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As Fast and Selective as Enzymatic Ligations: Unpaired Nucleobases Increase the Selectivity of DNA-Controlled Native Chemical PNA Ligation

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DNA-controlled reactions offer interesting opportunities in biological, chemical, and nanosciences. In practical applications, such as in DNA sequence analysis, the sequence fidelity of the chemical-ligation reaction is of central importance. We present a ligation reaction that is as fast as and much more selective than enzymatic T4 ligase-mediated oligonucleotide ligations. The selectivity was higher than 3000-fold in discriminating matched from singly mismatched DNA templates. It is demonstrated that this enormous selectivity is the hallmark of the particular ligation architecture, which is distinct from previous ligation architectures designed as "nick ligations". Interestingly, the fidelity of the native chemical ligation of peptide nucleic acids was increased by more than one order of magnitude when performing the ligation in such a way that an abasic-site mimic was formed opposite an unpaired template base. It is shown that the high sequence fidelity of the abasic ligation could facilitate the MALDI-TOF mass-spectrometric analysis of early cancer onset by allowing the detection of as little as 0.2% of single-base mutant DNA in the presence of 99.8% wild-type DNA.

Introduction

In DNA excision-repair processes, DNA ligases efficiently join nicked DNA duplexes that have been corrected to perfect complementarity, without resealing strand breaks that contain damaged or mismatched bases.[1–6] For example, in a *Tth* ligase reaction, ligation on matched templates occurs more than 1000-fold faster than ligation on single-base mismatched templates.[5] Such high discriminative powers are required in early detection of cancer, as here the challenge arises to detect acquired single-base mutations over the background of predominant wild-type sequences. The high sequence fidelity and the high rates of DNA-ligase-mediated reactions provide the basis for the powerful oligonucleotide ligation assay (OLA), in which single-nucleotide-specific formation of DNA ligation products serves as the most accurate means of analyzing DNA single-base mutations.[7]

The exceptional properties of enzymatic ligation systems as well as the desire to overcome restricted tolerance to modifications of substrates and media, stimulated chemists to explore the utility of chemical ligation methods. While elegant methods for achieving sequence-specific chemical ligation on DNA templates have been shown, the speed and accuracy of product formation were still lagging behind *Tth* enzyme-mediated ligations.[8–11] In addition, the extension of chemical techniques to genuine double-stranded DNA provided a major challenge. We have recently shown that the use of peptide nucleic acids (PNA), nonionic, biostable DNA analogues, and a native chemical-ligation-based chemistry[12,13] allowed product formation to occur even on double-stranded PCR DNA templates.[14] Previous ligation systems have been designed in analogy to nicked DNA duplex substrates of DNA ligase.[15–26] We now provide clear evidence that the fidelity of a chemical ligation syst'em, such as the PNA native chemical ligation, can be improved by more than one order of magnitude when avoiding contiguous base-stacking between the fragments to be ligated. A new 1,2-aminothiol-containing PNA monomer is presented that enables PNA fragment couplings with PNA thioesters. It is shown that the PNA native chemical ligation succeeds as rapidly and as selectively as the best performing DNA-ligase-mediated oligonucleotide ligation reactions.

Results and Discussion

In prior chemical DNA-ligation systems, the two fragments to be ligated, **1** and **2**, were aligned on the DNA template, **3**, so as to allow base stacking between bases at the ligation site (Scheme 1 A). We have recently introduced a PNA-based ligation in which base stacking between two DNA-bound PNA fragments, **5** and **6**, was hindered by allowing the ligation to form an abasic site in **8** (Scheme 1 B).[14,27,28] However, the enhanced flexibility of the reactive groups in such a ligation ar-

