

Technical Note

Locked Nucleic Acid



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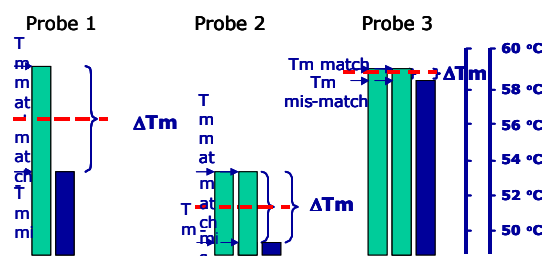
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The significance of T_m and ΔT_m for oligonucleotide primers and probes

Simultaneous use of more than one primer or probe can be challenging because the involved oligonucleotides must work under the same conditions. Of particular interest is the melting temperature (T_m) at which the hybridized oligonucleotides dissociate. In cases where probes are applied for specific detection of mutations or homologous sequences the ΔT_m is of importance. ΔT_m is here defined as the difference between T_m of the match and the T_m of the mismatch hybridizations. Generally, a large ΔT_m is required to ensure specific detection of the sequence of interest. In addition, a large ΔT_m permit more probes to be used simultaneously so that a higher degree of multiplexity can be achieved.

Figure 1A demonstrates common problems that are frequently encountered when attempting to use several probes simultaneously.

A



B

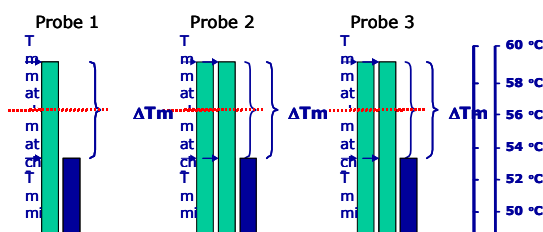


Figure 1 Simultaneous use of multiple probes. A. Badly designed probes containing only DNA that cannot be applied simultaneously. B Well designed probes with necessary LNA substitutions that may be used simultaneously. Red lines indicate necessary experimental temperature for each probe.

Probes 1 and 2 in Figure 1A, are not compatible due to a significant difference in T_m (melting temperature of match hybridization) even though they both have a considerable ΔT_m . This is in contrast to probes 1 and 3 (Figure 1A), which do have similar T_m but can still not be used together since the ΔT_m of probe 3 is too small to offer a proper discrimination between homologous and non-homologous sequences. Figure 1B demonstrates 3 well designed probes that may be operated in multiplex setting. All probes have similar T_m values and a significant ΔT_m , which ensure specific hybridization under the same stringent conditions, in this case approximately 56°C. It is often difficult to design probes and primers with similar melting temperature due to the variance in A/T and G/C content of their respective target sites. Probes aimed at A/T rich regions have low thermal stability (i.e. T_m) unless they are made exceedingly long which may reduce specificity. Certain base pair mismatches are difficult to resolve, in particular detection of single G/T mismatches that are known to contribute very little (if at all) to ΔT_m .

How can these important problems be solved?

The use of LNA (Locked Nucleic Acid), a DNA analogue, is the optimal way to adjust the stability of individual primers and probes while maintaining (or improving) their specificity. LNA can thus be used to solve problems related to multiplex use of primers and probes. LNA offers the possibility to adjust T_m and increase the ΔT_m at the same time. LNA increases T_m with 4-8°C/substitution and increases ΔT_m substantially (Table 1 and Figure 2).

T_m of LNA:DNA Duplexes	Perfect match	Single mismatch	ΔT_m
	3'-ACGACCAC-5'	3'-ACG <u>G</u> CCAC-5'	
LNA 8-mer 5'-TGCTGGTG-3'	71°C	45°C	26°C
DNA 8-mer 5'-TGCTGGTG-3'	35°C	25°C	10°C

Table 1. Comparison of LNA and DNA stability demonstrating the increased T_m and ΔT_m for LNA octamers.

