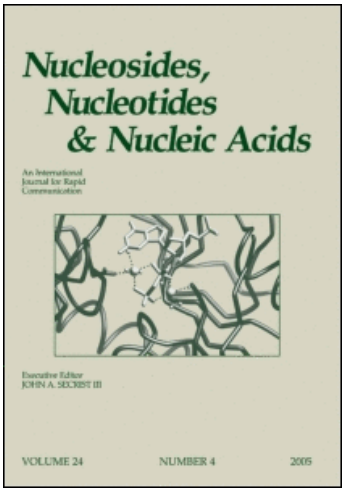

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2′-MeO-RNA CONTAINING OLIGODEOXYRIBONUCLEOTIDE PRIMERS CAN PREVENT TEMPLATE-INDEPENDENT BASE EXTENSION ON MICROARRAYS

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□ *DNA microarrays require tens of thousands of deoxyoligonucleotides to be registered in an addressable fashion through immobilization, so that they have the high-throughput capability of analyzing a large number of samples simultaneously in a minimal volume of each reagent. However, using immobilized DNA molecules on microarrays can impose certain technical problems for some assays. For example, high background noise has been observed in using immobilized oligonucleotide microarrays (DNA chip) for primer extension reactions. This noise may be associated with the reactions of secondary structures formed by the adjacent primers physically constrained on the surface. Single-base extension (SBE) of arrayed primers on a chip has been extensively used in mini-sequencing to examine single nucleotide polymorphisms (SNP). Some primers appeared to be extendable in the absence of any template and thus competed against the base extension directed by the assay target such as genomic DNA. In this article, a method is reported that is capable of reducing template-independent extension by the substitution of a 2′-methoxyribonucleotide in the otherwise oligodeoxyribonucleotide primer. The surrogate compound placed at the 5′-end of the putative secondary structure sequence of a given primer was able to inhibit template-independent extension and to improve data quality of surface-attached primer extension assays.*

Keywords Microarray; Genotyping; Primer extension; SNP

INTRODUCTION

Single base extension (SBE), or the mini-sequencing strategy,[1–5] has been adopted as an approach to query the nucleotide content at a particular locus, specifically in the field of genomic analysis. Single nucleotide polymorphisms (SNPs) are single point variations in genomic DNA and the most prevalent polymorphic markers for genetic studies of polygenic traits.[6,7]

Dedicated to the memory of John A. Montgomery.

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They have the potential to advance mapping and diagnosing of human disease genes. SBE has been developed into a high-throughput process through the use of multiplexing that allows multiple SNP sites to be queried simultaneously. Moreover, tag capture methods have been employed, which extend the use of chips by permitting primers used in SBE to be coupled with specific tags to be captured on the surface at known positions of addressable arrays.[4,5] This method allows multiplexed primer extension in solution, followed by solid-phase capture and readout. Each of these methods relies on the fidelity, efficiency, and accuracy of the primer extension reaction.

Difficulties have been observed during the analysis of this information because of background noise obscuring the signal. For example, the magnitude of noise relative to the expected signal should be low in order to accurately determine the identity of a polymorphic site. Noise in such a primer extension assay is often sequence-specific and primarily results from template-independent primer extension, particularly in platforms employing primers directly immobilized in high density on chips.

This off-template noise can be explained by the extension of the primers associated with secondary structures at the 3′-terminus, where a specific single base is extended in error. For example, a primer containing a palindrome segment can loop back on itself and may act as its own template, or two primers may interact sufficiently well so that one is capable of serving as template for the other (Figure 1). This arrangement results in spurious signal generation since the primer extension could occur regardless of a target nucleic acid, which would substantially confound the template-dependent signal.

Attempts have been made to reduce or eliminate unwanted primer interactions by conducting hybridization at elevated temperatures, in order to disrupt the interactions between the closely constrained primers on the array surface. However, elevated temperature may not be sufficient to overcome the anticipated primer/primer interaction.[8] The method that is described in this article (9) can reduce or even eliminate template-independent extension by modifying the oligodeoxyribonucleotide primer with

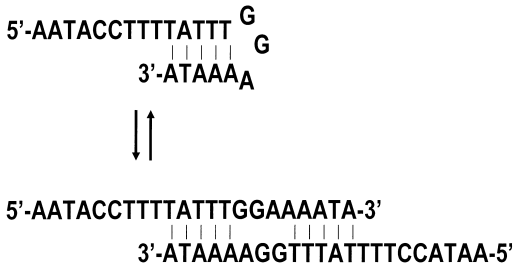


FIGURE 1 A surface-attached primer with internal complementary sequence may be in equilibrium of hairpin and primer dimer secondary structures.

an exogenous 2′-methoxyribonucleotide (2′-MeO-RNA). It is demonstrated that SBE on this modified primer was able to produce genuine signals without the interfering false positive noise.

