Chapter 18

Phosphate-Methylated Oligonucleotides as a Novel Primer for PCR and RT-PCR

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Abstract

This chapter introduces neutralized DNA (nDNA) as a novel design for the primers of PCR and RT-PCR by methylating phosphate groups of some oligonucleotides in their structures. It starts with an introduction of the nDNA which possesses an electrically chimeric neutral backbone as well as the proposed standards in designing nDNA as a novel primer for PCR and RT-PCR, concluded from various experimental results presented afterward. The primary content comprises empirical data from PCR to compare nDNA and unmodified DNA as primers in terms of ability to distinguish and amplify mismatch templates, activities of polymerase enzymes, melting temperature of double-stranded sequences, and the trials and discussions on various modified positions of the nDNA primers. In summary, nDNA exhibited outstanding performance as a primer for PCR and RT-PCR, compared to unmodified DNA, and is expected to be expanded in diverse applications which require enhanced specificity.

Key words nDNA, RT-PCR, PCR

Primer design plays the most important role to optimize the stability and specificity of template–primer hybridization in polymerase chain reaction (PCR), an effective technique to amplify the desired sequences for various bioanalysis purposes. There have been various nucleic acid (NA) analogs developed to overcome the limitations from negative charges of NA molecules to increase the sensitivity and specificity of DNA-DNA or DNA-RNA hybridization in vitro as well as their biological and double-stranded stability, which are easily degraded by nucleases in vivo [1]. Locked nucleic acid (LNA), which can improve the stability of the double-stranded structure [2–4], and peptide nucleic acid (PNA), with an electrically neutral backbone [5–7], are both widely exploited in molecular biology experiments or genetic technology. This chapter introduces phosphate-methylated DNA with an electrically chimeric neutral backbone [8] and proposes criteria in designing it as a primer for PCR and RT-PCR.

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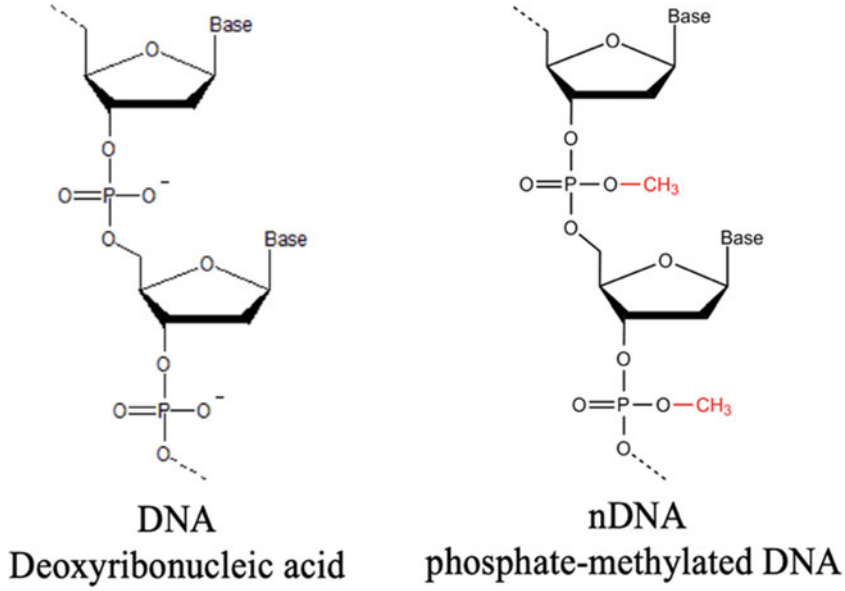


Fig. 1 Schematic diagram of the chemical structure of DNA and phosphate-methylated DNA (nDNA). Base represents one of four different bases: A, T, C, and G

The differences in structures of neutralized DNA and normal DNA are illustrated in Fig. 1. Methylation by CH_3 groups are employed to convert the negatively charged phosphodiester groups into a phosphotriester linkage and electrically neutralized the DNA sequence. This phosphate-methylated DNA consequently becomes neutralized DNA (nDNA) [8–11].

On the one hand, methylation of nDNA can reduce the electrostatic repulsion force between negative charges on the phosphate backbones of normal DNA sequences, which stabilizes the template–primer hybridization, because of neutralized characteristics. Modification of the primers with uncharged nucleotides in this chapter is expected to reduce the electrostatic repulsion between the double strands without scarifying the specificity. On the other hand, since the electrostatic repulsion force between negative charges on the phosphate backbone of the hybridized sequences is also shielded by the positively charged salt ions in the aqueous solution, hybridization with nDNAs possibly produces a stable double-stranded structure in solutions with low salt concentration due to their less negative charges, compared to that of normal DNAs [10, 12]. In addition to that, the presence of methyl groups also let two isomeric structures of Rp and Sp contribute to the hybridization structure and stability between nDNA and its target [13]. The steric hindrance from the methyl groups will be available if the phosphate groups are methylated at high density and the stacking of the bases will be destroyed if the methyl group is inward due to the double-strand formation of the Rp structure of nDNA.

