

Technical Note



Locked Nucleic Acid

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Determination of melting properties of LNA substituted oligonucleotides

The following document shows different approaches to measure the thermal stability (i.e. melting curves) of DNA and LNA substituted oligonucleotides.

Melting properties of oligonucleotides are routinely investigated at Exiqon A/S with three different methods. The most accurate method, which is used for generation of calibration data for the Exiqon LNA T_m-prediction algorithms (available at www.LNA-tools.com), is the standard UV spectrophotometric measurement based on the elicited hyperchromicity at 260 nm upon thermal denaturation (Example 1). However, this method is quite time consuming. An alternative method (Example 2) with a higher throughput is based on fluorescence measurement in a real-time PCR instrument. The decrease in fluorescence intensity from an intercalating fluorophore that specifically binds to double stranded DNA is followed. Finally, it has been observed that melting properties of surface attached oligonucleotides differs from their properties in solution. Therefore, T_m measurements were also performed by on-chip fluorescence analysis of surface bound capture probes (Example 3). This was done with a microscope setup, where it is possible to control the

temperature of the stage using a Peltier element. The presented data are only intended to serve as examples of experimental approaches to determine the melting properties of oligonucleotides. They do not represent a complete investigation of the effect of LNA substitutions on T_m.

Example 1:

Effect of terminal LNA substitutions in oligonucleotides investigated by UV-spectroscopy

LNA can be used to increase both the melting temperature and the discriminating power of PCR primers. In the following example, it is shown how a single LNA substitution in the penultimate position of an 18-mer can increase T_m with up to three degrees and concurrently increase ΔT_m with up to 4.7 degrees compared to the unmodified DNA primer (Table 1). It is worth noting that this significant increase in ΔT_m due to a single mismatch is obtained even though the mismatch is positioned next to the terminal nucleotide of the primer sequence. Figure 1 shows the melting curves corresponding to the data in Table 1. These results demonstrate the beneficial potential of LNA substitution in PCR primers. Similar effects are expected for probes for oligonucleotide ligation assays. It should be noted that helical distortions due to extensive use of LNA substitutions near the 3'-end of

| 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 1: Effect of single LNA nucleotide substitutions on the thermal stability of oligonucleotides. The position of the mismatch is indicated by red letters. DNA nucleotides are represented by lower case letters, whereas LNA substitutions are shown with capital letters. The melting properties were investigated by UV-spectroscopy. The corresponding melting curves are shown in Figure 1. The T_m was found as the temperature corresponding to the inflection point on the absorbance curves, which is most easily obtained from the peak value for the first derivative of the melting curves.