MATERIALS AND METHODS

Oligonucleotides were purchased from Operon Inc. (Alameda, Calif.) as salt free, but not HPLC purified products. They have been examined with molecular mass (ESI) and capillary electrophoresis (CE) to satisfy quality requirements in the microarray study. Tetramethylrhodamine (TAMRA)-labeled dideoxyribonucleotide triphosphates were purchased from NEN (Boston, MA). Klenow fragment of *E. coli* DNA polymerase I was purchased from New England BioLabs (Beverly, Mass.).

Arraying 5′-Disulfide-modified Oligonucleotides to Mercaptosilane-coated Slides

Mercaptopropyltrimethoxysilane coated microscope slides (25 × 75 mm, VWR) were prepared as previously described.[10] The oligonucleotides were dissolved in arraying buffer (500 mM Na_2CO_3, 0.02% SDS, pH 9.6) at 20 μM. Primers were printed at 20 μM on the slides with a Cartesian arrayer (Raleigh, N.C.). Each sample in each row was printed as quadruplicates. In this study, primers **1–8** were designed to have palindrome segments at the 3′-terminus. Primers **9** and **10** are actual SNP primers.

1. 5′-CTATGACTCTTAGGCC-3′
2. 5′-CTATGACTCTTAGGCC-3′
3. 5′-CTATGACTCTTAGGCC-3′
4. 5′-CTATGACTCTTAGCC-3′
5. 5′-CTATGACTCTTAGGTACC-3′
6. 5′-CTATGACTCTTAGGTACC-3′
7. 5′-CTATGACTCTTAGGTACC-3′
8. 5′-CTATGACTCTTAGGTACC-3′
9. 5′-AATACCTTTTATTTGGAAAATA-3′
10. 5′-AATACCTTTTATTTGGAAAATA-3′

The 2′-methoxyribonucleotide surrogate is illustrated by the underlined base shown in each oligonucleotide primer sequence.

The oligonucleotide templates of the primers are

11. 5′-TGATTACGGCCTAAGAGT-3′ (for primers **1–4**)
12. 5′-TGATTACGGTACCTAAGAGT-3′ (for primers **5–8**)
13. 5′-TAGCCTATTTTCCAAATAAAAGGT-3′ (for primers **9** and **10**)

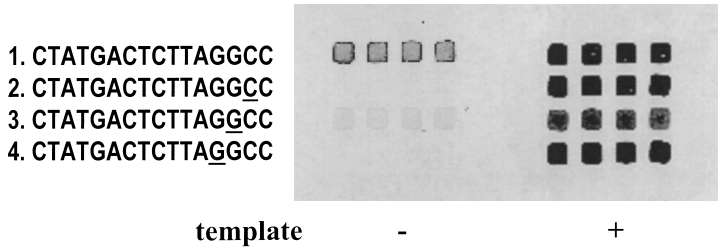
Primer Extension on Chips

Reactions were performed in wells separated with a vinyl gasket on the top of the glass slide (chip). The volume of the reaction mixture in each well was 10 μL of 1 $\times$ Klenow fragment buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol) containing 5 units of Klenow fragment of *E. coli* polymerase I and 10 nM TAMRA-ddNTP. The primer extension assays were carried out at 37°C for 20 min in (a) the presence of 1 μM of template and TAMRA-ddGTP, or (b) in the absence of template but with TAMRA-ddUTP. The slides were then washed with a washing buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween). The fluorescence images of TAMRA labeled spots were obtained on a GSI LUMONICS scanner (Billerica, Mass.). The excitation wavelength of the Green HeNe laser was at 543.5 nm. The fluorescence intensity was processed with Image-Pro Plus software and calculated with Microsoft Excel.