Hence, it is essential to consider the length of the neutralized sequence and the amount of the modified groups in the dsDNA stability perspective. In summary from our studies, the rule of thumb is that the two-nucleotide gap between the neutralized positions and the maximum methylated groups of one-fourth of the oligo length is the most important criteria in designing nDNAs primers [12]. Details are discussed in the following section.

First of all, we discuss the effects of nDNA modifications on the T_m value of dsDNA. The T_m value will significantly decrease if there is a mismatched base pair in hybridization of DNA/nDNA with its complementary strand (Table 1). The created significant difference of T_m value between perfect-matched hybridization and mismatched hybridization allows a wider temperature range for annealing temperature selection for PCR operation and correspondingly advances mismatched/perfect-matched recognition. The double-stranded structure is relatively unstable and favorably untwisted into single-stranded structure because there is no hydrogen bond formed in a couple of bases pairing without complement. The experiments on let-7 (lethal-7 gene, one of the initially discovered miRNAs [14]) were designed with different base mismatches to investigate the T_m and distinguishability of the nDNA on the various let-7 family. It can be inferred from Table 1 that C/T mismatch is the easiest to be discriminated since its ΔT_m (T_m, perfect match $-$ T_m, mismatch) is the biggest value regardless of varied salt concentrations [12].

Since the stability of the sequence during hybridization is affected by the steric hindrance created from methyl groups, it is also crucial to regulate this factor, especially for applications of nDNAs to detect base mismatches. In this system, the stability of the complementary sequences is deteriorated by the base mismatches. Modifying phosphate groups locating close to the mismatch positions, therefore, induces synergistic effect from steric hindrance (by CH_3) and mismatch instability and consequently enhances the ability to determine mismatch sequences [12].

Prior to performing PCR, it is necessary to consider the impact of the modification at the 3′ end of the nDNA primer on the ability of the enzyme reactivity in recognizing and synthesizing the primer–template complex. The region on the DNA template which interacts with the polymerase through the small groove of the duplex structure is reported about six to eight nucleotide length from the 3′ end of the primer [15], and amplification efficiency of the enzyme is also varied with the neutralized positions on the nDNA primer in PCR employing GoTaq and Pfu polymerase. Thus, modifying primer at the positions for low-yield amplification can obstruct the process with sequences containing mismatches and optimize the distinguishability between complementary pairs and mismatched sequences (Figs. 2, 3, and 4) [11]. As a result, combining the nDNA design for T_m and for polymerase's reactivity, the

Table 1
T_m and ΔT_m (T_m, perfect match − T_m, mismatch) values of double-stranded hybridization between n6,9 sequence and DNA at salt concentrations of (a) 1× SSC and (b) 0.1× SSC

(a)				
	nDNA (n6,9) 1× SSC		DNA 1× SSC	
	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
Perfect match	72.1	–	74.7	–
Mismatch 7T	62.9	9.2	66.2	8.5
Mismatch 7C	61.0	11.1	65.1	9.6
Mismatch 7G	64.7	7.3	68.8	5.8
Mismatch 15T	65.8	6.2	70.1	4.6
Mismatch 15A	69.9	2.1	70.5	4.1
Mismatch 15C	66.2	5.9	70.3	4.3
Mismatch 2A	67.2	4.9	70.5	4.2
Mismatch 2T	66.2	5.8	69.9	4.8
Mismatch 2C	67.1	5.0	70.7	4.0
(b)				
	nDNA (n6,9) 0.1× SSC		DNA 0.1× SSC	
	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
Perfect match	63.6	–	65.3	–
Mismatch 7T	51.4	12.2	56.4	8.9
Mismatch 7C	48.2	15.4	53.8	11.4
Mismatch 7G	53.2	10.4	59.3	5.9
Mismatch 15T	57.5	6.1	64.0	1.2
Mismatch 15A	59.6	4.0	60.2	5.0
Mismatch 15C	53.7	9.8	58.6	6.6
Mismatch 2A	59.2	4.4	62.6	2.6
Mismatch 2T	58.0	5.6	61.0	4.2
Mismatch 2C	56.9	6.6	60.4	4.9

specificity of PCR for amplification of target genes can be significantly improved.

In the PCR process, the temperature at which the double helix structure untwisted is not only an indicator for the stability of the DNA double strands but also important to determine the annealing temperature (T_a). In comparison between the melting points of double strands formed by complementary and mismatched templates, since the double helix structures are unstable due to mismatched sequences, the melting point of the primer-mismatched template is lower than that of the primer-complementary template.