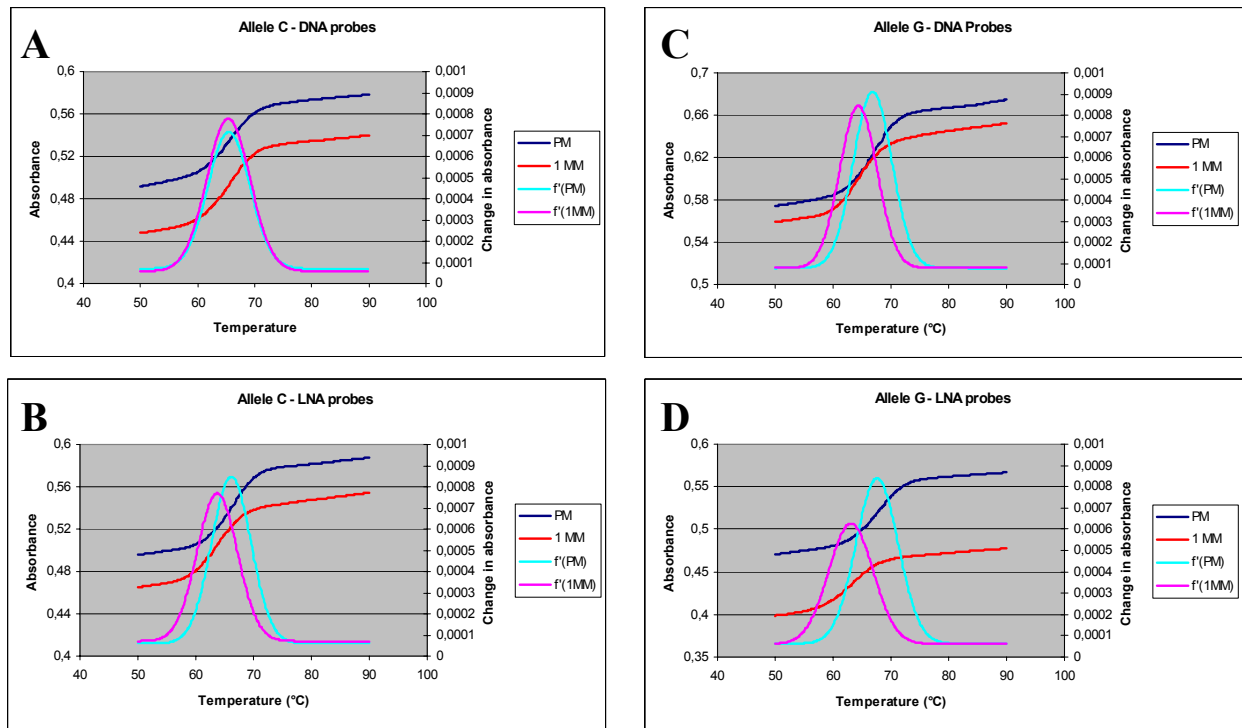


Figure 1: Melting properties of DNA and LNA substituted oligonucleotides. The melting curves in each panel A-D correspond to the entries A-D in Table 1. Blue and red lines indicate absorbance curves for perfect match and single mismatch duplexes respectively, whereas turquoise and pink show the first derivative of the perfect match and single mismatch duplexes respectively. The melting temperature, T_m , is defined as the temperature corresponding to the inflection point of the absorbance curve. The inflection point is easily determined as concurrent with the peak value of the first derivative. The peak position of the first derivative was therefore used to find the melting point of the oligonucleotides. Figure A and C: DNA oligonucleotides. Figure B and D: oligonucleotides with a single LNA substitution in the penultimate position. Melting curves were generated by measuring absorbance at 260 nm in a UV-spectrophotometer.

oligonucleotide primers may reduce amplification efficiency dramatically.

Method for T_m determination by UV-spectroscopy:

The analysis was performed by UV-spectroscopy (Perkin Elmer UV Lambda 40) measuring the absorbance at 260 nm of a duplex formed by a pair of the given oligonucleotides (1 μ M of each oligonucleotide in 100 mM NaCl, 10 mM Na-phosphate, 0.1 mM EDTA, pH 7.0). A final volume of 500 μ l was transferred to a 500 μ l quartz cuvette. Prior to recording of the melting curve, the sample was heated to 94 $^{\circ}$ C for five minutes and subsequently allowed to cool slowly to room temperature in order to obtain perfect annealing of the two constituent oligonucleotides. During measurement, the temperature was increased with 1 $^{\circ}$ C/min and the absorbance was recorded ten times every minute. The temperature in the cuvettes of the spectrophotometer was controlled by a Peltier element (Perkin Elmer PTP-6) with secondary water-cooling (Heto CBN8-30 and HMT200)

Example 2:

Melting properties of short oligonucleotides with multiple LNA substitutions investigated with a real-time thermocycler

It is generally assumed that the melting temperature of short DNA duplexes is reduced with approximately 1 to 1.5 $^{\circ}$ C per percent mismatch (Bonner et al.1973; Ausubel et al. 1987). It is therefore expected that the largest possible single-nucleotide mismatch discrimination is obtained with the shortest possible oligonucleotides. However, by shortening the probes, the melting temperature may be reduced below the practical limits for a given application. By substituting LNA for DNA at selected positions within these oligonucleotide sequences, it is possible to increase the melting temperature and furthermore improve the ΔT_m for single mismatch discrimination. For single nucleotide polymorphism (SNP) genotyping, LNA spiked 12-mer oligonucleotides are often used as probes. An example is shown in Table 2 and Figure 2, where 12-mer oligonucleotide probes were hybridized to 30-mer targets with either perfect match or a single mismatch centrally positioned in the target. For these relatively short oligonucleotides, a ΔT_m of more than 20 degrees is often observed even for a single mismatch. This remarkable level of discrimination is very rarely possible to

obtain with the longer DNA oligonucleotide probes required to get a similar thermal stability.

