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New Strategies for DNA Polymerase Library Screening

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ABSTRACT

Engineered enzymes are of increasing importance for a plethora of biotechnical applications. Especially DNA polymerases are workhorses in biochemical technologies in particular the polymerase chain reaction (PCR), cDNA cloning procedures, genome sequencing and in diagnostic applications. DNA polymerase mutant libraries can be used for the screening of non-standard reaction conditions or substrates e.g. the efficient amplification of difficult templates like ancient DNA. We are convinced that these fascinating enzymes can be optimized and costum-made for a specific application to result in more robust and reliable systems. To our knowledge, all known screening methods for DNA polymerase mutants are focused and thus limited to the screening of a single reaction or one new function. We developed improved strategies for multiplexed DNA polymerase screening that will be presented.

INTRODUCTION

To evolve and select costum-made DNA polymerases, we have previously demonstrated that the generation of a new DNA polymerase function or property is achievable through iterative screening of small libraries of DNA polymerase mutants derived by randomization of the respective genes.[1] We showed that the identified mutants find immediate applications and provide the basis for the development of new means for diagnostic technologies. Protein library expression can be conducted in multiwell plates and enzymes are subsequently screened directly after heat denaturation of host proteins and lysis without requiring further purification steps. In a first step the DNA polymerase activities can be monitored by SYBRgreen I mediated quantification of synthesized double stranded DNA after reaction termination. This setup links enzyme activity to a signal without requiring artificial substrates during reaction that might interfere with the enzymatic reaction. In a following step, the new pool of modified enzymes must be sampled by a suitable selection or screening method. A few primer extension based screening methods are established, like e.g. the Sybr-Green I assay[1], the molecular beacon approach[2], and the fluorescence polarization (FP) detection assay.[3] Self-replicating selection systems are an established selection method which can be employed.[4] However, all of these systems are only focused on the screening of a single reaction or one new function. Therefore we intended to establish a screening method for the multiplexed and parallel screening of DNA polymerase libraries. Microarrays have the potential to fullfill all requirements for screening in such a manner.

RESULTS AND DISCUSSION

Signal generation in microarray format relies on the incorporation of a signal e.g. a fluorophore. We first studied primer extension reactions in solution with a commercially available fluorophore labelled dUTP analogue (F3-dUTP) as surrogate of the native TTPs in solution. We found, that a ^{32}P-5'-end labelled primer is elongated to yield the full length product by the wild-type enzyme in the presence of all four natural dNTPs (Figure 1, lane 2).

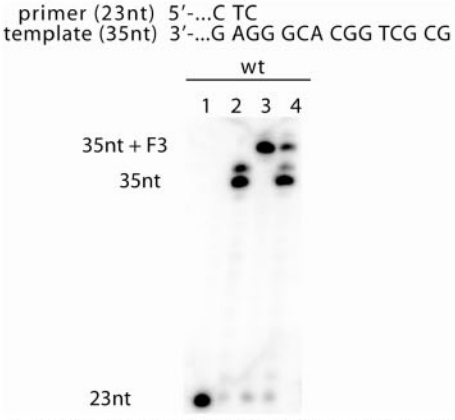

Figure 1 Radioactive primer extension reactions to assess the incorporation of F3-dUTP by a wild-type (wt) DNA polymerase (*Pfu exo*$^-$). A section of the primer template is shown in the figure. Lane 1: reactions without enzyme; Lane 2: reaction with all four native dNTPs; Lane 3: primer extension reactions with dCTP, dATP, dGTP and F3-dUTP; Lane 4 primer extension with all four dNTPs and 7.5% substitution of TTP by F3-dUTP. All reactions were conducted under the same conditions.