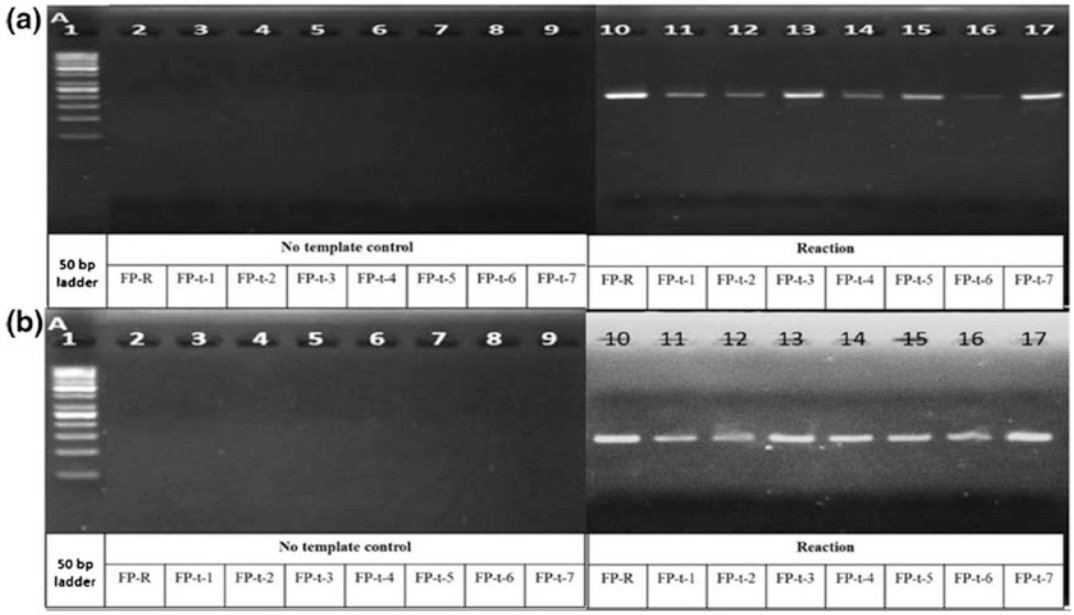
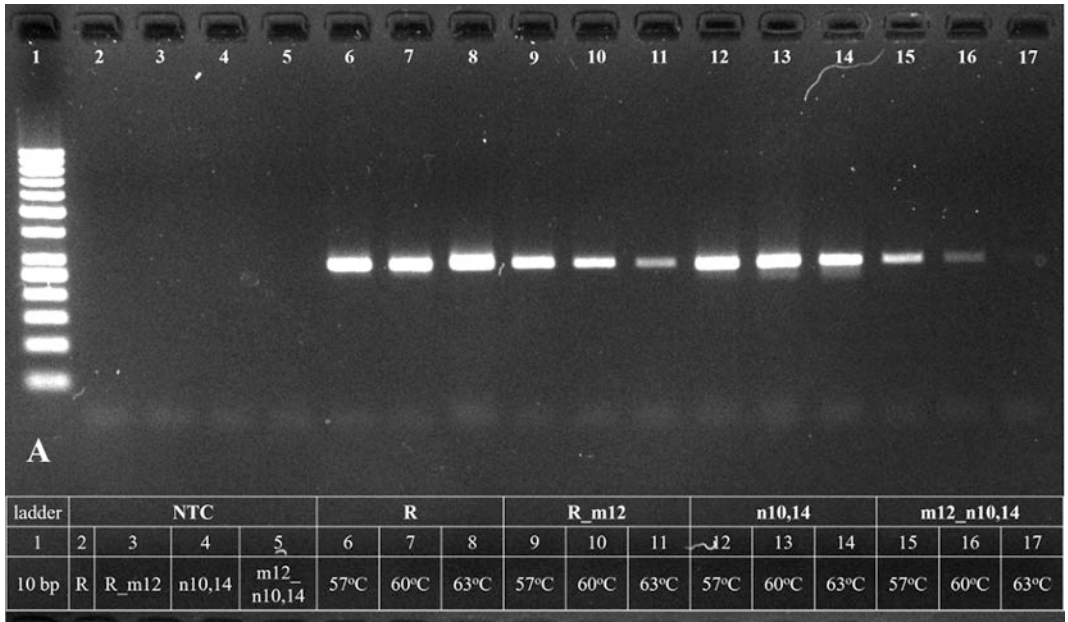