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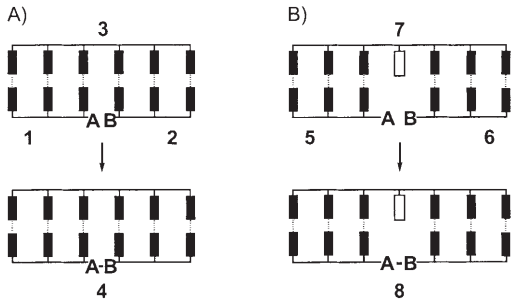
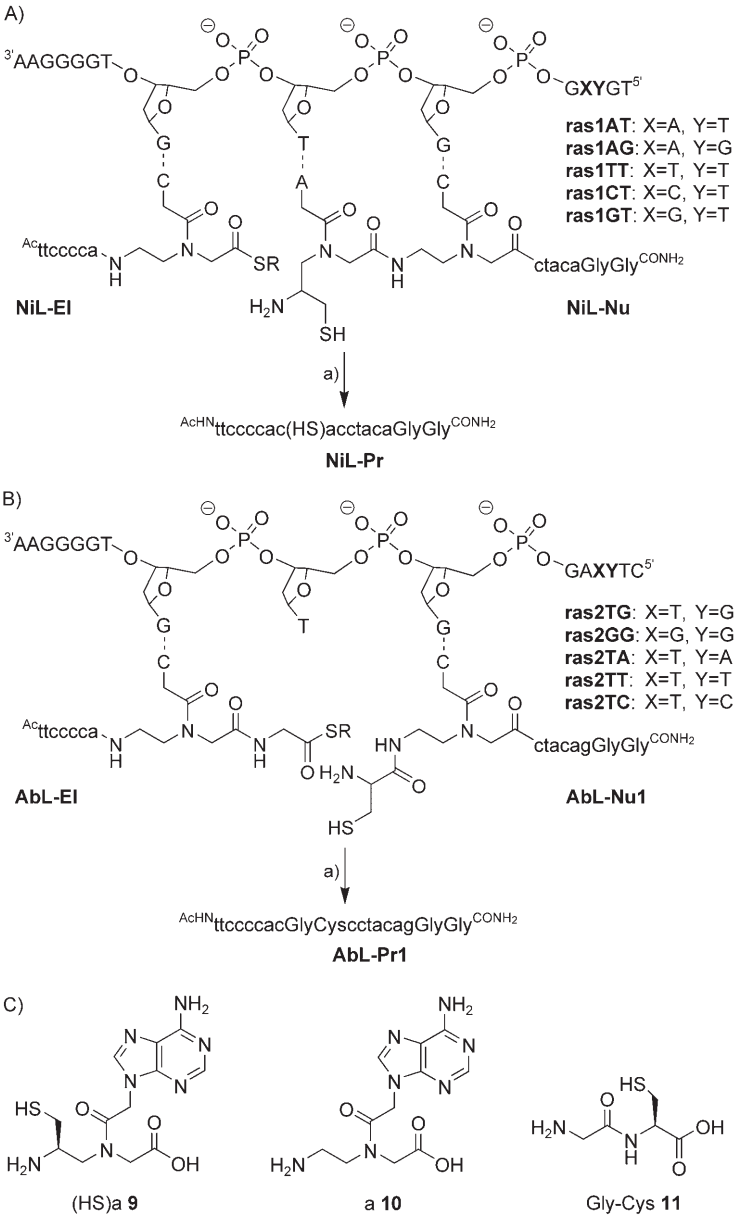


Scheme 1. DNA-controlled A) nick and B) abasic ligation of two nucleic acid conjugates **1** and **2** or **5** and **6**, respectively (A, B: reactive groups).

chitecture might result in reductions of the effective molarity, thereby diminishing the sequence selectivity and/or rate accelerations exerted by the template.[26] On the other hand, it is conceivable that the disruption of the cooperativity of "unselective" base stacking would lead to enhancements of sequence fidelity due to the increased contribution of more selective Watson–Crick hydrogen bonding.

