Fast Detection of Single Nucleotide Polymorphisms (SNPs) by Primer Elongation with Monitoring of Supercritical-Angle Fluorescence

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We describe the rapid detection of single nucleotide polymorphisms (SNPs) by real-time observation of primer elongation. The enzymatic elongation of surface-bound primers is monitored by detecting the increase of surface-bound fluorescence caused by the incorporation of Cy5-labelled deoxycytidine 5′-triphosphate residues (Cy5-dCTPs) into the corresponding strand. In order to discriminate against the fluorescence from unbound Cy5-dCTPs, the detection volume was restricted to the surface by collecting supercritical-angle fluorescence. The efficiency of enzymatic double-stranded DNA synthesis is governed by the complementarity of the primer and template. An SNP in the sequence of the primer obstructs its elongation increasingly with decreased distance of the mismatch to the 3′ end of the primer. By real-time fluorescence detection during primer elongation, SNPs can be detected within a few minutes, which is significantly faster than in experiments where the fluorescence is measured after completion of the reaction. We demonstrate the efficiency of the method by detecting an SNP in the ErbB2 *gene that is involved in causing a higher risk of breast cancer.*

Introduction

The decoding of the human genome by the Public Human Genome Project[1] and the private group of Venter[2] revealed that the sequence not only contains over 3.2 billion bases but also 30000–40000 genes with a broad spectrum of genetic variation, mostly caused by single nucleotide polymorphisms (SNPs). An SNP occurs every 2 kilobases on average but can also occur with higher frequency in some areas of the genome.[3–5] These variations play an important role in the outbreak of hereditary diseases such as Alzheimer's[6] or multiple sclerosis[7] and are of particular interest in pharmaceutics due to their influence on drug response.[8,9] For these reasons, new genotyping methods are required that combine high throughput and sensitivity to identify SNPs.[10] Existing methods are either based on probe–target hybridisation[11,12] or enzymatic reactions such as cleavage,[13,14] digestion,[15] minisequencing[16,17] and primer elongation.[18] The most commonly used methods are hybridisation on DNA chips[19,20] and in solution (Taqman, molecular beacons).[21,22] Probe annealing is often accompanied with the problem of cross-hybridisation, that is, probe–target pairing can occur even though the sequences are not fully complementary. Consequently mismatches are frequently overlooked. Patil et al. found that, by hybridisation, only 65% of the SNPs investigated were detectable within 97% accuracy.[23]

For detection by hybridisation the mismatch needs to be located near the centre of the probes, which are 20–40 nucleotides long. In contrast, for SNP methods based on primer elongation the mismatch needs to be located near the end of the probe (primer). The advantage of primer elongation lies in the high sensitivity of the enzyme towards mismatches, thereby allowing SNP detection with high accuracy.[18] Primer elongation is commonly carried out in solution and, after sample purification, is measured by MALDI-TOF mass spectrometry.[24–27] Sample-purification steps can be circumvented by using the Good assay, whereby DNA charge tagging increases the sensitivity of the MALDI-TOF measurement.[28] Drawbacks of SNP detection by MALDI-TOF mass spectrometry are the high cost of the instrumentation and the considerable time consumption, as measurement is not started until completion of the enzymatic primer elongation. In the template-directed dye–terminator incorporation (TDI) assay, the primer is tagged with a dye. This primer is elongated by only one base, a dye-tagged dideoxynucleoside 5′-triphosphate (ddNTP), which causes a measurable fluorescence decrease due to energy transfer between the dyes.[29,30] In this case, the synthesis of a dye-tagged primer is necessary, which increases the cost. In all SNP-detection methods the relevant DNA sequence needs to be extracted from the genome by PCR. Comparisons of the methods can be found in refs. [31–34].

In this study, we report a new method to detect SNPs by primer elongation at a glass/water interface. At this interface, the primer is annealed to surface-bound single-stranded DNA (ssDNA). A polymerase synthesises a complementary strand containing Cy5-labelled deoxycytidine 5′-triphosphate residues (Cy5-dCTPs) with an efficiency that strongly depends on the sequence of the primer. The major advantage of immobilizing the primer is the possibility of observing the formation of fluo-

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  DOI: 10.1002/cbic.200400044

rescently tagged double-stranded DNA (dsDNA) in real time, thereby revealing information about the primer sequence within a few minutes. Using this technique, we found that the increase of surface-bound fluorescence is dependent on the occurrence of SNPs and their location in the primer sequence.