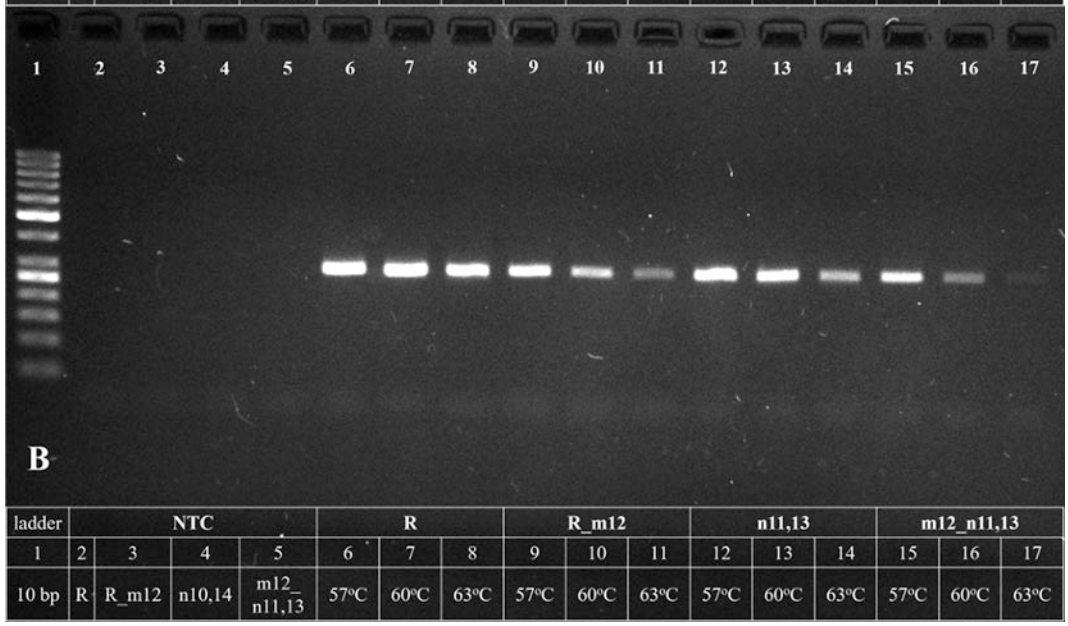
Fig. 2 PCR by (**a**) GoTaq polymerase and (**b**) Pfu DNA polymerase with nDNA modified at different positions as the primer [13]. FP-t-*n* (*n* = 1–7): *n* indicates (*n* + 1) position where the nucleotide was modified, counted from the 5′ end to the 3′ end. (**a**) Lane 1: 50 bp ladder, lane 2–9: products without a template, lane 10–17: amplification of templates, lane 2 and 10: R, lane 3 and 11: t-1, lane 4 and 12: t-2, lane 5 and 13: t-3, lane 6 and 14: t-4, lane 7 and 15: t-5, lane 8 and 16: t-6, lane 9 and 17: t-7. (**b**) lane 1: 50 bp ladder, lane 2–9: products without a template, the order is the same as (**a**) with lane 10–17 [11]

The difference in the melting points is, therefore, exploited to distinguish complementary and mismatched sequences. It is also feasible to regulate the melting point by adjusting T_a to improve the specificity of the primer and the target gene. Thus, paying attention to T_a (the significance of being above or below the melting point) for choosing the appropriate value can obtain the product with the optimal specificity. Low-yield PCR will be obtained if the T_a is higher than the melting point because of difficulty in primer–template hybridization, whereas too low T_a results in nonspecific products due to a large number of base mismatches (Fig. 3).

Figure 5 below illustrates the influence of nDNA primer on the melting point of the double strands. On the one hand, neutralizing the primer with uncharged nucleotides increases the primer-complementary strand hybridization. On the other hand, the impacts of steric hindrances from the methyl groups destabilize the double strands and reduce their melting point. These two features are useful for determining their melting point [11, 12]. The variation in melting points of complementary hybridization is insignificant, whereas modifying the primers with neutralized nucleotides remarkably raises the instability of mismatched hybridization. These data present that, in comparison with unmodified ones, nDNA primers exhibit higher specificity not only to



ladder	NTC				R			R_m12			n10,14			m12_n10,14		
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
10 bp	R	R_m12	n10,14	m12_ n10,14	57°C	60°C	63°C	57°C	60°C	63°C	57°C	60°C	63°C	57°C	60°C	63°C



ladder	NTC				R			R_m12			n11,13			m12_n11,13		
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
10 bp	R	R_m12	n10,14	m12_ n11,13	57°C	60°C	63°C	57°C	60°C	63°C	57°C	60°C	63°C	57°C	60°C	63°C

Fig. 3 PCR of perfect-matched and mismatched DNA/nDNA primers at different binding temperatures. n indicates the nucleotides modified with methyl phosphotriester (MPTE), while the m locates the position of mismatch (all are numbered nucleotide positions from the 5′ end to the 3′ end). The amplification reaction volume was 10 μl, including 0.2 μl of DNA (1.95×10^{-4} ng/μl), 0.3 μl of each primer (10 μM), 2 μl of 5× SYBR Green, 0.8 μl $MgCl_2$ (25 mM), 0.8 μl of dNTP (2.5 mM), 0.05 μl of DNA polymerase (5 U/μl), and 5.55 μl of Nuclease-Free water. PCR reaction was performed at Veriti 96-well Thermal Cycler (Applied Biosystem, Singapore) and consisted of initial activation at 95 °C within 5 min, followed by 30 cycles at 95 °C (30 s), 57–63 °C (30 s), 72 °C (30 s), and eventually held at 72 °C within 5 min [13]

