxicities since the mutated enzyme is inefficient in inactivating 5-FU.[13] We spotted all possible six different primer probes and conducted primer-extension reactions in the presence of only one, two or three of the respective templates (Figure 3A).

In the case of the wild-type enzyme only poor discrimination ratios (2:1–5:1) were obtained due to errant extension of noncognate primer probes. By using the M2 enzyme the specific template showed primer extension predominantly with increased discrimination ratios (14:1–20:1), which were in the same range found in the first studies with the Farber sequence (compare Figures 2 and 3; Table 1). No significant fluorescence was detected at locations where noncognate primer probes were spotted.

Taken together, we have shown that the Pfu DNA polymerase mutant M2 is more accurate than the corresponding wild-type enzyme in arrayed primer extensions. Recently, we have reported that single nucleotide discrimination with primer extension or PCR can be achieved and increased by chemical modification of the 3′-terminal nucleotide of the primer probe.[6] Here we report that mutant DNA polymerases in combination with unmodified primer strands are able to fulfil the same demands on solid support, and thus, obviate

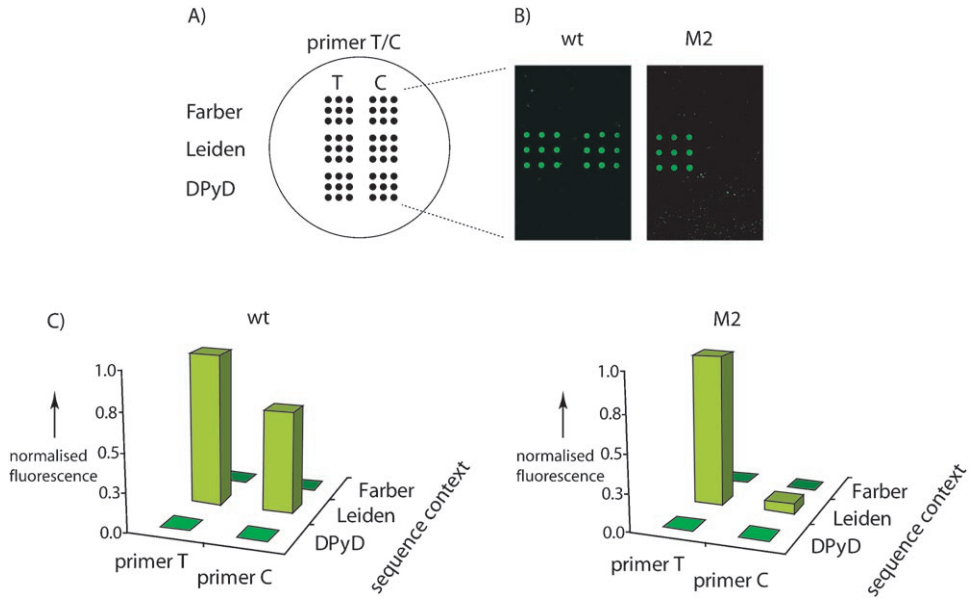


Figure 3. A) Microarray spotting design of three different sequences (Farber, Leiden and DPyD) and two different 3′ primer termini, T or C, for generating a match and mismatch situation. B) Fluorescence images obtained after microarrayed primer extension with Leiden A template as a representative experiment. Primer-extension reactions were carried out with wild-type (wt) and M2 enzyme. All reactions were conducted under the same conditions, with the same amount of enzyme, template and dNTPs. All other results obtained from other templates are shown in Table 1 and in the Supporting Information. C) Bar charts of normalized fluorescence intensities; data were normalised with respect to the highest fluorescence intensity (match situation).

Table 1. Discrimination ratios[a] of wild-type and M2 Pfu DNA polymerase in three different sequence contexts.

Sequence context	Wild type $F_{match}/F_{mismatch}$[a]	M2 $F_{match}/F_{mismatch}$[a]
Farber A	2.5 ± 0.2[b]	14 ± 2[b]
Leiden A	1.5 ± 0.5[b]	14 ± 1[b]
DPyD A	4.6 ± 0.3[b]	20 ± 3[b]

[a] The ratio between the two fluorescence intensities $F_{match}/F_{mismatch}$ (primer probe T:primer probe C) after primer-extension reaction in the presence of F3-dUTP was defined as the discrimination ratio; [b] error was calculated from standard deviations of replicates and error propagations from the averaged calculated ratios ($F_{match}/F_{mismatch}$).

the need for chemical modifications of primer probes. The entire microarray experiment, starting from the primer-extension reaction (20 min reaction time), including washing steps and readout of the microarray, was finished within 40 min without further optimization of parameters. The system might be enhanced by optimizing reaction buffer composition and washing conditions. In addition, the two-fold lower activity of the depicted Pfu DNA polymerase mutant M2 compared to the wild-type enzyme[8] might be improved by further mutation. Nevertheless, the accuracy and simplicity of the demonstrated approach by using a mutant DNA polymerase and unmodified DNA primer probes was demonstrated. The system depicted herein could provide the basis for further advancements in microarrayed nucleic-acid diagnostics.