Ligation reactions

We explored the two distinct ligation architectures by using native chemical ligation of PNA on mutant *ras*-gene segment **ras1AT** as a paradigm. The evaluation of a nick PNA ligation (Scheme 2A) required a new PNA monomer **9**, which was designed as a structural analogue of a PNA adenine monomer, **10**, that contained the 1,2-aminothiol structure needed for native chemical ligation-like reactions (Scheme 2C).[29] The abasic ligation format was put into practice by allowing PNA glycine thioester **AbL-El** to react with cysteinyl-PNA **AbL-Nu1**. Upon ligation, a PNA–dipeptide hybrid **AbL-Pr1** is formed in which the central dipeptide (highlighted in **11**) serves as the isosteric replacement of a PNA monomer, such as adenine **10**. The ligation probes **NiL-El** and **NiL-Nu** (nick ligation) and **AbL-El** and **AbL-Nu1** (abasic ligation) were allowed to react in the presence of perfectly matched and singly mismatched DNA and in the absence of DNA. HPLC analysis revealed that neither nick ligation (Figure 1A) nor abasic ligation (Figure 1B) proceeded in the absence of DNA template (< 0.2% product after 60 min). The addition of matched DNA templates led to strong increases in the rates and yields of product formation. For example, in the abasic ligation on matched DNA **ras2TG**, 20% yield of product **AbL-Pr1** was obtained after 90 s. An apparent second-order rate constant of 2470 $L\,mol^{-1}\,s^{-1}$ was determined, which is faster by almost two orders of magnitude than previous nonenzymatic DNA ligation reactions.[8,11,15] Interestingly, nick ligation of **NiL-El** and **NiL-Nu** proceeded even faster than the abasic PNA ligation, as evidenced by the 1.6-fold enhanced second-order rate constant of 3973 $L\,mol^{-1}\,s^{-1}$.



Scheme 2. A) Nick PNA ligation of PNA conjugates **NiL-El** and **NiL-Nu** on matched DNA template **ras1AT** and singly mismatched templates **ras1AG-GT**. B) Abasic PNA ligation of PNA conjugates **AbL-El** and **AbL-Nu1** on matched DNA template **ras2TG** and singly mismatched templates **ras2GG-TC**. a) 1 μM probes (1:1 molar ratio), 1 μM template, 10 mM NaCl, 10 mM NaH_2PO_4 (pH 7.4), saturated benzyl mercaptan at 25 °C. R = $SCH_2C_6H_5$. C) In nick PNA ligation, the thiol group adenine monomer **9** replaces the "conventional" adenine PNA monomer **10**. In the abasic ligation architecture, the formed Gly-Cys dipeptide **11** serves as isosteric replacement of adenine monomer **10**.

Most noticeable were the results of the ligation experiments on singly mismatched templates. While abasic ligation proved inefficient (less than 0.2% yield of **AbL-Pr1** after 60 min), nick ligation still proceeded remarkably well on mismatched template **ras1AG**. The initial rates of ligation indicated that abasic ligation of **AbL-El** and **AbL-Nu1** on matched DNA **ras2TG** occurred 3450-fold faster than on singly mismatched DNA **ras2GG**. In contrast, only sevenfold rate differences were determined when analyzing matched and mismatched nick ligation of **NiL-El** with **NiL-Nu**. Ligation experiments on templates **ras1TT**, **ras1CT**, **ras1GT**, **ras2TA**, **ras2TT**, and **ras2TC** were per-

formed in order to evaluate the roles of the position and identity of mismatched base pairs. Ligation of **NiL-El** and **NiL-Nu** in the nick architecture on mismatched templates **ras1TT**, **ras1CT**, and **ras1GT** furnished product **NiL-Pr** in 6.0, 4.5, and 5.5% yield, respectively (Table 1). In contrast, abasic ligations on mismatched DNA templates **ras2TA**, **ras2TT**, and **ras2TC** were virtually indistinguishable from template-free controls (<0.2% ligation product). These results suggest that abasic ligation proceeded with higher sequence selectivity than nick ligation irrespective of the identity and position of mismatched bases. It is interesting to note that the selectivity of up to 270-fold rate differences obtained in nick PNA ligation resembles the orders of match/mismatch discrimination reported for other DNA ligation systems that featured contiguously paired template bases.[8,16,25]