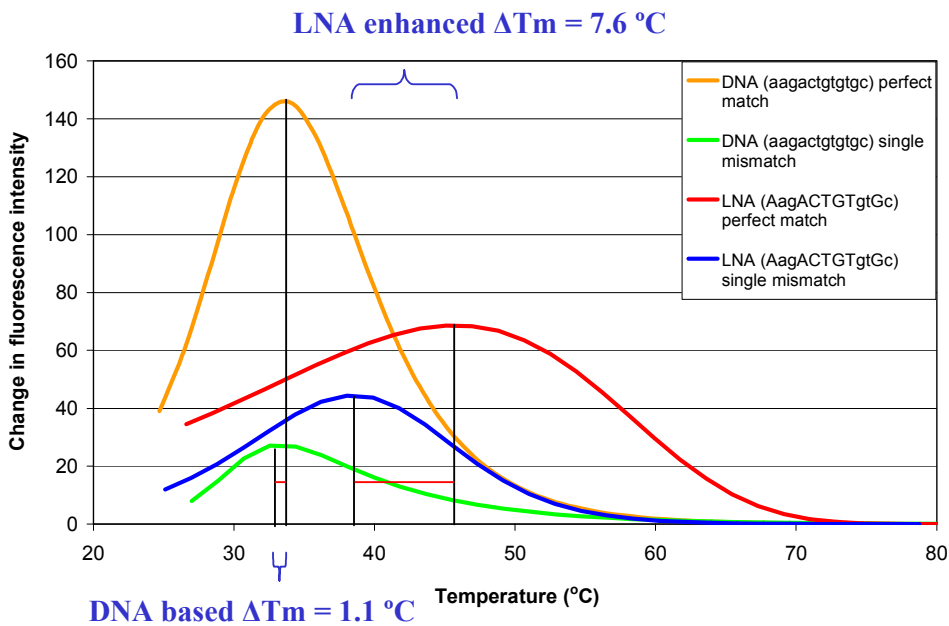


Figure 2. Example of how LNA substitutions can increase ΔT_m . The curves show the first derivative of 4 melting profiles. The curve peaks corresponds to the measured T_m values. As can be seen, the ΔT_m has been increased significantly after incorporation of LNA in the probe.

As LNA can substitute for DNA during standard oligonucleotide synthesis, LNA can be placed at optimal positions in any probe sequence in order to adjust T_m (Figure 3). Furthermore, LNA placed at a few strategic positions may significantly enhance ΔT_m as demonstrated in Figure 3.

ΔT_m can be increased from $1,1^\circ\text{C}$ to $7,6^\circ\text{C}$ just by substituting 5 of the DNA nucleotides with LNA as shown in Figure 3. This increase in ΔT_m is enough to enable accurate detection of single base mismatches. It is also demonstrated that a high ΔT_m can be obtained at different

