

Analysis of DNA-microarrays produced by inverse in situ oligonucleotide synthesis

Markus Beier¹, Jörg D. Hoheisel*

Functional Genome Analysis, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 506, D-69120 Heidelberg, Germany

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Abstract

5'-Phosphoramidites protected by 2-nitrophenylethyl (NPE) and 2-(4-nitrophenyl)ethoxy carbonyl (NPEOC) functions were employed for in situ synthesis of oligonucleotides in 5' → 3' direction on flat glass surfaces. By this inverse synthesis format, the oligonucleotides are attached to the solid support via their 5'-ends while the free 3'-hydroxyl groups are available as substrates for enzymatic reactions such as elongation by polymerases, thereby adding another feature to the portfolio of chip-based applications. Having a fluorescence dye present at the first base during synthesis, the quality of the oligonucleotides was analysed quantitatively by capillary electrophoresis after release from the solid support. With about 95% yield per condensation, it was found to be equivalent to synthesis results achieved on CPG support. The chip-bound oligonucleotides could be extended enzymatically upon hybridisation of a DNA-template. Surprisingly, however, only 63% of the oligonucleotides were elongated in polymerase reactions, while oligonucleotides that were released from the support behaved normally in standard PCR amplifications. This rate of 63% nevertheless compares favourably with an extension rate of only 50%, which was achieved under identical conditions, if pre-fabricated oligonucleotides of identical sequence had been spotted to the glass support. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

DNA-microarrays made of oligonucleotide probes have become an essential tool for functional analyses in biology and a diagnostic assay

system of ever increasing importance for a wide range of bio-technical and bio-medical applications (The Chipping Forecast, 1999; Ferea and Brown, 1999). Such chips are produced either by the deposition of pre-fabricated oligonucleotides (e.g. Proudnikov et al., 1998) or an in situ synthesis directly on the solid support. For the latter approach, several means exist for the direction of synthesis. The currently still best known is photolithographic control based on light-sensitive protection groups (Fodor et al., 1993; McGill et

* Corresponding author. Tel.: +49-6221-424680; fax: +49-6221-424682.

E-mail addresses: markus.beier@febit.de (M. Beier), j.hoheisel@dkfz-heidelberg.de (J.D. Hoheisel).

¹ Present address: FeBIT Ferrarius Biotechnology GmbH, Käfertalerstraße 190, D-68167 Mannheim, Germany.

al., 1997; Beier and Hoheisel, 2000). However, arrays of high complexity can also be manufactured by other, non light-directed means such as printing technologies (Ermanntraut et al., 1997; Morozov and Morozova, 1999) and electrochemical patterning (Southern, 1996; Sosnowski et al., 1997), for example. Usually, synthesis takes place in 3' → 5' direction, thus producing oligonucleotides that are attached to the support via their 3'-terminus. Therefore, only the 5'-ends of the oligonucleotides are accessible for subsequent enzymatic reactions.

For several applications of DNA-microarrays—already established as well as new ones—it is advantageous or even essential, however, to have the 3'-ends available for enzymatic reactions. Detection of polymorphisms, for example, can be based on the ability of a polymerase to extend an oligonucleotide probe upon annealing of a complementary DNA-fragment (Dubiley et al., 1999; Hacia and Collins, 1999). Typically, this methodology has a high specificity, since numerous polymerases exhibit a strong dependency of their incorporation activity on the accuracy of the base pairing (Hirschhorn et al., 2000; LaForge et al., 2000; Pastinen et al., 2000). Other applications are the creation of microarrays containing double-stranded DNA probes (Bulyk et al., 1999) and approaches to highly parallel DNA-sequencing (Ronaghi et al., 1998). We also intend to use such arrays for the analysis of differential splicing events, copying all RNA-molecules of a given cell type to a solid support by using gene-specific, chip-bound oligonucleotides as primer molecules.

Currently, microarrays with 5'-attached oligonucleotides are mainly produced by spotting pre-fabricated oligonucleotides. Alternatively, Kwiatkowski et al. (1999) published a technique by which in situ synthesised oligonucleotides were inverted on the chip subsequent to their synthesis, thereby yielding the free 3'-ends. Direct synthesis in 5' → 3' direction was mentioned by Case-Green et al. (1999) but no quantification of yields or any other quality measures were given. Since a direct in situ synthesis offers the highest degree of flexibility in array design and involves the least num-

ber of processing steps, we opted for the last procedure using 5'-phosphoramidites whose nucleobases were protected by functional groups known to perform well in 3' → 5' synthesis on flat glass surfaces (Weiler and Hoheisel, 1996).