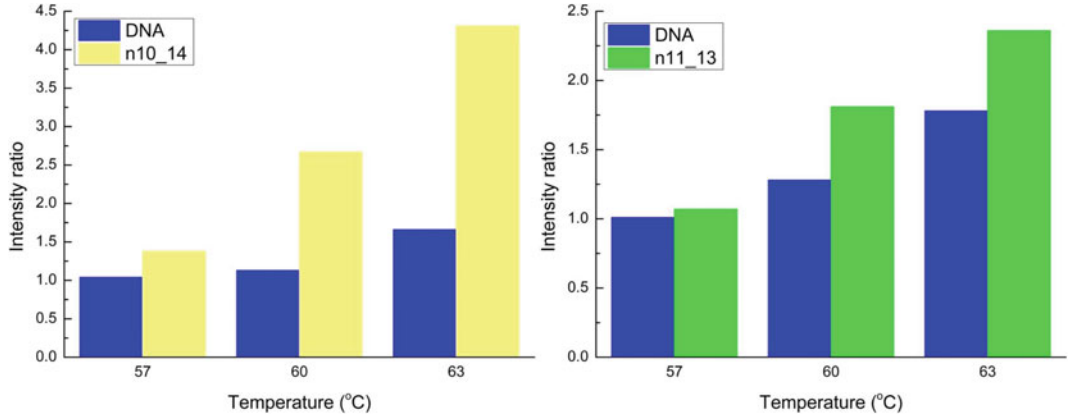


Fig. 4 Comparison of various designed nDNA with DNA as PCR primers at different binding temperatures [13]

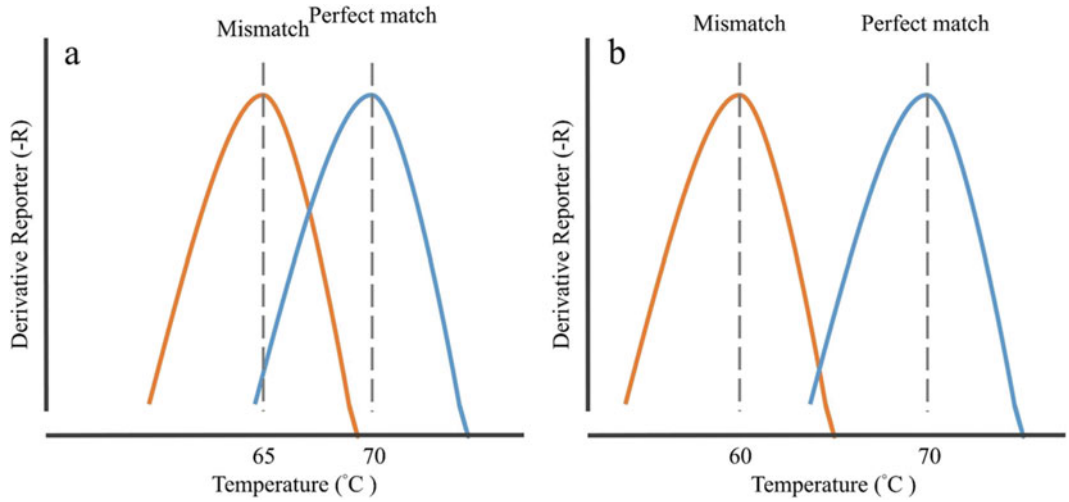


Fig. 5 (**a**) Unmodified DNA and (**b**) schematic diagram of the changes in melting points of the template-nDNA duplex

recognize target genes but also less likely to amplify the mismatched templates and generate more no-specific products at the same binding temperature, because of lower melting points between the modified primers and the mismatched templates.

Another example of using nDNA as primer design is for pUC19 and KRAS gene and the stem-loop primers in reverse transcription PCR used to detect single-nucleotide polymorphism (SNP). The sequence of pUC19, KRAS, and let-7 is listed in Table 2. Specifically, the study of distinguishing let-7a and let-7c is applications of nDNA as primers for RT-PCR. The experiments on pUC19 were designed to investigate the impact of modifying the primers at the 3′ end position on the ability of the polymerase in recognizing and synthesizing primer–template complexes. The empirical results concluded that the neutralized primers were

Table 2
Investigation of nDNA as forward primer for recognition of KRAS gene by complementary and mismatched sequence. T_m and ΔT_m of the designed forward primers [16]

	FP	FP_n3,11	FP_n5,11	FP_n7,11
NTC	48.69	47.49	46.9	46.75
Perfect match	71.73	70.38	69.88	70.18
Mismatch	63.95	62.6	62.01	62.16
ΔT_m	7.78	7.78	7.87	8.02