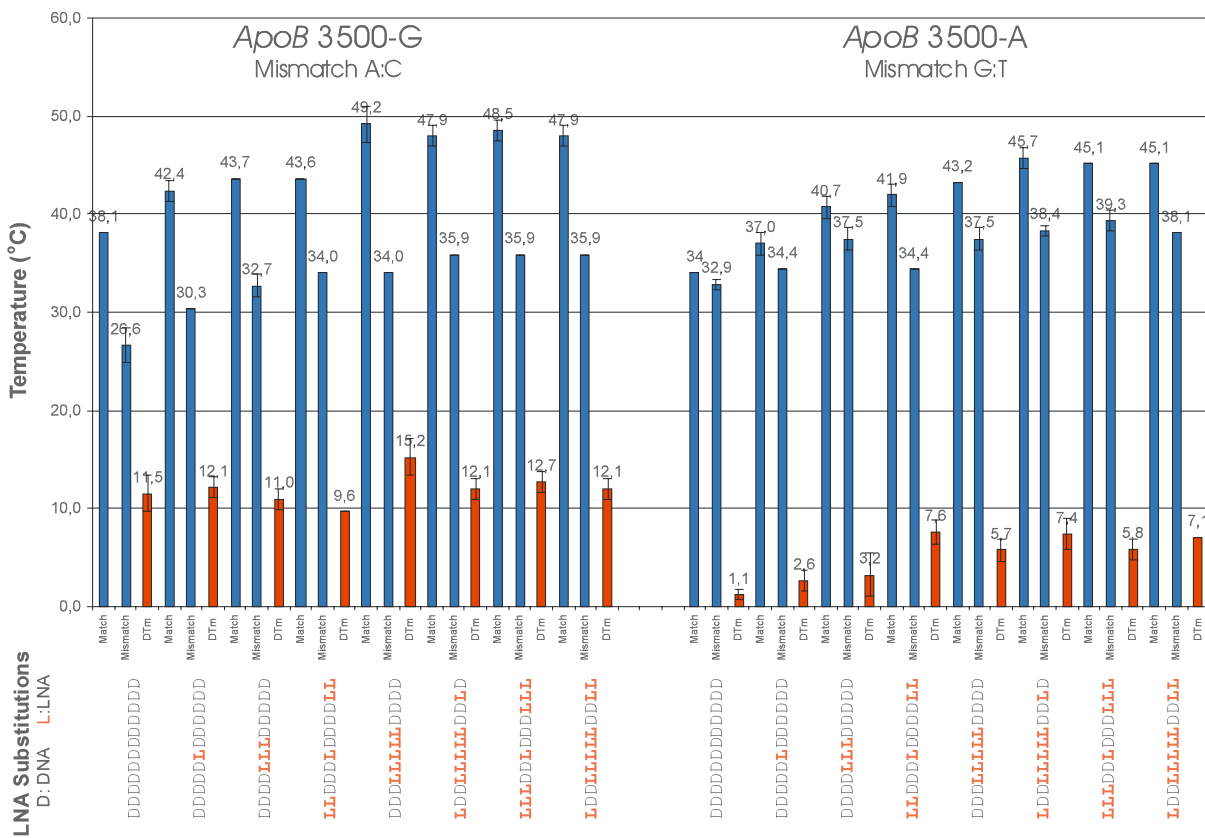


Figure 3. T_m and ΔT_m values obtained by on-chip melting of target DNA in microarray hybridizations. Probes with different LNA substitutions were analyzed for their ability to resolve a single centrally positioned mismatch (T-G and A-C). For each design variant of the 12-mer probes, the T_m of perfect match and single mismatch were measured. Each triplet of bars contains the T_m of match (left blue bar), T_m of mismatch (central blue bar), and the ΔT_m (red bar). In the sequences below the columns, the positions of LNA substitutions are indicated with red capital letters for the different capture probes.

T_m values (compare T_m and ΔT_m of 38.1°C and 11.5°C for pure DNA with 49.2°C and 15.2°C after 5 LNA substitutions).

Figure 4 demonstrates how LNA can be used to optimize and trim capture probes to work together in a multiplex hybridization experiment. The probes are designed to detect a single nucleotide polymorphism (SNP) in the ApoB gene. As can be seen, the two DNA probes perform poorly together because the ΔT_m is too small for the probe detecting allele 2. The low discrimination is probably due to the relative stability of a G:T mismatch. However, by incorporating LNA in the probe sequences the ΔT_m of the probes were enhanced by 38% and 300%, respectively. The resulting probes can be used together at 40°C.