Stability of binary and ternary complexes

The high reaction rates and the >3000-fold match/mismatch selectivities obtained with abasic ligation are without precedence in chemical nucleic acid ligation formats. It is commonly assumed that the fidelity of a given DNA-controlled chemical-ligation reaction is governed by the selectivity of probe hybridization. The $T_M = 37\,°C$ measured for a DNA duplex containing matched thiol probe **NiL-Nu** and the $T_M = 42\,°C$ measured for thiol probe **AbL-Nu1** indicated that, at 25 °C, both probes are bound by the template (Table 2 A). Hybridization of both thiol probes **NiL-Nu** and **AbL-Nu1** with mismatched DNA **ras1AG** and **ras2GG** failed to give sigmoid melting curves; this suggests that formation of singly mismatched duplexes is a less favoured event at 25 °C. However, in ligation reactions, both thioester probes **NiL-El** or **AbL-El** and thiol probes **NiL-Nu** or **AbL-Nu1** must bind simultaneously. Differences in cooperative stabilization in contiguously stacked (nick) nonligated complexes as opposed to abasic-site-containing complexes might account for the different ligation selectivities. We therefore determined the stability of nonligated ternary complexes. The lengths of the DNA template and the thioester probes were extended in order to allow the resolution of the two hybridization events by analysis of biphasic melting curves (see Supporting Information). The concomitant hybridization of **NiL-PNA** probe ($T_M = 75\,°C$) in the nick complex enhanced the affinity of thiol probe **NiL-Nu** for template **ras3T** by $\Delta T_M = 2\,°C$ from $T_M = 39\,°C$ in the binary complex (**NiL-Nu·ras3T**, Table 2 B) to $T_M = 41\,°C$ in the ternary complex (**NiL-Nu·ras3T·NiL-PNA**, Table 2 C). Just as high was the increase of the template affinity of abasic ligation probe **AbL-Nu1** that was brought about by simultaneous hybridization of probe **AbL-PNA** (**AbL-Nu1·ras3T**, $T_M = 43\,°C$; **AbL-Nu·ras3T·AbL-PNA**, $T_M = 45\,°C$). Ternary hybridization including mismatched DNA templates (**NiL-Nu·ras3G·NiL-PNA**, **Nu·ras3G·AbL-PNA**) failed to give biphasic melting curves (Supporting Information). The identical cooperative stabilization ($\Delta T_M = 2\,°C$) conferred by simultaneous hybridization of two ligation probes in both ligation architectures, the higher stability of nonligated abasic ligation complexes, and the observation that hybridization is extremely fast and certainly not rate-limiting apparently contradict the notion of probe binding being the only determinant of ligation fidelity. There must be additional parameters than the selectivity of probe binding that determine the sequence fidelity of DNA-controlled chemical ligation reactions. At present, the origin of the superior ligation fidelity of abasic PNA ligation remains unclear. We do, however, note that the abasic PNA-ligation format also provides high match/mismatch discriminations when using other PNA ligation chemistries (data not shown). It is conceivable that the positive effect of forming abasic sites might be common to chemical DNA-controlled nucleic acid ligations in general, provided that binding at the template does not impose steric constraints on transition-state geometry.