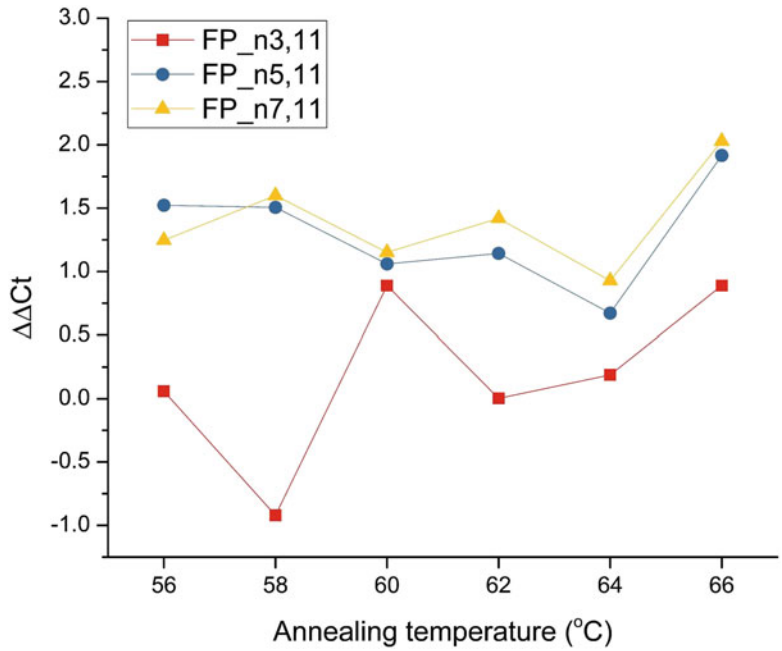


Fig. 6 Investigation of nDNA as forward primer for recognition and amplification of template (complementary and mismatched KRAS gene) by PCR [16]

recognized and amplified by the enzyme, and modifying the primers at the positions producing low-yield amplification can increase the distinguishability between complementary and mismatched duplexes (Figs. 2, 3, and 4) [11].

In order to navigate the modified positions providing the optimal ability in discriminating the complementary sequences from the mismatched ones, the primers used in the figures below were neutralized at the positions on the left and right sides of the mismatch, following the aforementioned rules in designing nDNA primers [16]. Figure 6a reveals the highest ΔΔCt (ΔCt,

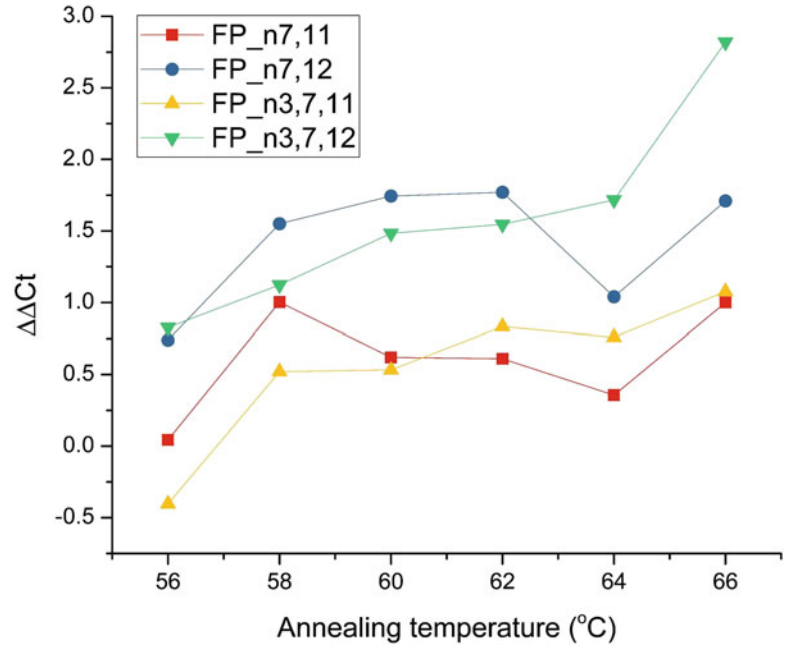


Fig. 7 Amplification by PCR to investigate the ability of recognizing KRAS gene by complementary and mismatched forward primers neutralized at various positions [16]

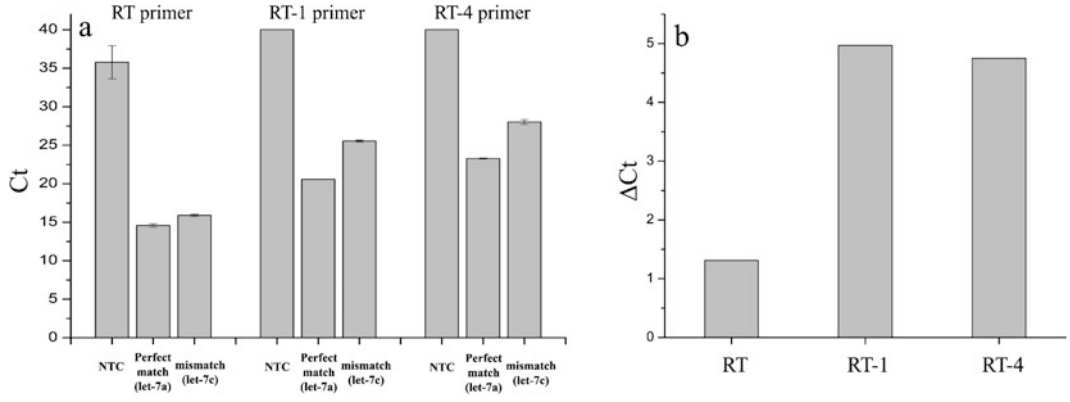


Fig. 8 (**a**) PCR by nDNA modified at different positions on the RT primer. (**b**) Difference in recognition ability of nDNA modified at different positions on RT primer (ΔCt = Ct, mismatch – Ct, perfect match) [16]