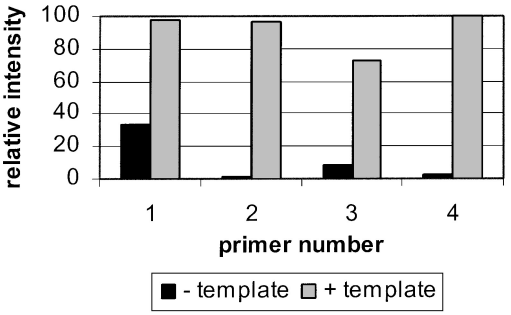
RESULTS AND DISCUSSION

Several methods have been previously described that attempt to reduce the noise associated with template-independent extension. These include inserting the abasic C_3 linker O-$CH_2CH_2CH_2$- in the problematic primer,[11] probing the SNP from the opposite strand,[12] or performing the extension reaction at elevated temperature with thermal stable enzymes.[3] However, none of these approaches offers a satisfactory solution to the problem. Different from the background noise in general, this noise is sequence-specific and is more detrimental to the determination of SNPs, because the false positive signal misleads the anticipated genotype. The noise may result from either primer extension of a hairpin structure or a more probable dimer structure of one primer interacting/annealing to another primer at the 3′-terminus. The method described herein is to use an unnatural nucleotide substrate of DNA polymerase as a surrogate nucleotide to block or substantially inhibit the unwanted off-template extension of a primer in the reaction. The ultimate purpose is to make it more difficult for the DNA polymerase to extend the primer, given that the primer is held in its secondary structure. Therefore, the resulting signal in the primer extension assay will simply represent an authentic interrogation of a specific SNP of interest.

To test the effectiveness of this method for reducing unwanted template-independent primer extension, a set of oligonucleotides was designed to contain a palindromic segment at the 3′-terminus, for which template-independent noise has been well documented in SNP assays. Primer **1**, 5′-CTATGACTCTTAGGCC-3′, has a palindrome GGCC at the 3′-terminus (Figure 2A). A palindrome, as small as four base pairs in this primer, begins to exhibit off-template extension signal. The small size of the palindrome



(A)



(B)

FIGURE 2 (A) Primers **1–4** were extended with TAMRA-ddTTP in the absence of a template and were extended with TAMRA-ddGTP in the presence of a template. The replacement of 2′-MeO-RNA is illustrated by the underlined base shown in each sequence. (B) Histogram of the relative TAMRA intensities derived from the results of template-independent (black) and -directed (gray) extension of primers **1–4**.

suggests that the four-base-long segment may not be able to form an otherwise highly strained hairpin structure, but may instead form a partially annealed dimer structure. In the absence of any template, the secondary structure facilitated the DNA polymerase to extend a single base T at the 3′-terminus of the primer to base pair with A on the other template-serving primer (Figure 3). In contrast, the template directs the primer to extend a single base G at the 3′-terminus, so that the signals of incorporating these two bases clearly distinguishes the source of the extended products of the same primer.

The signal intensity from the template-independent extension of the unmodified deoxyoligonucleotide primer **1** was as high as 35% of that observed for the template-directed extension (Fig. 2B). With replacement of a 2′-MeO-RNA at the palindrome segment in chimeric primers **2**, **3**, and **4**, the template-independent extension was substantially reduced. However, the modification did not impair the template-directed extension. By scanning the position of the 2′-MeO-RNA in the region of the palindrome, it was

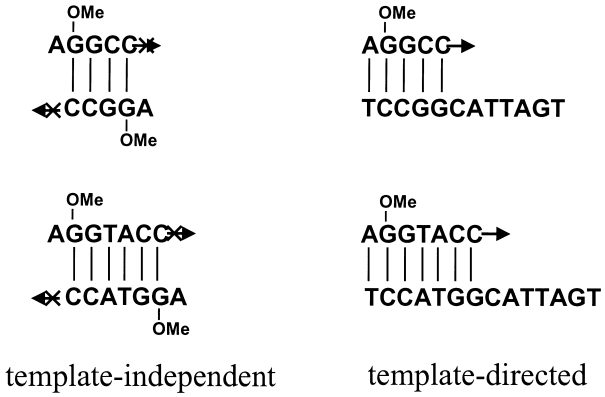
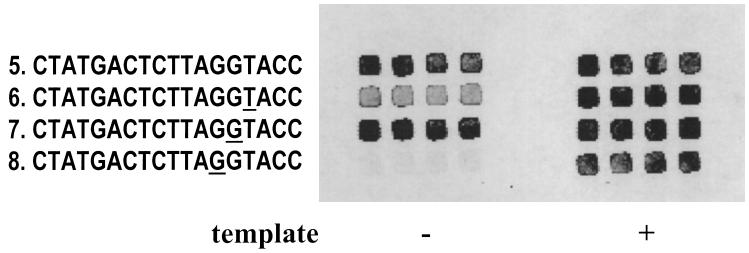


FIGURE 3 Replacement with 2′-MeO-RNA at the 5′-end of the palindrome makes primer **4** (top) and **8** (bottom) more difficult along the modified primer than on external template.