Towards detecting acquired single-base mutations present as minor compounds

In order to be considered as true alternatives to commonly used ligase-catalyzed reactions, chemical-ligation strategies must proceed as rapidly and as selectively as enzymatic ones. Moreover, in a "real-world" scenario, ligations must succeed on genuine double-stranded DNA, which is usually obtained by PCR. We have recently shown that native chemical PNA ligation allowed the single-base-mutation analysis of double-stranded PCR DNA.[13] The results of the present study demonstrated the high selectivity and rate of abasic PNA ligation. These features should allow the design of very precise and very fast single-nucleotide polymorphism detection assays. Highest selectivities are demanded by assays aiming to detect acquired single-base mutations present as a minority of the DNA sample. Codon 12 *ras* wild-type and mutant templates were mixed in varying ratios to emulate early cancer onset. Figure 2 shows that single-nucleotide-specific ligation products can be detected by MALDI-TOF mass spectrometry after only 20 min reaction time in spite of high backgrounds of wild-type DNA.[30] The formation of mutant ligation product **AbL-Pr1** ($m/z = 4260$) and, hence, occurrence of mutant DNA was detected when present as a mixture of **ras4G**(wt)/**ras4T**(mt) DNA in 9:1 and 99:1 ratios (Figure 2 A). Even 0.2% of mutant DNA was still detectable (signal-to-noise ratio = 32) in the presence of 99.8% of wild-type DNA, while virtually no signal appeared on pure wild-type DNA (signal-to-noise ratio <3). In a duplex assay, the formation of a wild-type specific ligation product can serve as internal positive control. Figure 2 B shows the ligation products formed in a reaction of thioester **AbL-El** with competing mutant thiol probe **AbL-Nu1** and wild-type thiol probe **AbL-Nu2**. Wild-type DNA **ras4G** was the major component of the template mixture, and, indeed, wild-type-specific ligation product **AbL-Pr2** was the dominating peak in all instances. The smallest detectable proportion of the mutant template was 0.2% (signal-to-noise ratio = 6); this testifies to the high selectivity of native chemical PNA ligation.

Conclusion

In conclusion, we have provided evidence that the ligation architecture is of critical importance for the sequence selectivity of chemical PNA ligation. It was shown that the fidelity of a

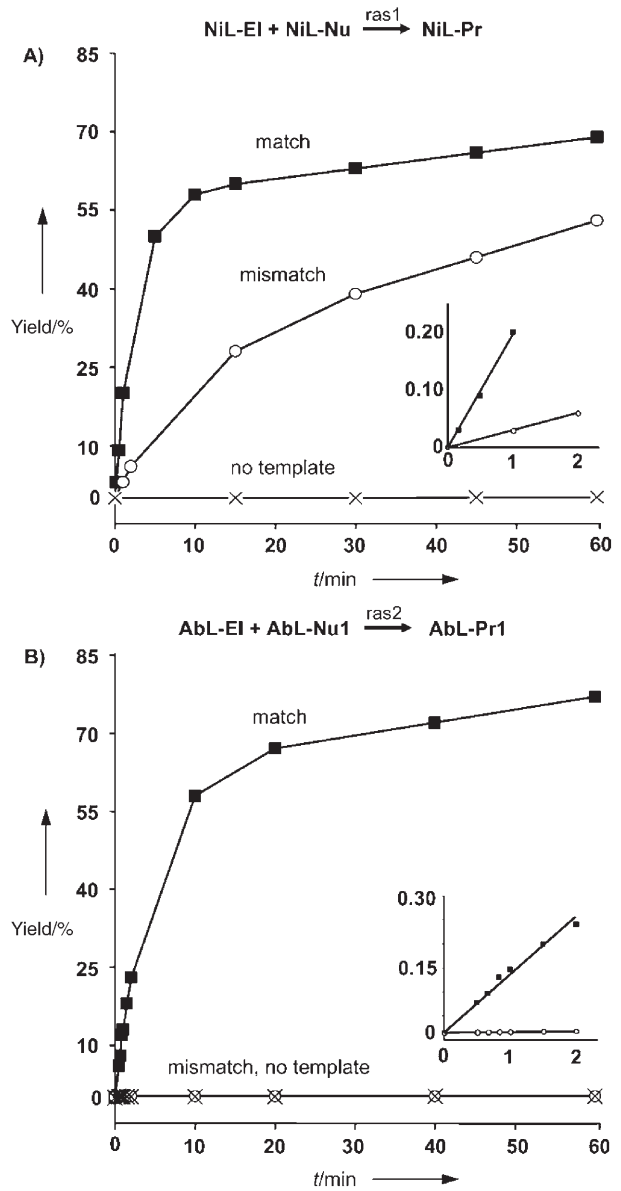


Figure 1. Time course of A) nick and B) abasic PNA ligation of PNA oligomers **NiL-El** and **NiL-Nu** or **AbL-El** and **AbL-Nu1** in the presence of matched DNA template **ras1AT** or **ras2TG** (■), mismatched DNA template **ras1AG** or **ras2GG** (○) and without DNA template (×) as revealed by HPLC analysis. Insets show early time courses.