Results and Discussion

To detect the incorporation of the Cy5 label into the dsDNA in the presence of high concentrations of fluorescently labelled nucleotides in solution, it is necessary to confine the detection region strictly to the interface. This was accomplished by the detection of supercritical-angle fluorescence (SAF) with the custom-made biosensor described in ref. [35]. Briefly, the coverslip/analyte interface is illuminated orthogonally from below through the glass with the focussed beam of a HeNe laser. A parabolic glass lens collects the SAF signal, that is, the fluorescence emitted into the angular region above the critical angle of refraction, which amounts to $\approx 61°$ for a glass/water interface. By detecting only supercritical emissions, the detection volume is restricted to a surface distance well below 100 nm and bulk fluorescence is for the most part rejected.[36] The surface confinement obtained by using the SAF emissions is even more efficient than that achieved with common biosensors based on total-internal-reflection fluorescence (TIRF).[37] A schematic diagram of the SAF biosensor is shown in Figure 1. The

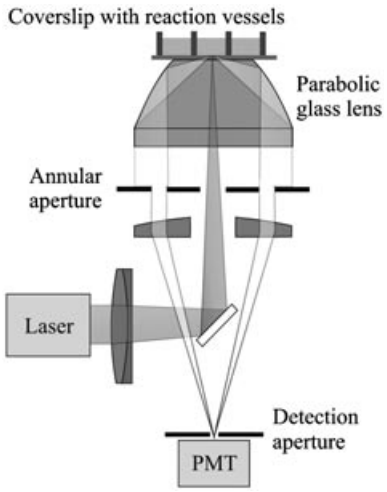


Figure 1. *Schematic diagram of the SAF biosensor. PMT = photomultiplier tube.*

core element of the setup is a parabolic lens that captures the fluorescence at large surface angles, in principle, up to 90° with respect to the surface normal.[38] It has been demonstrated that, with this element, SAF collection and TIRF excitation can be combined in order to further reduce the detection volume.[39,40] This characteristic allows the real-time detection of labelled dNTPs incorporated during complementary-strand synthesis of surface-bound dsDNA.

As the template for primer annealing, we immobilised ssDNA that was 80 nucleotides long (for sequences of DNA strands, see Table 1) on to aminosilane-coated coverslips. An additional C_6 spacer was introduced between the DNA strand and the 5′-end-labelled amino group to minimise interaction between the polymerase and surface (for the linker's chemical composition, see Table 1). The primers (21 bases long) were chosen to have either a corresponding sequence to the unbound end of the template or to contain mismatches at different sequence positions. The remaining sequence of the template was chosen to have no correspondence to the primer sequence and to contain five guanine residues. In the case of successful corresponding strand synthesis, five Cy5-dCTPs are expected to be incorporated into the dsDNA at the interface.[41]

Table 1. *Sequences of primers and target DNA used in these studies.*

Name	Sequence (5′–3′)
SNP-1	TTA GAG GTA ATA AGA GAT GA**A**
SNP-2	TTA GAG GTA ATA AGA GAT G**GT**
SNP-3	TTA GAG GTA ATA AGA GAT **C**AT
SNP-4	TTA GAG GTA ATA AGA GA**A** GAT
SNP-5	TTA GAG GTA ATA AGA G**TT** GAT
Mismatch-2 & 3	TTA GAG GTA ATA AGA GAT **AG**T
Corresponding primer	TTA GAG GTA ATA AGA GAT GAT
DNA target	TTT TTT TTT TTT TTC GCT CTC CCT TGT CTC TCT CTG CCC CCC CCC GTT TTT TTT TGT TT**A TCA TCT CTT ATT ACC TCT AA**
ErbB2-G-Valine	ACC CAC TCC TGT GTG GAC CTG GAT GAC AAG GGC TGC CCC GCC GAG CAG AGA GCC AGC CCT CTG ACG TCC ATC **GTC TCT GCG GTG GTT GGC ATT**
ErbB2-A-Isoleucine	ACC CAC TCC TGT GTG GAC CTG GAT GAC AAG GGC TGC CCC GCC GAG CAG AGA GCC AGC CCT CTG ACG TCC **ATC ATC TCT GCG GTG GTT GGC** ATT
Primer-Valine	AAT GCC AAC CAC CGC AGA GAC
Primer-Isoleucine	AAT GCC ACC CAC CGC AGA GAT
Chemical composition of the linker	$-O-P(O_2)-(CH_2)_6-NH_2$