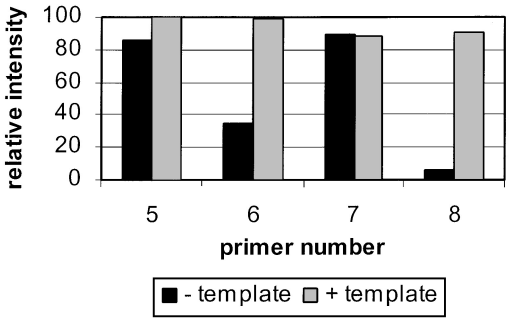
discovered that the position rather than the distance of the surrogate nucleotide in the palindrome from the 3′-terminus of the primer was the most important factor in blocking DNA polymerase from extending the primers based on their secondary structures. The template-independent extension was inhibited to less than 5% of the template-directed extension in primer **4**, which has the 2′-MeO-RNA at the 4th base from the 3′-terminus, but only 15% of the template-directed extension in primer **3**, which has 2′-MeO-RNA at the 3rd base from the 3′-terminus (Figure 2B). The secondary structure revealed that the palindrome in primer **4** has the surrogate right next to the to-be extended base pair while the surrogate in primer **3** is one more base away from the extending terminus. The interference by the surrogate for DNA polymerase to bypass **4** should be greater than **3** given they served as the templates for off-template extension.

The modification becomes ineffective if 2′-MeO-RNA is located more than two bases upstream from the 5′-side of the palindrome (data not shown). The significant reduction of the template-independent extension of primer **2** may primarily be due to the steric hindrance to form a stable palindrome because the 2′-MeO modified nucleotides on both strands are so close to each other near the 3′-terminus[13,14] to form an energetically favorable secondary structure. As a result, template-independent extension was not significant. In practice, the modified nucleotide was usually not placed too closely to the 3′ terminus because it may affect the function of the enzyme for appropriate template-directed extension.

This primer design principle was further demonstrated in a second example. Oligonucleotides **5–8** have a six-base 5′-GGTACC-3′–3′-CCATGG-5′, palindrome at the 3′-terminus (Figure 4A). The additional two base pairs helped to create a more stable palindrome secondary structure than the four-base pairs in primer **1**, and thus increased the template-independ-



(A)



(B)

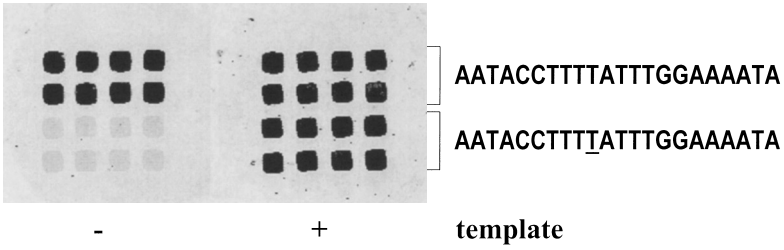
FIGURE 4 (A) Primers **5–8** were extended with TAMRA-ddTTP in the absence of a template and were extended with TAMRA-ddGTP in the presence of a template. The replacement of 2′-MeO-RNA is illustrated by the underlined base shown in each sequence. (B) Histogram of the relative TAMRA intensities derived from the results of template-independent (black) and -directed (gray) extension of primers **5–8**.