2. Materials and methods

All chemicals and solvents were purchased from Fluka (Germany) or SDS (France), unless stated otherwise, and used without additional purification. DNA-grade acetonitrile was from Proligo (Germany). 3'-*O*-DMTr-deoxynucleotide 5'-*O*-phosphoramidites protected by 2-nitrophenylethyl (NPE) and 2-(4-nitrophenyl)ethoxycarbonyl (NPEOC) functions (Fig. 1) were from Chemogen (Germany). N⁴-fluoresceine labelled succinoyl deoxycytidine was a kind gift from W. Pfeleiderer (University of Constance).

2.1. Support

DNA-array synthesis was performed either on microscopic glass slides (Menzel Gläser; Germany) or polypropylene material derivatised as reported in detail earlier (Beier and Hoheisel, 1999). For the purpose of checking the quality of the oligomers synthesised on the arrays, the first nucleotide—coupled to the support by a cleavable succinate linkage—was labelled with fluoresceine. Loading of the slides was performed by agitating the derivatised support twice for 2 h in a solution made of 0.13 mM fluoresceine-labelled succinate, 0.45 mM *N*-methylmorpholine and 0.51 mM *O*-[(ethoxycarbonyl)cyanomethylenamino]-*N,N,N',N'*-tetramethyluronium tetrafluoroborate (TOTU) in dichloroethane/acetonitrile (2:1). Subsequently, the support was incubated with a 1:1 mixture of capping solutions A and B of PE-Biosystems (USA) for 30 min and washed with dimethylformamide (DMF), methanol, acetone and diethylether. For the parallel synthesis in columns, CPG beads (500 Å, CPG Inc., USA) were used; they were derivatised as reported by Stengle and Pfeleiderer (1990).

2.2. DNA synthesis

On-chip DNA synthesis was controlled by an Eppendorf D200 DNA-synthesiser and directed by a synthesis chamber described earlier (Weiler and Hoheisel, 1996). Phosphoramidite coupling took place for 3 min using a 50 mM solution of the respective phosphoramidite in acetonitrile; 0.5 M pyridine hydrochloride in acetonitrile was used as the activator of choice. After completed synthesis, the NPE/NPEOC groups—and concomitantly also the phosphate protecting groups—were removed by immersion of the solid support overnight in 1 M 1,8-diazabicyclo-(5.4.0)-undec-7-ene (DBU) in acetonitrile. For the release of succinoyl-linked oligomers from the support, the DBU treatment was followed by a 1 h incubation in concentrated

aqueous ammonia at ambient temperature. The released oligonucleotides were lyophilised and used without further purification.

2.3. Hybridisation

Hybridisations to the oligonucleotide-arrays were carried out with 200 nM target-DNA labelled with either Cy3 or Cy5 (Ark Scientific, Germany) in 600 mM NaCl, 60 mM Na citrate, 7.2% (v/v) Na sarcosyl (SSARC buffer). Typically, 50 μ l of the hybridisation solution were applied to a microscope slide and spread evenly by a coverslip. The hybridisation temperature was 15 °C for 10–15-mer molecules, and 20 °C for longer fragments. Subsequently, the slides were briefly washed with cold SSARC buffer and ethanol to remove remain-