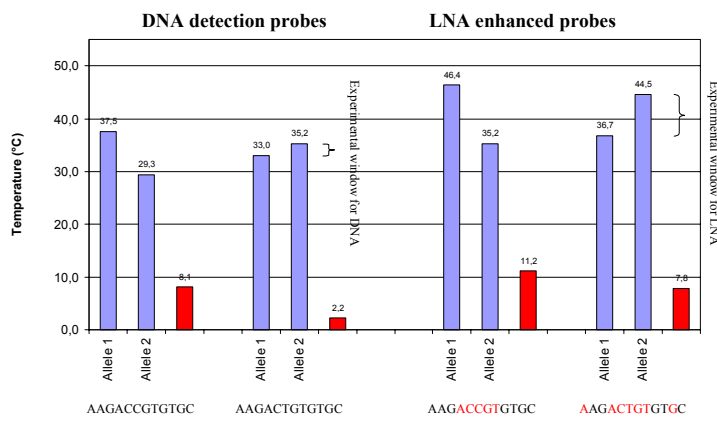


Figure 4. LNA is the enabling molecule in the design of compatible probes. The nucleotides of Allele 1 and 2 in the central mismatch position are G and A, respectively. The assay thus require a detection of the notoriously difficult G:T mismatch that is almost as stable as an A:T match. The red letters in the sequence of the probes denote the LNA substitutions.

LNA in microarray experiments

LNA substitutions can improve the performance of oligonucleotide probes used for microarray experiments as shown in figure 3. The T_m properties of surface attached oligonucleotides were investigated with a fluorescence microscope based experimental setup, in which the temperature of the microscope stage was controlled with a peltier element. The effect of different LNA substitutions on the ability of microarray-attached probes to distinguish between single mismatches was investigated for the single nucleotide polymorphism ApoB3500 as shown in Figure 3. For larger scale microarray experiments, where many probes are required to function together, it is even more important to be able to adjust the melting properties of the individual probes by including LNA substitutions.

LNA in PCR primers

The specificity of PCR primers may also be enhanced by using LNA substitutions in the primer sequences, as has been demonstrated for allele specific PCR (www.proligo.com). Often, only a low ΔT_m can be obtained with DNA primers, especially if the mismatch position is located near the 3'-end of the primer. In these cases it is very important that the hybridization between the 3'-end of the primer and the target sequence is destabilized as much as possible, in order to leave the 3'-end of the primer dangling. With LNA, it is possible to increase ΔT_m , thus making the discrimination between alleles more effective as shown in Table 2. It has furthermore been shown that a more specific amplification can be obtained for multiplex PCR. The high thermal stability of LNA containing duplexes has one notable disadvantage: LNA sequences has a very high propensity to form LNA:LNA duplexes, so LNA should not be used in cases where self complementarity can occur. Likewise, partial complementarity between the different primer sequences must be avoided, as even short stretches of LNA:LNA duplexes (= 4 bp) are surprisingly stable.

Example	Probe Sequence	Target Sequence	T_m (°C)	ΔT_m
A	agtccccgcttccagtca	tgactggaagcggggact	65.4	-0.2
		tcactggaagcggggact	65.6	
B	agtccccgcttccagtCa	tgactggaagcggggact	68.1	4.7
		tcactggaagcggggact	63.4	
C	agtccccgcttccagtga	tcactggaagcggggact	66.1	2.2
		tgactggaagcggggact	63.9	
D	agtccccgcttccagtGa	tcactggaagcggggact	66.9	2.6
		tgactggaagcggggact	64.3	

Table 2: Effect of single nucleotide LNA substitutions on the melting properties of oligonucleotides. The position of the mismatch is indicated with red, and LNA substitutions are shown with capital letters. The melting properties were investigated by UV-spectrophotometry. The T_m was found as the maximum of the first derivative of the thermally induced hyperchromicity.