ent noise to more than 90% of the template-directed extension signal in primer **5**. As expected, a single substitution of a 2′-MeO-RNA at the 6th nucleotide from the 3′-terminus effectively inhibited template-independent extension of the primer **8** and reduced the noise to less than 10%. Replacement at the 4th or 5th nucleotide in primers **6** and **7** did not as effectively block extension as in **8** (Figure 4B). The reason that primer **6** had less noise than primer **7** could again be attributed to the steric hindrance of forming a more favorable secondary structure since 2′-MeO-modified moieties on both strands were close to each other at the center of the palindrome, which made primer **6** less susceptible to template-independent extension than primer **7**. Hence, the template-independent extension on the problematic primer can be moderated by either interrupting the formation of the plaindrome or blocking the DNA polymerase from bypassing the palindrome secondary structure. The latter approach is more straightforward and effective. It appeared that 2′-MeO-RNA positioned right at the 5′ end of the palindrome was most effective to inhibit template-

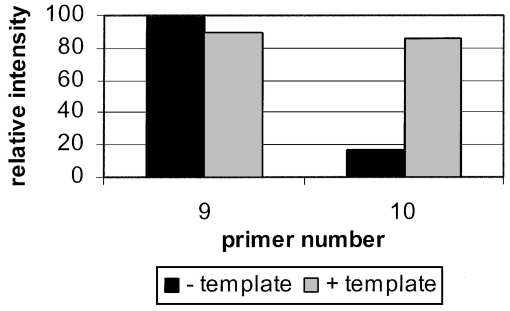
independent extension but had little effect on the desired template-directed extension.

In a third experiment, an actual SNP primer having a strong off-template false positive noise was used to validate this primer design principle (Figure 5A). The primer 5′-AATACCTTTT**T**ATTTGGAAAATA-3′ is in equilibrium of two possible secondary structures as shown in Figure 1. The thymine in bold T at the 5′-end of the palindrome secondary structure was replaced with a 2′-MeO-T (underlined). Template-independent extension on the modified primer 5′-AATACCTTTT<u>T</u>ATTTGGAAAATA-3′ was subsequently reduced to less than 20% of that of the unmodified primer (Figure 5B). The modification was 13 nucleotides away from the 3′-terminus, and did not disturb the template-directed extension.

Of all the illustrated examples, template-independent extension was significantly suppressed by the substitution of a 2′-MeO-RNA at the 5′-end of a palindrome to block DNA polymerase I to bypass the palindrome. It is known that the Klenow fragment of *E. coli* DNA polymerase I does not



(A)



(B)

FIGURE 5 (A) Primers **9** and **10** were extended with TAMRA-ddATP in the absence of a template and were extended with TAMRA-ddGTP in the presence of a template. The replacement of 2′-MeO-RNA is illustrated by the underlined base shown in the sequence. (B) Histogram of the relative TAMRA intensities derived from the results of template-independent (black) and -directed (gray) extension of primers **9** and **10**.

function well on RNA templates.[15] The 2′-methoxy-modified nucleotide surrogate challenges the DNA polymerase to overcome the 2′-methylated RNA on template serving strand as the primer precedes base extension. Substitution of a greater number of nucleotides with the 2′-MeO-RNA counterparts in the vicinity of the 5′-end of the palindrome, was able to further strengthen the inhibition of the DNA polymerase to precede off-template primer extension.[9] The ribose of an RNA is in a different sugar pucker conformation from the 2′-deoxyribose of a DNA. Besides the adverse conformation, the bulky 2′-methoxyl introduced in the minor groove of the palindrome by the surrogate could disrupt the normal function of a highly proficient DNA polymerase.

In conclusion, a method has been developed to inhibit template-independent primer extension on DNA chips. 2′-MeO-RNA was able to stall DNA polymerase I from extending the 3′-terminus when the primer was modified at the 5′-end of the proposed secondary structure. Replacement of 2′-MeO-RNA did not change the base sequence of the primer and is not expected to interrupt hybridization of the primer to any target templates, and therefore has no effect on desired template-directed primer extension. This method can be used as a general strategy for reducing template-independent false signals and may prevent primer-dimer formation in PCR under the similar hypothesis.[16,17]