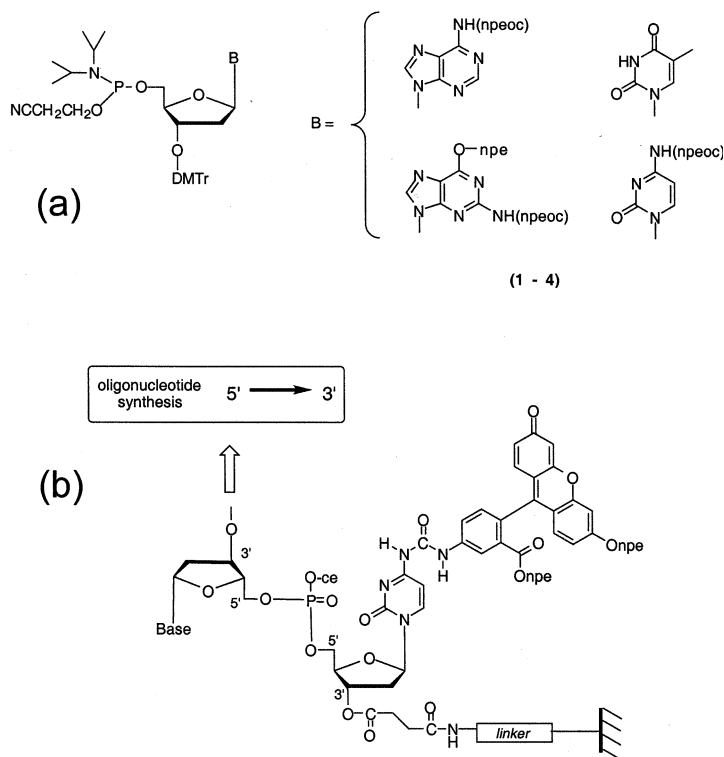


Fig. 1. Schematic presentation of the nucleoside derivatives used. In (a) the 3'-O-DMTr-5'-O-phosphoramidite monomers are shown which have their exocyclic amino groups protected by NPE/NPEOC groups. For a quantitative control of synthesis yield, a linker-bound but cleavable and fluoresceine-labelled dC unit (b) was used; upon cleavage from the support, it remained attached to the 5'-end of the released oligonucleotides.

ing salt residues and finally dried under a stream of nitrogen. Fluorescence detection was performed on a ScanArray 3000 system (GSI Lumonics, USA). Quantification of signals was done with the IMAGENE software package (BioDiscovery, Canada). Subsequently, the target-DNA was stripped off the chip by two incubations of 30 s at 95 °C in 2.5 mM Na₂HPO₄, 0.1% (v/v) SDS.

2.4. Analysis by capillary electrophoresis

Individual, fluoresceine-labelled synthesis products were analysed on a PACE-5000 capillary electrophoresis system equipped with laser-induced fluorescence detection (Beckman, USA). Electrophoretic separation occurred in an ss-DNA-100 capillary (injection at 10 kV for 40 s, separation at 20 kV) using the replaceable ss-DNA-100R gel matrix (Beckman, USA) and ready made Tris–borate/urea buffer from the same supplier.

2.5. On-chip primer extension

Template-DNA was denatured at 95 °C for 5 min and immediately chilled on ice. Hybridisation to the glass slide took place in EasiSeal reaction chambers (Hybaid, Germany) of 25 µl volume, which were mounted directly on the slide and filled with a buffer of 16.5 mM Tris–HCl, pH 7.5, 12.5 mM MgCl₂, 12.5 mM DTT and 0.4 mM of each deoxynucleotide triphosphate. After 10 min, either 1 U µl⁻¹ Klenow fragment of DNA polymerase I (New England Biolabs, Germany) or 0.4 U µl⁻¹ *Taq* polymerase was added for primer extension. Incubation was overnight at 37 or at 55 °C, respectively, placing the slide in a custom-built PCR machine for temperature control. Subsequently, the arrays were washed with water and the template was completely removed by a 30 s treatment with 2.5 mM Na₂HPO₄, 0.1% (v/v) SDS at 95 °C. Successful extension of the chip-bound oligomers was detected by hybridising oligonucleotides known to be complementary to the newly synthesised 3'-end sequences and thus specifically binding to the elongated molecules only.

3. Results

3.1. Inverse in situ synthesis

For the in situ synthesis of high-quality oligonucleotides in 5' → 3' direction, 5'-*O*-phosphoramidites with the base-protecting groups 2-(nitrophenylethyl) (NPE) and 2-(4-nitrophenyl)-ethoxycarbonyl (NPEOC) (Stengele and Pfeleiderer, 1990) were used (Fig. 1). Such synthesis done in the standard 3' → 5' direction had yielded on arrays a stepwise coupling efficiency of more than 98% (Weiler and Hoheisel, 1996) much outperforming other monomers. Prior to in situ synthesis, the glass support was derivatised with a succinate-linker to which a fluorescently labelled dC unit was attached (Fig. 1). Oligonucleotide synthesis was done by mounting the slide in a synthesis chamber, which was based on a design by Maskos and Southern (1993). DNA synthesis was performed as described (Weiler and Hoheisel, 1996) but for the use of pyridine hydrochloride as activator instead of tetrazole. Depending on the channel size, approximately 100–300 µl of 50 mM phosphoramidite solution were applied for each coupling step so that the coupling reagents were in great excess over the growing nucleotide chain on the glass surface.