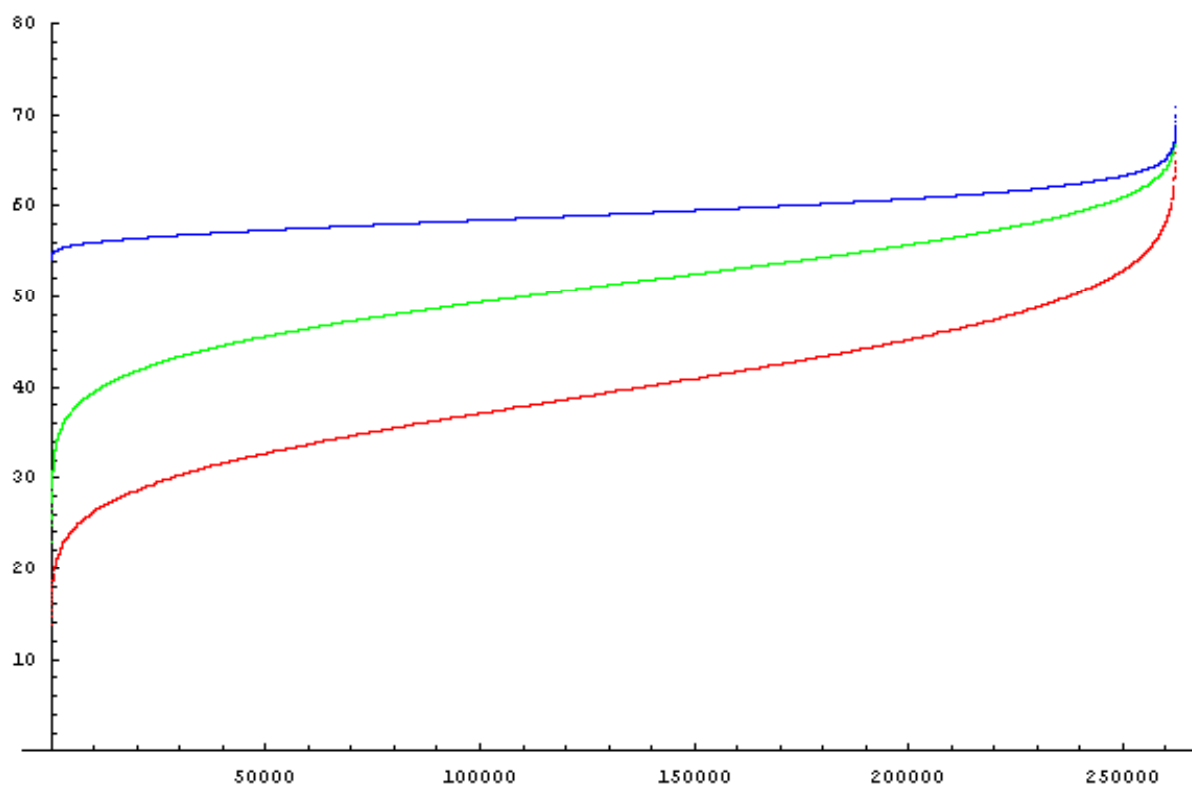


Figure 5: Graphical representation of the ordered distribution of melting temperatures of all possible 9-mer oligonucleotides consisting of either pure DNA (red line), LNA T substituted (green line) or A and T substituted (blue line). The T_m -values of the single oligonucleotides has been predicted with the Exiqon T_m prediction tool (available at www.LNA-tools.com).

Normalization of thermal stability by universal LNA A and T nucleotide substitutions

High affinity nucleotide analogs such as LNA can be also used universally to equalize the melting properties of oligonucleotides with different AT and CG content. The increased affinity of LNA adenosine and LNA thymidine corresponds approximately to the normal affinity of DNA guanine and DNA cytosine. An overall substitution of all DNA-A and DNA-T with LNA-A and LNA-T, will result in melting properties that are nearly sequence independent but only depend on the length of the oligonucleotide. This may be important for design of oligonucleotide probes used in large multiplex analysis and likewise for applications using random oligonucleotides, where differences in stability often lead to strong biases. The effect of LNA A and T substitutions has been evaluated by predicting the T_m value (link to the T_m -prediction tool) of all possible 9-mer oligonucleotides with different universal substitutions. The distribution of the 262.000 T_m -values is shown in Figure 5, where a very homogeneous T_m value is observed for the universally LNA A and T substituted oligonucleotides (blue curve). The standard deviation of the melting temperature for all 9-mers drops from 7.7°C for pure DNA (red curve) to only 2.2°C for LNA A and T substituted oligonucleotides (blue curve). This equalizing effect may also be utilized for photomediated on-chip synthesis of oligonucleotides.

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