nDNA – ΔCt, DNA) of FP_n5,11 and FP_n7,11, indicating the maximal efficiency in distinguishing mismatched and complementary strands of these two types of modified primers. However, the lowest T_m in detecting complementary sequences of FP_n5,11 suggested that the its neutralized positions destabilized the hybridization. Neutralized locations in FP_n7,11 and their spaces, consequently, become optimal to modify nDNA primers. Figure 7

Table 3
List of DNA and nDNA sequences used in the experiment. Lowercases n are nucleotides modified with methyl phosphotriester (MPTE), while the red letters are mismatch [12, 14, 16]

Name	Sequence (5'-3')
Let7	
Target DNA	TGAGGTAGTAGGTTGTATAGTT
Probe DNA	ACAACCTACTACCTCAAA
Mismatch 7T	TGAGGTTGTAGGTTGTATAGTT
Mismatch 7C	TGAGGTCGTAGGTTGTATAGTT
Mismatch 7G	TGAGGTGGTAGGTTGTATAGTT
Mismatch 15T	TGAGGTAGTAGGTTTTATAGTT
Mismatch 15A	TGAGGTAGTAGGTTATATAGTT
Mismatch 15C	TGAGGTAGTAGGTTCTATAGTT
Mismatch 2A	TAAGGTAGTAGGTTGTATAGTT
Mismatch 2T	TTAGGTAGTAGGTTGTATAGTT
Mismatch 2C	TCAGGTAGTAGGTTGTATAGTT
n6,9 probe	ACAACC^{n}TAC^{n}TACCTCAAA
pUC19	
FP_1_R	AGTCCAACCCGGTAAGACAC
FP_1_t-1	AGTCCAACCCGGTAAGACA^{n}C
FP_1_t-2	AGTCCAACCCGGTAAGAC^{n}AC
FP_1_t-3	AGTCCAACCCGGTAAGA^{n}CAC
FP_1_t-4	AGTCCAACCCGGTAAG^{n}ACAC
FP_1_t-5	AGTCCAACCCGGTAA^{n}GACAC
FP_1_t-6	AGTCCAACCCGGTA^{n}AGACAC
FP_1_t-7	AGTCCAACCCGGT^{n}AAGACAC
RP_R	GGGCCTCTTCGCTATTACGC
FP_R	TTAGCTCACTCATTAGGCAC
FP_n10,14	TTAGCTCACT^{n}CATT^{n}AGGCAC
FP_n11,13	TTAGCTCACTC^{n}AT^{n}TAGGCAC
FP_R_m12	TTAGCTCACTCCTTAGGCAC
FP_m12_n10,14	TTAGCTCACT^{n}CCTT^{n}AGGCAC
FP_m12_ n11,13	TTAGCTCACTC^{n}CT^{n}TAGGCAC

KRAS	
FP	GCCACCAGCTCCAACTAC
FP_n3,11	GCC^{n}ACCAGCTC^{n}CAACTAC
FP_n5,11	GCCAC^{n}CAGCTC^{n}CAACTAC
FP_n7,11	GCCACCA^{n}GCTC^{n}CAACTAC
FP_n7,12	GCCACCA^{n}GCTCC^{n}AACTAC
FP_n3,7,11	GCC^{n}ACCA^{n}GCTC^{n}CAACTAC
FP_n3,7,12	GCC^{n}ACCA^{n}GCTCC^{n}AACTAC
RP	GCCTGCTGAAAATGACTGAAT
Let-7	
Let-7a RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC AACTAT
RT-1	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC AACTA^{n}T
RT-4	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC AA^{n}CTAT
FP	GTATACTGAGGTAGTAGGTTG
RP	GTGCAGGGTCCGAGGT

compares the performance of varied nDNA in polymerization, with higher ΔΔCt (FP_n7,11 and FP_n3,7,11) presents better efficiency, verifying the pUC19 experiment mentioned above. Neutralizing the primers at positions which can result in low-yield amplification optimizes the discrimination of the primers to the complementary and mismatched templates.

Although the expression levels of specific miRNAs relate to respective disease and therefore can be employed as biomarkers for disease diagnosis, short chain and high similarity of sequences within a family obstruct the determination [16]. Figure 8 displays the feasibility in amplifying the template as well as considerable improvements in identifying let-7a and let-7c by nDNA designed on the stem-loop primers of the reverse transcription (RT) process [16]. The specificity of RNA recognition is also anticipated to be improved by introducing nDNA as forward primer and adjusting the hybridizing temperature of the PCR processes.