"conventional" nick ligation system can be increased by more than one order of magnitude when performing the ligation such that an abasic site was formed. The abasic PNA ligation is as selective against single-base mismatches as the best performing *Tth* DNA ligase and exceeds the fidelity of previous chemical DNA ligations by one order of magnitude. The rates of native chemical PNA ligation surpass those of other chemical-ligation techniques used in DNA analysis by two orders of magnitude and are in the range of velocities of enzymatic ligation reactions. This chemical-ligation system could therefore offer a true alternative to enzymatic oligonucleotide ligation in DNA and RNA-mutation analysis.

Experimental Section

General procedures and materials: Analytical HPLC was run on a Merck–Hitachi Elite LaChrom chromatograph with an RP-C18A5μ "Polaris" column (PN 2000–250×0.46, Varian). Detection of the signals was achieved with a photodiode array detector at wavelengths $\lambda = 260$ and 280 nm. Eluents A (0.1% TFA in water + 1% MeCN) and B (0.1% TFA in MeCN + 1% water) were used in a linear gradient at 55 °C with a flow rate of 1 mL min^{-1}. Gradient: 3% B→30% B in 30 min. Preparative HPLC was performed on a Gilson Nebula 321 Series chromatograph with a semipreparative column (VP 250/10 Nucleosil (100–7) HD or SP 125/10 Nucleodur Gravity (5μ; Macherey&Nagel, Düren). Flow rate 3.5 or 6 mL min^{-1} at 55 °C.

MALDI-TOF mass spectra were measured on a Voyager-DE Pro Biospectrometry Workstation from PerSeptive Biosystems (Foster City, USA). A solution of sinapinic acid (10%) in MeCN/1%TFA (1:1) was used for generating the probe-matrix mixture. The concentrations of the stock solutions of the oligonucleotides were determined by measuring the optical density at $\lambda = 260$ nm on a Varian Cary 100 UV/Vis spectrometer. The specific absorption coefficients ε of the oligonucleotides were calculated by using the nearest-neighbor method.

DNA was purchased from MWG (Ebersberg, Germany) at high-purity, salt-free quality. Water was taken from a Milli-Q® Ultrapure Water Purification System (Millipore Corp.).

T_M measurements: UV melting curves were measured at 260 nm by using a Varian Cary 100 spectrometer equipped with a peltier block. A degassed aqueous solution of NaCl (10 mM) and NaH_2PO_4 (10 mM) adjusted to pH 7.0 by using NaOH (2 M) was used as buffer. The oligonucleotides were mixed at 1:1 stoichiometry, and the solutions were adjusted to a final duplex concentration of 1 μM. Prior to analysis, the samples were heated to 85 °C at a rate of

Table 1. Properties of nick and abasic ligation on matched and singly mismatched DNA templates.

	Nick ligation				Abasic ligation			
DNA template		yield [%][a]	initial rate [$M s^{-1} \times 10^{-11}$]	selectivity[b]	DNA template	yield [%][a]	initial rate [$M s^{-1} \times 10^{-11}$]	selectivity[b]
ras1AT	(match)	70	326		**ras2TG** (match)	76	206	
ras1AG	(mismatch)	53	50	1:7	**ras2GG** (mismatch)	<0.2[c]	<0.06	>1:3450
ras1TT	(mismatch)	6.0	1.6	1:204	**ras2TA** (mismatch)	<0.2[c]	<0.06	>1:3450
ras1CT	(mismatch)	4.5	1.2	1:272	**ras2TT** (mismatch)	<0.2[c]	<0.06	>1:3450
ras1GT	(mismatch)	5.5	1.4	1:233	**ras2TC** (mismatch)	<0.2[c]	<0.06	>1:3450

[a] Yields after 60 min. [b] Ratio between the initial rates of product formation on matched and mismatched DNA. [c] Smallest reliable yield to be determined (HPLC traces are presented in Figure S2 in the Supporting Information).