When the natural TTP was substituted for F3-dUTP a reaction product was formed that migrated slower (lane 3) than the original band derived from experiments comprising TTP in denaturing polyacrylamide gel electrophoresis. The retardation can be well explained by the additional size of the chromophore. Noteworthy, the respective bands were fluorescent when read out of the dried gel was performed with a fluorescence imager. Furthermore, we could show that F3-dUTP is able to

compete with TTP incorporation since even in the presence of only 7.5% F3-dUTP in the presence of TTP the slower migrating band was detectable after analysis (lane 4).
In the next step, we transferred the concept of fluorophor incorporation based primer extension on solid support. Therefore we immobilized primer probes on *1,4*-phenylene diisothiocyanate–activated (PDITC) glass slides via aminoalkyl linkage at their 5´-termini[5] and performed comparative primer extension reactions with wild-type *Pfu* DNA polymerase as well as with a mutant enzyme M2 that exhibits significantly increased selectivity in extending a matched primer template duplex versus its mismatched counterpart.[6] For immobilization, we spotted the relevant 5´-terminal aminohexyl modified oligonucleotide (20nt) as nine replicate blocks on the PDITC-activated glass slides. To demonstrate that both enzymes are not only able to extend the immobilized primer but also functional to discriminate between the matched and mismatched cases, we spotted the respective primer probe blocks with the respective 3'-terminal nucleobase thymine (T) or cytosine (C) directly neighbouring each other (see Figure 2, A).

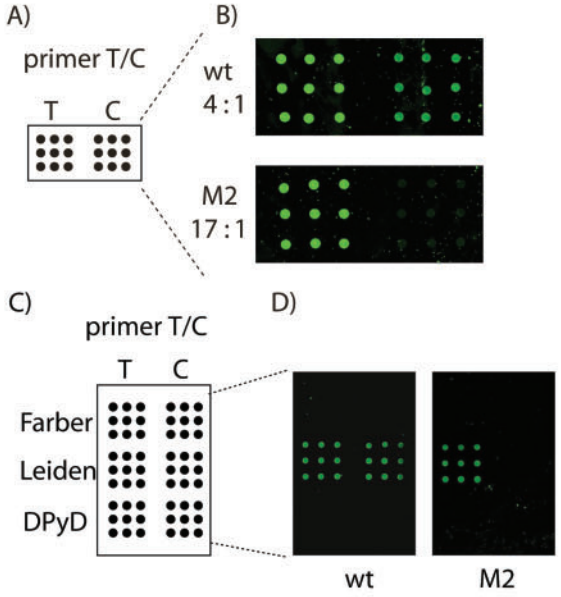

Figure 2 Primer extension in a selective microarray format. **A)** Microarray spotting design, primer probes with 3´terminal T or C were spotted in 3x3 blocks directly neighbouring each other. **B)** Functional comparison between wild-type (wt) and a mutant (M2) *Pfu* DNA polymerase. Primer extension reactions were carried out with wild-type enzyme and M2. All reactions were conducted under equal conditions on the same slide with the identical amount of enzyme, template and dNTPs. **C)** Microarray spotting design of three different primer probes (Farber, Leiden, DPyD[5]). and two different 3´primer termini T or C for generating matched and mismatched cases. **D)** Resulting fluorescence images after microarrayed primer extension reaction with Leiden A template as a representative experiment. Primer extension reactions were carried out with wild-type enzyme (wt) and M2. All reactions were conducted under equal conditions on the same glass slide with the same amount of enzyme, template and dNTPs.

Both enzymes showed sufficient primer extension reaction and incorporation of the fluorescent labelled F3-dUTP on the surface. The primer elongation reaction with wild-type *Pfu* DNA polymerase only resulted in poor discrimination properties with match (T-probe) to mismatch (C probe) ratios (see Figure 2, B). However, using M2 the discrimination ratio increased significantly (see Figure 2, B). 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. Indeed the current template (in this case Leiden A) is adressed from the respective immobilized primer spots and selectively elongated (see Figure 2, C).
Currently we are expanding our microarrayed primer extension system with regards to the screening of DNA polymerase mutant libraries. This will enable us to probe for several new enzyme properties in a multiple and simultaneous manner. The new method will be reported in the poster presentation.

CONCLUSION

In brief, we demonstrated that selective primer extension reactions can be catalysed by DNA polymerases on a microarray. Additionally they can be used for the detection of single nucleotide variations in a microarrayed primer extension system[5]. The system depicted herein could provide the basis for further advancements in microarrayed nucleic acid diagnostics and opens up the opportunity to screen DNA polymerase libraries for several new enzyme properties in a multiplexed and parallel manner.

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