Currently, nDNA can be synthesized with neutralized nucleotides at any position by chemical method, based on the standards discovered in the experiments above, for various PCR systems and/or genetic testing. In addition to applications in primer design, nDNA is expected to be widely exploited for a variety of biological and medical testing platforms to provide enhanced specificity of test results (Table 3).

References

1. Kawane K, Motani K, Nagata S (2014) DNA degradation and its defects. Cold Spring Harb Perspect Biol 6:a016394. https://doi.org/10.1101/cshperspect.a016394
2. Obika S, Nanbu D, Hari Y, Morio K, In Y, Ishida T, Imanishi T (1997) Synthesis of 2′-O,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C_3, -endo sugar puckering. Tetrahedron Lett 38 (50):8735–8738. https://doi.org/10.1016/S0040-4039(97)10322-7
3. Levin JD, Fiala D, Samala MF, Kahn JD, Peterson RJ (2006) Position-dependent effects of locked nucleic acid (LNA) on DNA sequencing and PCR primers. Nucleic Acids Res 34:e142. https://doi.org/10.1093/nar/gkl756
4. Mouritzen P, Nielsen AT, Pfundheller HM, Choleva Y, Kongsbak L, Moller S (2003) Single nucleotide polymorphism genotyping using locked nucleic acid (LNA™). Expert Rev Mol Diagn 3:27–38. https://doi.org/10.1586/14737159.3.1.27
5. Egholm M, Buchardt O, Nielsen PE, Berg RH (1992) Peptide nucleic acids (PNA). Oligonucleotide analogs with an achiral peptide backbone. J Am Chem Soc 114:1895–1897
6. Wang J, Palecek E, Nielsen PE, Rivas G, Cai X, Shiraishi H, Dontha N, Luo D, Farias PAM (1996) Peptide nucleic acid probes for sequence-specific DNA biosensors. J Am Chem Soc 118:7667–7670. https://doi.org/10.1021/ja9608050
7. Ray A, Norden B (2000) Peptide nucleic acid (PNA): its medical and biotechnical applications and promise for the future. FASEB J 14:1041–1060. https://doi.org/10.1096/fasebj.14.9.1041
8. Chen WY, Chen HC, Yang YS, Huang CJ, Chan HWH, Hu WP (2013) Improved DNA detection by utilizing electrically neutral DNA probe in field-effect transistor measurements as evidenced by surface plasmon resonance imaging. Biosens Bioelectron 41:795–801. https://doi.org/10.1016/j.bios.2012.10.010
9. Hu WP, Tsai CC, Yang YS, HWH C, Chen WY (2018) Synergetic improvements of sensitivity and specificity of nanowire field effect transistor gene chip by designing neutralized DNA as probe. Sci Rep 8:12598. https://doi.org/10.1038/s41598-018-30996-4
10. Kuo TC, Wu MW, Lin WC, Matulis D, Yang YS, Li SY, Chen WY (2020) Reduction of interstrand charge repulsion of DNA duplexes by salts and by neutral phosphotriesters—contrary effects for harnessing duplex formation. J Taiwan Ins Chem Eng 110:1–7. https://doi.org/10.1016/j.jtice.2020.02.023
11. Li TL, Wu MW, Lin WC, Lai CH, Chang YH, Su LJ, Chen WY (2019) Designed phosphate-methylated oligonucleotides as PCR primers for SNP discrimination. Anal Bioanal Chem 411:3871–3880. https://doi.org/10.1007/s00216-019-01865-4
12. Chen WY, Matulis D, Hu WP, Lai YF, Wang WH (2020) Studies of the interactions mechanism between DNA and silica surfaces by isothermal titration calorimetry. J Taiwan Ins Chem Eng 116:62–66. https://doi.org/10.1016/j.jtice.2020.11.019
13. Coenen AJJM, Henckens LHG, Mengerink Y, van der Wal S, Quaedflieg PJLM, Koole LH, Meijer EM (1992) Optimization of the separation of the Rp and Sp diastereomers of phosphate-methylated DNA and RNA dinucleotides. J Chromatogr 596:59–66. https://doi.org/10.1016/0021-9673(92)80202-6
14. Roush S, Slack FJ (2008) The let-7 family of microRNAs. Trends Cell Biol 18:505–516. https://doi.org/10.1016/j.tcb.2008.07.007
15. Vinogradova OA, Pyshnyi DV (2010) Selectivity of enzymatic conversion of oligonucleotide

probes during nucleotide polymorphism analysis of DNA. Acta Nat 2:36–53

16. Hong CY, Chen WY "Studies of improving detection specificity of single nucleotide variation and miRNAs by phosphate methylated oligoDNA primers" Master Thesis of Department of Chemical and Materials Engineering, National Central University, July, 2021