For the spotting of SNPs, we employed two different procedures referred to as method 1 and method 2. In method 1, primer, dNTPs and polymerase (exonuclease-free Klenow fragment) were added consecutively. After 30 min of primer annealing, Cy5-dCTPs, dATPs, dGTPs and dTTPs were added and 10 min later synthesis was started by addition of the polymerase (dATP = deoxyadenosine 5′-triphosphate, dGTP = deoxyguanosine 5′-triphosphate, dTTP = deoxythymidine 5′-triphosphate). In method 2, all reagents, that is, primer, polymerase and dNTPs, were added at once, thereby allowing simultaneous primer annealing and formation of dye-labelled dsDNA.

Figure 2 shows typical time courses of the surface fluorescence obtained with method 1. After the hybridisation we measured a signal of ≈ 4 kHz (photoelectrons per second), which equals the background intensity obtained with a blank glass/water interface. Addition of the dNTP mix (dATP, dGTP, dTTP, and Cy5-dCTP) caused a rapid increase of the fluorescence to 100 ± 40 kHz and the signal remained at this level afterwards. This count rate can be attributed to the relatively

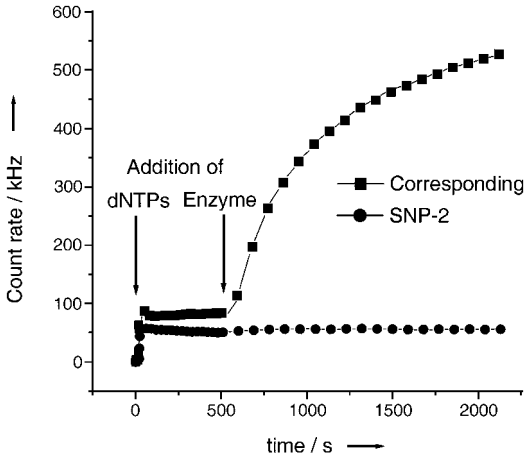


Figure 2. *Time course of the SAF intensity with method 1.*

high concentration of Cy5-dCTPs (3.3×10^{-7} mol L^{-1}) showing a nonspecific interaction with the coated surface. Addition of the polymerase leads to a substantial increase in the fluorescence signal over approximately 30 min in cases of primer elongation or to no further increase when a primer is used with an SNP at position 2 (in the 3′→5′ direction, SNP-2). We repeated the experiment by using a primer with a mismatch at position 1 (SNP-1), a primer with mismatches at positions 2 and 3 (Mismatch-2&3) and with the corresponding primer. The use of the corresponding primer led to a fluorescence increase of more than 400 kHz (see Figure 3), whereas a different sequence caused no or only minor increase in the signal. Accordingly, SNPs in the primer are detected with high reproducibility. Within a few minutes after addition of the enzymes, we were able to render an accurate judgement about the presence of a mismatch. With method 1, the time needed for an entire experiment was approximately 1 h.

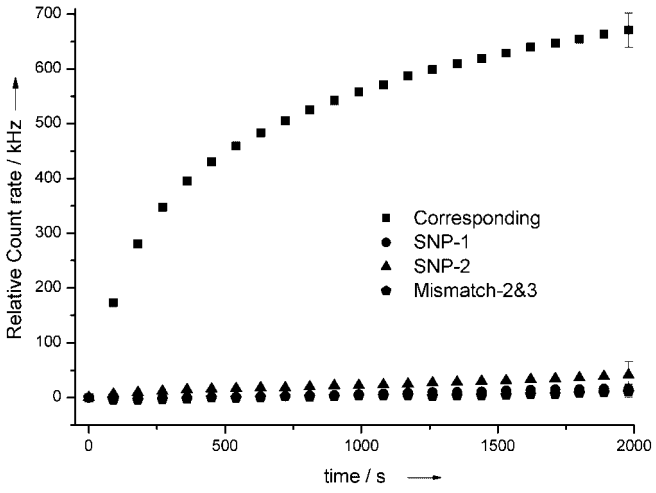


Figure 3. *SAF intensity after addition of polymerase for the corresponding primer and those with mismatches at position 1, position 2 and positions 2 and 3. Each experiment was repeated three times. The count rate obtained prior to primer elongation was subtracted.*