3.2. Oligomer quality

Subsequent to synthesis, the oligonucleotides were deprotected by a DBU-treatment. For an assessment of quality, some oligomers were released by incubation in concentrated aqueous ammonia, lyophilised and taken up in water. The fluorescently labelled dC unit remained attached to the 5'-end of the released oligonucleotides. Therefore, separation by capillary electrophoresis and laser-induced fluorophore detection produced a band pattern that instantly indicated the molar relationship between full-length molecules and shorter derivatives. In Fig. 2, a typical example is shown for a 20-mer sequence, compared to the synthesis result obtained with the same chemistry on CPG support. The 5'-fluorescence label proved critical because of the high sensitivity and con-

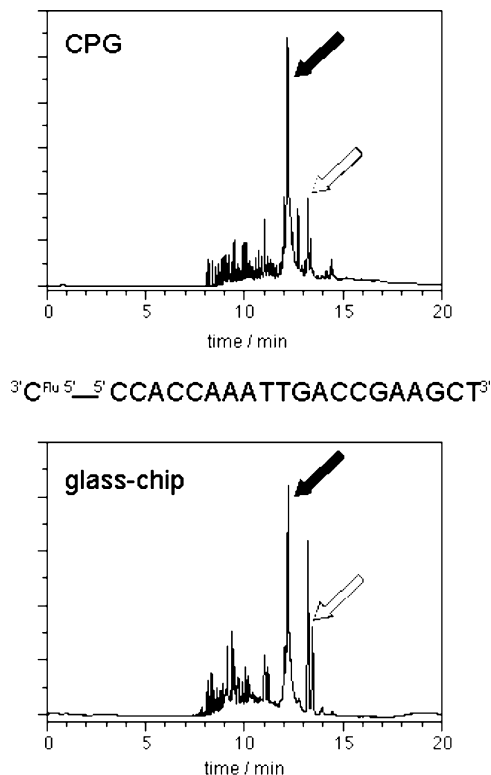


Fig. 2. Assessment of synthesis quality. The depicted 20-mer sequence was synthesised both on CPG support and the surface of a microscopic glass-slide. Subsequent to release from the respective support, the unpurified oligomers were separated by capillary electrophoresis and quantitatively detected via the 5'-attached fluorophore. The black arrows indicate the peaks of the full-length molecules; the white arrows highlight the peaks produced by remains of the succinate linker.

comitant high accuracy of detection; in addition, the recorded signal intensities were directly proportional to the actual amounts of the individual oligonucleotide derivatives, as opposed to detection by UV-light absorption. From a quantitative analysis of the peak sizes, a stepwise synthesis yield of 95% could be determined both for synthesis on CPG and flat glass support. This number could be confirmed for the CPG synthesis by a quantitative trityl assay. While this result may seem unsurprising, since the protection groups were known to work well with 3'-phosphoramidites, such a result could not have been taken for granted because of the nature of the flat glass

support. Syntheses with other monomers exhibited a sharp drop in yield, if synthesis took place on glass slides rather than CPG support (data not shown).

Apart from the signals of the oligomer molecules, two additional peaks eluted from the capillary electrophoresis between minutes 13 and 14 (Fig. 2). They originate from the remains of the succinate linker. Comparing material derived from glass-chips or CPG support, there was more of this signal relative to the amount of oligonucleotide in the analysis of the glass-slide material. On the glass-slide only part of the surface was actually used for oligonucleotide synthesis but the linker molecules were released by the ammonia treatment also from areas untouched by synthesis. The succinate peaks indicate the actual loading of the glass-slide with oligonucleotide. By comparison of the peak sizes to those obtained from slides on which no oligomer synthesis had been performed prior to the ammonia treatment, it could be concluded that nearly all linker molecules were extended during the oligonucleotide synthesis process.