Table 2. Melting temperatures of the nick and abasic ligation probes in complex with target DNA.[a]

		Nicked Ligation				Abasic Ligation			
A)	**ras1XY**	3′AAGGGGTGTGG**XY**GT5′			**ras2XY**	3′AAGGGGTGTGGA**XY**TC5′		
		(HS)acctacaGlyGly^{CONH_2}				Cyscctacag GlyGly^{CONH_2}		
		NiL-Nu				**AbL-Nu1**		
		ras1AT		**ras1AG**		**ras2TG**		ras2GG
	T_M/°C	37		n.s.[b]	T_M/°C	42		n.s.[b]
B)	**ras3T**	3′CCCGTGAGAAGGGGTGTGGATGTC5′			ras3T	3′CCCGTGAGAAGGGGTGTGGATGTC5′		
		(HS)acctacaGlyGly^{CONH_2}				Cyscctacag GlyGly^{CONH_2}		
		NiL-Nu				**AbL-Nu1**		
	T_M/°C	39			T_M/°C	43		
C)	**ras3X**	3′CCCGTGAGAAGGGGTGTGGA**X**GTC5′			**ras3X**	3′CCCGTGAGAAGGGGTGTGGA**X**GTC5′		
		AcHN gggcactcttccccac^{CONH_2}(HS)acctacaGlyGly^{CONH_2}				AcHN gggcactcttccccacGly^{CONH_2} Cyscctacag GlyGly^{CONH_2}		
		NiL-PNA	**NiL-Nu**			**AbL-PNA**	**AbL-Nu1**	
		ras3T,G	**ras3T**	**ras3G**		**ras3T,G**	**ras3T**	**ras3G**
	T_M/°C	75	41	n.s.[b]	T_M/°C	75	45	n.s.[b]

[a] Conditions: 1 μM probe and template, 10 mM NaCl, 10 mM NaH_2PO_4 (pH 7.0). Denaturation curves were determined at 260 nm from 5 to 85 °C at a rate of 1.0 °C min^{-1}, data interval 0.1 °C. [b] n.s. = not sigmoidal.