In order to speed up the detection of SNPs, we adopted method 2. In this case, primer, dNTPs and polymerase were applied to a template-coated coverslip simultaneously. This approach is reasonable since hybridisation reactions under comparable conditions were found to be completed within approximately 5 min.[41] A possible drawback of method 2 is that the intensity increase observed after addition of Cy5-dCTPs could superpose on the increase caused by primer elongation. The rapid, nearly cascaded jump in the intensity means this problem can be easily circumvented by neglecting the data obtained during the first minute after adding the dNTPs. Consequently, the measurement was started 60 s after pipetting of the reagents. We investigated primers of corresponding sequence and those with SNPs at positions 1–5 and repeated each experiment three times. Figure 4 gives the averaged fluorescence intensity minus background fluorescence (count rate of first data point) for primer elongation by using method 2. Again, greatest elongation efficiency was observed with the matching primer. Introduction of one mismatch interferes with primer elongation increasingly from positions 5–1. Our results therefore even give the correct order of the SNP position, although this does not comply until the data are averaged. Compared to method 1, a slower intensity increase was obtained with method 2, as a result of simultaneous primer annealing and enzymatic elongation. Nevertheless, with method 2 a mismatch at positions 1–3 was detected in every single experiment after a measurement time of only 5 min.

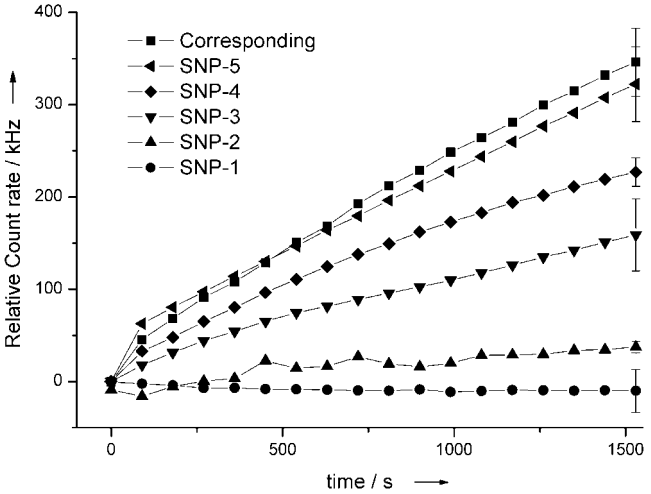


Figure 4. *Primer elongation with method 2. The plots give the average fluorescence intensity for the corresponding primer sequence and for primers with SNPs at positions 5–1. Each experiment was repeated three times. The intensity obtained immediately after addition of the reagents was taken as the background value and subtracted.*

In cases where the mismatch was shifted too far towards the centre of the primer, the annealing step could be interfered with. However, with 21 bases, the primers are long enough to bind to the template even when mismatches are introduced near the 3′ end. The observed signal increase connected with shifting the mismatch towards the centre proves

that the signal is dominated by polymerase activity and not by hybridisation efficiency.

Efficient genotyping methods should cope with low amounts of genetic material. We quantified the concentration of primer DNA needed for SNP spotting. For this, we applied method 2 to primer elongation and varied the concentration of the fully corresponding primer. As may be seen from Table 2, concentrations in the nanomolar region are sufficient to yield a distinct signal. Higher primer concentrations do not lead to a strong signal increase due to the saturation of the templates. In order to further reduce the need for material, it is, for example, possible to increase the fluorescence intensity by high-density labelling.[42–44]

Table 2. *Dependency of SAF intensity on primer concentration (corresponding primer). The fluorescence increase was measured 33 min after adding polymerase.*

Concentration [M]	Relative fluorescence
7.0×10^{-7}	1.00
7.0×10^{-8}	0.87
7.0×10^{-9}	0.67
1.4×10^{-9}	0.13
7.0×10^{-10}	0

We applied our approach by spotting an SNP involved in human disease. The SNP occurring in the *ErbB2* gene is responsible for an increased onset risk of breast cancer.[45–47] Breast cancers with valine encoded in the responsible *ErbB2* codons show an overexpression of ErbB2. The DNA sequences for the expression of valine and isoleucine only differ by one base. This SNP can have severe consequences. Women with valine instead of isoleucine at position 655 in the amino sequence of ErbB2 have a higher risk of breast cancer.