REFERENCES

1. Syvanen, A.C.; Aalto-Setala, K.; Harju, L.; Kontula, K.; Soderlund, H. A primer-guided nucleotide incorporation assay in the genotyping of apolipoprotein E. Genomics, **1990**, 8, 684–692.
2. Pastinen, T.; Kurg, A.; Metspalu, A.; Peltonen, L.; Syvanen, A.C. Minisequencing: A specific tool for DNA analysis and diagnostics on oligonucleotide arrays. Genome Res. **1997**, 7, 606–614.
3. Tõnisson, N.; Kurg, A.; Lõhmussaar, E.; Metspalu, A. Arrayed primer extension on the DNA chip—Method and applications. In *Microarray Biochip Technology*, Schena, M. (ed.), Biotechniques Books, Eaton Publishing, **2006**, 247–263.
4. Fan, J.B.; Chen, X.; Halushka, M.K.; Berno, A.; Huang, X.; Ryder, T.; Lipshutz, R.J.; Lockhart, D.J.; Chakravarti, A. Parallel genotyping of human SNPs using generic high-density oligonucleotide tag arrays. Genome Res. **2000**, 10, 853–860.
5. Hirschhorn, J.N.; Sklar, P.; Lindblad-Toh, K.; Lim, Y.M.; Ruiz-Gutierrez, M.; Bolk, S.; Langhorst, B.; Schaffner, S.; Winchester, E.; Lander, E.S. SBE-TAGS: An array-based method for efficient single-nucleotide polymorphism genotyping. Proc. Natl. Acad. Sci. USA, **2000**, 97(22), 12164–12169.
6. Sachidanandam, R.; Weissman, D.; Schmidt, S.C.; Kakol, J.M.; Stein, L.D.; Marth, G.; Sherry, S.; Mullikin, J.C.; Mortimore, B.J.; Wiley, D.L.; et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature **2001**, 409, 928–933.
7. Venter, J.C.; Adams, M.D.; Myers, E.W.; Li, P.W.; Mural, R.J.; Sutton, G.G.; Smith, H.O.; Yandell, M.; Evans, C.A.; Holt, R.A.; et al. The sequence of the human genome. Science **2001**, 291, 1304–1351.
8. Fotin, A.V.; Drobyshev, A.L.; Proudnikov, D.Y.; Perov, A.N.; Mirzabekov, A.D. Parallel thermodynamic analysis of duplexes on oligodeoxyribonucleotide microchips. Nucleic Acids Res. **1998**, 26, 1515–1521.
9. Zhao, X.; Gelfand, C.A.; Swenson, R.E. Primer extension using modified nucleotides. U.S. Patent Application Publication, Pub. No.: US2003/0190627 A1, 2003.

10. Rogers, Y.H.; Jiang-Baucom, P.; Huang, Z.J., Bogdanov, V.; Anderson, S.; Boyce-Jacino, M.T. Immobilization of oligonucleotides onto a glass support via disulfide bonds: A method for preparation of DNA microarrays. Anal. Biochem. **1999**, 266, 23–30.
11. Nikiforov, T.T.; Rendle, R.B.; Goelet, P.; Rogers, Y.H.; Kotewicz, M.L.; Anderson, S.; Trainor, G.L.; Knapp, M.R. Genetic Bit Analysis: A solid phase method for typing single nucleotide polymorphisms. Nucleic Acids Res. **1994**, 22, 4167–4175.
12. Pastinen, T.; Raitio, M.; Lindroos, K.; Tainola, P.; Peltonen, L.; Syvanen, A.-C. A system for specific, high-throughput genotyping by allele-specific primer extension on microarrays. Genome Res. **2000**, 10, 1031–1042.
13. Kawai, G.; Yamamoto, Y.; Kamimura, T.; Masegi, T.; Sekine, M.; Hata, T.; Iimori, T.; Watanabe, T.; Miyazawa, T.; Yokoyama S. Conformational rigidity of specific pyrimidine residues in tRNA arises from posttranscriptional modifications that enhance steric interaction between the base and the 2′-hydroxyl group. Biochemistry **1992**, 31, 1040–1046.
14. Lesnik, E.A.; Freier, S.M. What affects the effect of 2′-alkoxy modifications? 1. Stabilization effect of 2′-methoxy substitutions in uniformly modified DNA oligonucleotides. Biochemistry **1998**, 37, 6991–6997.
15. Joyce, C.M.; Steitz, T.A. Function and structure relationships in DNA polymerases. Annu. Rev. Biochem. **1994**, 63, 777–822.
16. Rychlik, W. Selection of primers for polymerase chain reaction. Mol. Biotechnol. **1995**, 3, 129–134.
17. Brownie, J.; Shawcross, S.; Theaker, J.; Whitcombe, D.; Ferrie, R.; Newton, C.; Little, S. The elimination of primer-dimer accumulation in PCR. Nucleic Acids Res. **1997**, 25, 3235–3241.