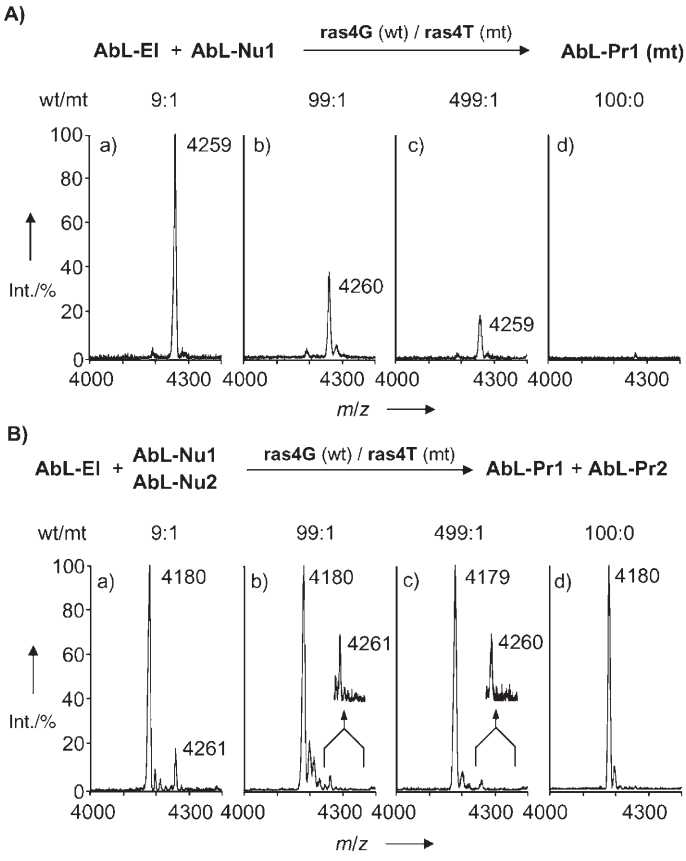


Figure 2. MALDI-TOF/MS analysis of abasic ligation in the presence of 28-mer wild-type DNA **ras4G** = 3′-CGTGAGAAGGGGTGTGGA**G**GTCGCGGAT-5′ and 28-mer mutant DNA **ras4***T* = 3′-CGTGAGAAGGGGTGTGGA**T**GTCGCGGAT-5′ in the presence of A) **AbL-El** and **AbL-Nu1**(mt) and B) **AbL-El**, **AbL-Nu1**(mt), and **AbL-Nu2**(wt) = CyscctcagGly^{CONH_2}. Peak heights are calibrated on signal-to-noise ratios. Conditions: 1 μM probes, sum of mutant + wild-type DNA: 1 μM, 2 mg mL^{-1} sodium 2-mercaptoethanesulfonate, 10 mM NaCl, 10 mM NaH_2PO_4 (pH 7.4) at 25 °C, t = 20 min, (m/z: 4260 **AbL-Pr1**, 4179 **AbL-Pr2** = ^{Ac}ttccccacGlyCyscctcagGly^{CONH_2}).

1 °C min^{-1} and cooled within 3 h to a starting temperature of 5 °C. T_M values were defined as the maximum of the first derivative of the melting curve.

PNA ligations: Aqueous DNA or PNA stock solutions (1–5 mM) were prepared. The ligation buffer was comprised of an aqueous solution of NaCl (10 mM) and NaH_2PO_4 (10 mM) and was freshly prepared prior to use. The pH was adjusted to 7.4 by using NaOH (2 M) solution. Subsequent manipulations were carried out while avoiding unnecessary exposure to oxygen. The ligation buffer was placed in Eppendorf tubes and the appropriate amount of PNA thioester conjugate and template were added together with benzylmercaptan (4%) or sodium 2-mercaptoethanesulfonate (2%) for conversion into the desired thioester. The corresponding cysteine-PNA conjugates were added, and the reaction mixture (1:1:1 molar ratio) was allowed to react for the denoted time by vortexing at 25 °C. For measurements of the initial rates, the benzylmercaptan was separated from the reaction mixture by centrifugation and removed after the conversion into the benzylthioester prior to the addition of the cysteine-PNA conjugate.

HPLC analysis: If not already done (measurement of the initial rates), the benzylmercaptan was separated from the reaction mixture by centrifugation. After disposal of the benzylmercaptan layer, the reaction was quenched by adding TFA (10%). After 30 min, the solvent was removed in vacuo, the residue was dissolved in water containing MeCN (1%) and TFA (0.1%), and analyzed by analytical HPLC.

Yields were determined according to:

$$\text{Yield} = \frac{PA_{product}}{PA_{thiol} + PA_{thioester} + PA_{hydrolyzed\ thioester} + PA_{product}} \times 100\,\%$$

where $PA_{product}$ = peak area corresponding to ligation product **NiL-Pr** or **AbL-Pr1**, PA_{thiol} = peak area corresponding to PNA probe **NiL-Nu** or **AbL-Nu1**, $PA_{thioester}$ = peak area corresponding to benzylmercaptan converted PNA probe **NiL-El** or **AbL-El**, and $PA_{hydrolyzed\ thioester}$ = peak area corresponding to hydrolyzed PNA probe **NiL-El** or **AbL-El**.

MALDI-TOF/MS analysis: The chemical ligation was quenched by adding TFA (1%). The reaction was extracted (~10×) by using a water-equilibrated RP-C18 OMIX® 100 μL micro pipette (Varian). After the reaction mixture had been washed with water (1×50 μL) and eluted with water/MeCN 1:1 (4×50 μL), the solvent was evaporated under reduced pressure. The residue was dissolved in TFA (5 μL, 10%), and five spots were placed on the MALDI-plate. Matrix: sinapinic acid.

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Keywords: DNA · native chemical ligation · peptide nucleic acids · single-nucleotide polymorphism · template synthesis

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