For detection of this particular SNP, we immobilised a 93-base chemically synthesised sequence of the *ErbB2* gene (ErbB2-G-Valine) that would express valine at the position of interest.[48] We applied method 2 by adding the mixture of primer, polymerase and dNTPs as described above. The employed primers where either complementary to valine (Primer-Valine) or isoleucine (Primer-Isoleucine) at the end position (position 1). We performed three real-time measurements for each primer and obtained a significant fluorescence increase for Primer-Valine and no increase for Primer-Isoleucine each time. Figure 5 shows the averaged data of the experiments.

Additionally, we demonstrated that an equivalent result can be obtained by immobilizing the sequence expressing isoleucine (ErbB2-A-Isoleucine), which only differs from ErbB2-G-Valine by one base. As shown in Figure 6, this time the fluorescence increase is only observed with Primer-Isoleucine.

Although the natural DNA sequence provides 20 incorporation sites for Cy5-dCTP, four times more than our artificially designed target, the fluorescence increase caused by primer elongation was approximately 50% lower. The fluorescence signal cannot serve as a direct measure of the incorporated dyes, because quenching of proximate dyes reduces the fluorescence yield. The chosen dye distance with a Cy5-dCTP incorporation every 10 bases in the artificial DNA strand is higher than the average distance of guanines occurring in natural DNA. However, the reduced signals proved to be clearly sufficient to detect SNPs.

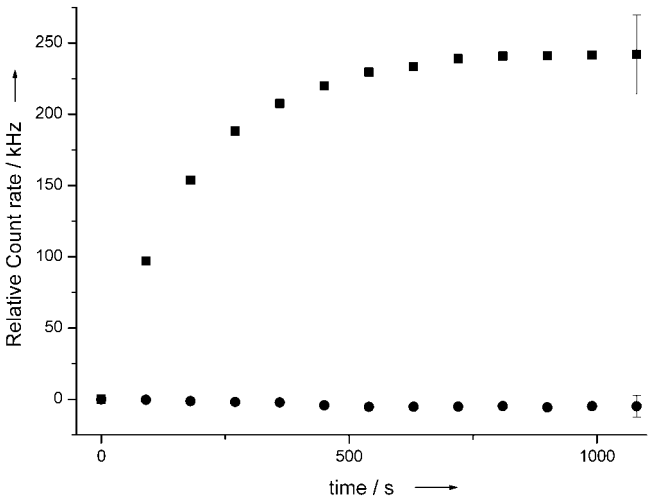


Figure 5. *Primer elongation of the immobilised ErbB2-G-Valine sequence. The plots give the average fluorescence intensity for the corresponding Primer-Valine sequence (squares) and the SNP Primer-Isoleucine sequence (circles). Each experiment was repeated three times. The intensity obtained immediately after addition of the reagents was taken as the background value and subtracted.*

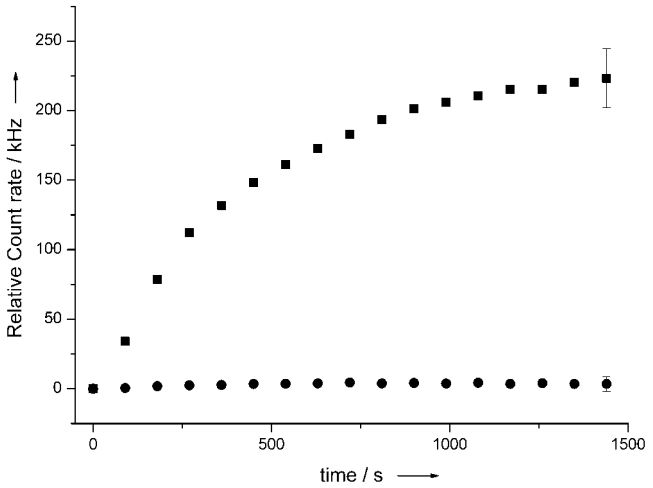


Figure 6. *Primer elongation of immobilised ErbB2-A-Isoleucine sequence. The plots give the average fluorescence intensity for the corresponding Primer-Isoleucine sequence (squares) and the SNP Primer-Valine sequence (circles). Each experiment was repeated three times. The intensity obtained immediately after addition of the reagents was taken as the background value and subtracted.*

Conclusion

We have introduced a rapid detection method for SNPs by primer elongation at a solution/coverslip interface. By transfer-