3.3. On-chip primer extension

Oligonucleotides that were identical in sequence to commercially available yeast-specific primers were synthesised on glass-slides. Released from the support, they were used in standard PCR amplifications next to the respective primer from a commercial source. In all cases, no difference in performance could be observed.

For on-chip elongation experiments, primers were synthesised in a strip format as depicted in Fig. 3. Control hybridisations under stringent conditions with fluorescently labelled DNA fragments of either complementary or non-complementary sequence resulted in hybridisation of the specific molecules only (data not presented). Subsequent to the removal of this control DNA, the glass slides were incubated with unlabelled template-DNA in two reactions chambers that were mounted at either end of the synthesis zone and filled with a mixture of nucleotide triphosphates at appropriate buffer conditions. After a 10 min period, the polymerase was added, followed by an

overnight incubation. Subsequently, any DNA duplex was denatured at high temperature, thereby washing off the annealed template-DNA while the covalently attached primer molecules could not be removed by this treatment. Primer extension was tested for by simultaneously hybridising to the entire glass slide surface a Cy5-labelled oligonucleotide complementary to the

oligonucleotide sequence originally synthesised on the chip and a Cy3-labelled molecule identical in sequence to the 5'-end of the template DNA. The latter could only bind upon extension of the chip-bound oligomers during the polymerase reaction (Fig. 3). In absence of any DNA-template during the polymerase reaction or upon addition of a fragment that could not hybridise to the oligonu-