Experimental Section

Materials: Aminopropyl silylated glass slides were obtained from Genetix, DNA oligonucleotides were synthesised by IBA (Göttingen, Germany). Other materials included self-seal reagent (BioRad, München, Germany), glass cover slips (Menzel-Gläser, Braunscweig, Germany), dNTPs and F3-dUTP (a Rhodamine-B derivative, $\lambda_{ex\,max} = 555$ nm, $\lambda_{em\,max} = 580$ nm; Fermentas, St. Leon Rot, Germany), BSA, crown capped pyridine, 1,4-phenylene diisothiocyanate (Fluka), acetone (Prolabo, Fontenay sous Bois, France), acetonitrile (Sigma–Aldrich), ammonium hydroxide and *N,N*-dimethylformamide (Acros, Geel, Belgium). Spotting of primer probes was conducted with a Nanoplotter 2.0 system (GeSiM, Großerkmannsdorf, Germany). Reactions on glass slides were performed in a microarray Peltier Thermal Cycler 200 (MJ Research). Microarray-image data were acquired with a GenePix Personal 4100A microarray scanner (Molecular Devices). ESI-MS data were obtained by using an Esquire 3000$^+$ (Bruker Daltonics).

Activation of glass slides and spotting of amino-modified oligonucleotides to glass slides: Aminopropyl-silylated glass slides were derivatized with 1,4-phenylene diisothiocyanate (0.2%, *w/v*) in a pyridine/dimethylformamide (10%, *v/v*) solution for 2 h at room temperature. The slides were subsequently washed several times with dimethylformamide and acetone, dried under a stream of nitrogen and stored desiccated until spotting. Spotting of 5′-amino-modified primer probes (20 μM; ~4 nL per spot) in sodium phosphate buffer (150 mM, pH 8.5) was performed between 19–22 °C and 70–77% humidity. The slide tray was cooled during the spotting procedure at 10 °C. After the spotting process the slides were incubated at room temperature in a closed petri dish over a saturated NaCl solution, overnight. Subsequently, the slides were blocked in NH_4OH solution (10%) for 30 min; this was followed by washing steps with water. The slides were dried under a stream of nitrogen and stored at 4 °C until further use.

DNA primer and template sequences: The integrity of all primer probes was evaluated by ESI-MS. DNA oligonucleotide sequences for primer extension in solution were as follows: primer (23 nt): 5′-d(GAC CCA CTC CAT CGA GAT TTC TC)-3′, single-stranded template (35 nt): 5′-d(GCG CTG GCA CGG GAG AAA TCT CGA TGG AGT GGG TC)-3′. Sequences employed in arrayed primer extension were: Farber primer T/C (20 nt), 5′-$NH_2(CH_2)_6$-d(CGT TGG TCC TGA AGG AGG A**T**/**C**)-3′, Leiden primer T/C (25 nt), 5′-$NH_2(CH_2)_6$-d(CAA GGA CAA AAT-ACC TGT ATT CCT **T**/**C**)-3′, DPyD primer T/C (25 nt), 5′-$NH_2(CH_2)_6$-d(GTT TTA GAT GTT AAA TCA CAC TTA **T**/**C**)-3′, Farber templates A/G (90 nt), 5′-d(CCG TCA GCT GTG CCG TCG CGC AGC ACG CGC CGC CGT-GGA CAG AGG ACT GCA GAA AAT CAA CCT **A**/**G**TC CTC CTT CAG GAC-CAA CGT ACA GAG)-3′, Leiden templates A/G (98 nt), 5′-d(GAC ATC-ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT-AAG AGC AGA TCC CTG GAC AGG C**A**/**G**A GGA ATA CAG GTA TTT TGT-

CCT TG)-3′, DPyD templates A/G (120 nt), 5′-d(AAA GCT CCT TTC-TGA ATA TTG AGC TCA TCA GTG AGA AAA CGG CTG CAT ATT GGT GTC-AAA GTG TCA CTG AAC TAA AGG CTG ACT TTC CAG ACA AC**A/G** TAA-GTG TGA TTT AAC ATC TAA AAC)-3′.

Primer extension and arrayed-primer extension: Wild-type and mutant Pfu DNA polymerase were obtained as described.[8] The mixtures for the primer-extension reactions in solution contained reaction buffer (20 mM Tris-HCl, pH 8.8, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% (*v*/*v*) Triton-X100, 0.01 mg mL^{-1} BSA), dATP, dGTP, dCTP (200 μM each), single stranded template (200 nM), 5′-^{32}P-labelled primer (150 nM) and Pfu DNA polymerase (100 nM) in a total volume of 20 μL. dTTP and F3-dUTP concentration was varied (200 μM dTTP, 185 μM dTTP + 15 μM F3-dUTP (7.5%), 200 μM F3-dUTP). The mixtures were denatured for 2 min at 95 °C, annealed at 55 °C and the reaction was initiated by addition of DNA polymerase. After 20 min at 72 °C primer extension was stopped by addition of gel-loading buffer (20 μL, 80% formamide, 20 mM EDTA). Product mixtures were separated by denaturing PAGE (12%). Gels were analysed with a Molecular Imager by phosphor imaging and with the Cy3-fluorescence channel. The arrayed primer-elongation reactions contained reaction buffer (20 mM Tris-HCl, pH 8.8, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% (*v*/*v*) Triton-X100, 0.01 mg mL^{-1} BSA), dATP, dGTP, dCTP (200 μM), dTTP (185 μM), F3-dUTP (15 μM), single stranded template (500 nM), BSA (0.1%), 0.5x self-seal reagent and the corresponding Pfu DNA polymerase (200 nM). The reaction solution (3.4 μL per reaction) was placed on the spotted area and covered with round cover slips (10 mm diameter). Primer extension was carried out with a thermocycler by using the following temperature steps: 95 °C for 225 s, 55 °C for 60 s and 72 °C for 20 min. The reaction was stopped by cooling the slides to 4 °C, and the slides were subsequently washed twice under gentle agitation in 0.1 × SSC buffer (sodium chloride/sodium citrate) with SDS (0.1%) for 5 min and three times with water (5 min each). The slides were then dried under a stream of nitrogen directly before readout with a GenePix microarray scanner machine.

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