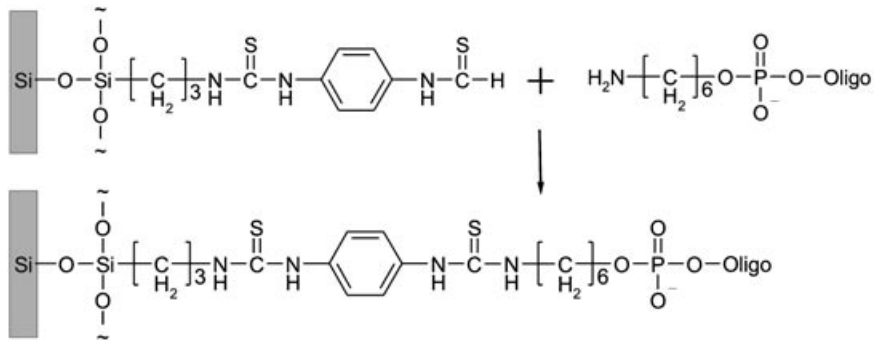
ring the method to the surface, we are able to measure the enzymatic reaction in real time. The SAF biosensor provides extreme surface-selective detection and high sensitivity. Compared to hybridisation, the presented method allows SNP detection with higher accuracy and higher speed. By using temperature gradients[49] or electrical fields,[50] the accuracy of the hybridisation method can be improved, but the cost and complexity of the systems grow likewise, whereas the method described here involves easy handling and provides reliability.

A commercial system like the Affymetrix GeneChip requires 16–24 h of hybridisation before read-out, whereas real-time measurements allow the detection of SNPs within a few minutes. This time need does not include the preparation time for immobilizing the single-stranded target DNA. However, for the screening of a particular SNP in the sequence of a multitude of DNA samples (primers), the preparation step ($\approx$1.5 h) can be paralleled and therefore makes a minor contribution to the overall time need.

Currently, we are enhancing the system for quasisimultaneous detection on DNA chips.[51] For screening large libraries of DNA samples it is not necessary to obtain smooth binding curves for every spot. Rather, a few kinetic data points will suffice to judge the occurrence of a mismatch.

Experimental Section

Fixation of template DNA on aminated cover slips (Genorama SAL, Asper Biotech, Estonia): The cover slips were glued onto a measuring cell containing six reaction chambers. A spotting solution (Genorama, Asper Biotech) containing amino-labelled ssDNA (7×10^{-7} M; Microsynth, Switzerland) was applied (see Scheme 1). After 1 h, the solution was removed and the chambers were treated for 30 min with ammonia solution (1%; Suprapur, Merck, Germany), then rinsed three times with hot twice-distilled water.



Scheme 1. *Reaction scheme for the coupling of aminated ssDNA to a coverslip coated with 3-aminopropyltrimethoxysilane pretreated with 1,4-phenylene-diisothiocyanate. Oligo = oligonucleotide.*

Annealing (method 1): The primer (10^{-7} M) solvated in the hybridisation buffer for PCR/DIG ELISA (150 μL; Roche, Germany) was added and exposed for 30 min. After removal of the solution, the chambers were washed twice with cold twice-distilled water.

Elongation (method 1): A mixture of Cy5-dCTP (3.3×10^{-7} M; Amersham biosciences Pa55021), dATP, dGTP and dTTP (3.3×10^{-6} M each; MBI Fermentas, Germany) was solvated in Klenow reaction buffer 10x (150 μL; consisting of 0.5 M tris(hydroxymethyl)aminomethane (Tris)/HCl (at pH 7.5), 0.1 M $MgCl_2$, 0.1 mM dithiotreitol, 0.5 mg mL^{-1} bovine serum albumin) and pipetted into the chamber. After 10 min, elongation was started by addition of 1 Unit (defined by supplier) of exonuclease-free Klenow fragment (70057Y, Amersham biosciences) solvated in Klenow reaction buffer (10 μL).

Annealing and elongation in one step (method 2): All reagents (see method 1) were added at once.

Acknowledgements

The authors would like to thank Dr. Cornelia Fritsch, Max-Planck-Institute for Biophysical Chemistry, Göttingen (Germany), for helpful advice concerning biological questions.

Keywords: biosensors · DNA · enzymes · primer elongation · single nucleotide polymorphisms

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Received: February 10, 2004
Early View Article
Published online on November 8, 2004