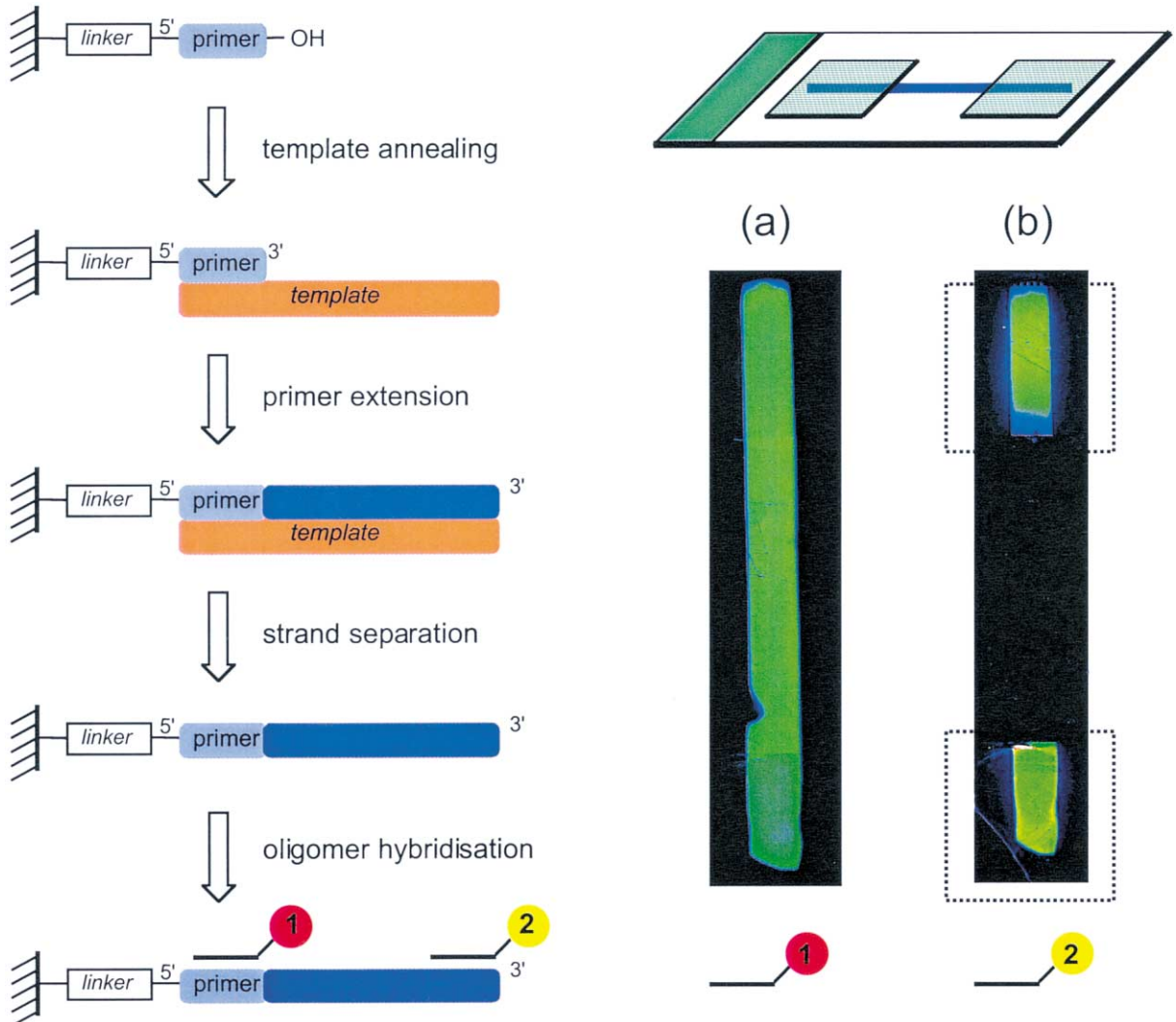


Fig. 3. On-chip polymerase reaction. Left, the principle of primer extension is shown. The oligonucleotide $d(T)_9$ was synthesised in a channel of 3 cm length. Upon annealing of a template, primer extension was carried out in small reaction chambers mounted at either end of the oligonucleotide stripe. Upon removal of the template strand, the entire slide was simultaneously hybridised with a pair of oligonucleotide probes complementary to the primer itself and to the extended molecule, respectively. The signal in panel (a) was obtained with the Cy5-labelled oligomer $d(A)_{16}$; in (b) the signal of the Cy3-labelled oligonucleotide $d(CTATAGT-GAGTCGTATTA)$ is shown that was identical in sequence to the very 5'-end of the DNA-template.

cleotide primers, no such signal could be observed.

The proportion of extended primer could be calculated from a comparison of the signal intensities obtained with the two oligomer probes. This assay was also carried out on slides onto which pre-fabricated, 5'-amino-linked oligonucleotides of identical sequence had been spotted (Beier and Hoheisel, 1999). At a primer loading that produced identical signals upon hybridisation with a complementary probe, 63% of the in situ synthesised oligonucleotides were extended by polymerases while only a rate of 50% was achieved on the spotted molecules. There is no obvious explanation why molecules of identical sequence and at conditions, under which they display the same affinity to hybridisation targets, exhibit this difference in accessibility to an enzymatic reaction. An effect of shorter oligonucleotide derivatives can be largely ruled out. Spotted and in situ synthesised oligomers were of very similar quality, as determined by capillary electrophoresis. Also, the two Cy3- and Cy5-labelled probes were hybridised simultaneously only after the extension reaction. If shorter derivatives would have added to the eventual total, they should also contribute to the primer-specific signal. Possibly, the chemicals involved in the in situ synthesis procedure reduce the eventual interaction of oligonucleotides and the surface compared to spotted material. Alternatively, part of the spotted molecules could be attached via their 3'-hydroxyl group rather than the 5'-amino, although the latter had been found to be much more reactive under the conditions used (Beier and Hoheisel, 1999).

4. Discussion

The availability of DNA-microarrays containing oligonucleotides with a free 3'-terminus opens up a variety of potential applications not possible without. Using NPE/NPEOC-protected phosphoramidites, the yield per condensation during in situ synthesis in 5' → 3' direction was about 95%. By virtue of the reversed orientation, the 5'-ends of all molecules are well defined, irrespective of the eventual length of an oligonucleotide. For

applications that do not rely on an identical length of all molecules of an oligonucleotide population, such as the preparation of double-strand DNA on microarrays by on-chip PCR or the reverse transcription of RNA molecules using gene-specific primers attached to the array, even long oligomer primers can be utilised. For the determination of polymorphisms, the yield limits the applicability with increasing oligonucleotide length. However, such assays can be performed with primers as short as 12-mers, in which case still more than 50% of the molecules are full-length. With the discrimination power of specific base incorporation versus background being well beyond 10-fold if not 100-fold (Hirschhorn et al., 2000; LaForge et al., 2000; Pastinen et al., 2000), with the background signal being split between the four bases, if a mixture of nucleotides is used simultaneously, and in consideration of the fact that most truncated oligonucleotides have a significantly reduced duplex stability, the overall stringency should be sufficient for most such analyses.

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