The melting properties described here were investigated by measuring the fluorescence intensity of the intercalating fluorophore SYBR[®] Green I when it was bound to double stranded oligonucleotides. When the temperature was increased and the two oligonucleotide strands started to dissociate, the fluorophores were no longer intercalating and therefore lost their fluorescence. Melting of the two strands could therefore be quantified by the ensuing drop in fluorescence. The temperature dependence of the observed fluorescence intensity was measured with a real time PCR instrument as described below. It should be noted that some intercalating fluorophores have been shown to destabilize oligonucleotide duplexes, notably those that are not perfectly matched. This may in some cases affect the measured melting temperature.

Method for generating melting curves with a real-time PCR instrument:

The experiments presented here were performed on a single color real-time PCR instrument (Opticon, MJ-Research) by measuring the fluorescence elicited by intercalation of SYBR[®] Green I in DNA duplexes. T_m of the oligonucleotides were measured in 25 μ L degassed 0.3x standard sodium citrate buffer (SSC) with 0.1% Tween 20. The final oligonucleotide concentration was 2 μ M of each oligo and SYBR[®] Green I was added to a final concentration of 1x according to the manufacturers guidelines. The samples were initially incubated at 95°C for 5 minutes and then cooled slowly during 15 min to 15°C. Melting curves were subsequently generated by increasing the temperature from 15 to 95 °C. Temperature was increased in steps of 1°C and kept constant for 30 sec before fluorescence was measured.

| EQ No | Oligo Name | Probe Sequence | Target Sequence | Match | T_m (°C) | ΔT_m |
|-------|--------------|-----------------------|---|-------|------------|--------------|
| 8664 | CYP2D6+2480C | tgccAgcca G Cg | tgcata t cccagc g ctggctggcaaggtcc | PM | 59 | 18 |
| | | | tgcata t cccagc g ctggctggcaaggtcc | 1 MM | 41 | |
| 8665 | CYP2D6+2480T | gCcagcca A Cgc | tgcata t cccagc g ctggctggcaaggtcc | PM | 66 | 18 |
| | | | tgcata t cccagc g ctggctggcaaggtcc | 1 MM | 48 | |
| 8668 | CYP2D6+2575C | cTcgggg G gGct | gacc c agcccagcc c ccccgagac c tgact | PM | 68 | 15 |
| | | | gacc c agcccagcc a ccccgagac c tgact | 1 MM | 53 | |
| 8669 | CYP2D6+2575A | ctCgggg T ggCt | gacc c agcccagcc a ccccgagac c tgact | PM | 63 | 16 |
| | | | gacc c agcccagcc c ccccgagac c tgact | 1 MM | 47 | |
| 8680 | CYP2D6+3288G | ggtCACac C cAg | acatcg t ccccctg g gtgtgacccat a tga | PM | 64 | 17 |
| | | | acatcg t ccccctg a gtgtgacccat a tga | 1 MM | 47 | |
| 8681 | CYP2D6+3288A | gTCaCa C TcAgg | acatcg t ccccctg a gtgtgacccat a tga | PM | 63 | 10 |
| | | | acatcg t ccccctg g gtgtgacccat a tga | 1 MM | 53 | |

Table 2: Melting properties of LNA substituted 12-mer oligonucleotides. The position of the single mismatch is indicated with red letters. LNA substitutions are shown with capital letters. The corresponding melting curves are shown in Figure 2. The 12-mer probes were hybridized to 30-mer targets with either perfect match (PM) or a centrally positioned single mismatch (1 MM). The melting properties were investigated by measuring fluorescence of the intercalating fluorophore in a real time PCR instrument. The T_m was found as the temperature corresponding to the peak of the first derivative of the fluorescence curve using an algorithm implemented in the instrument software.

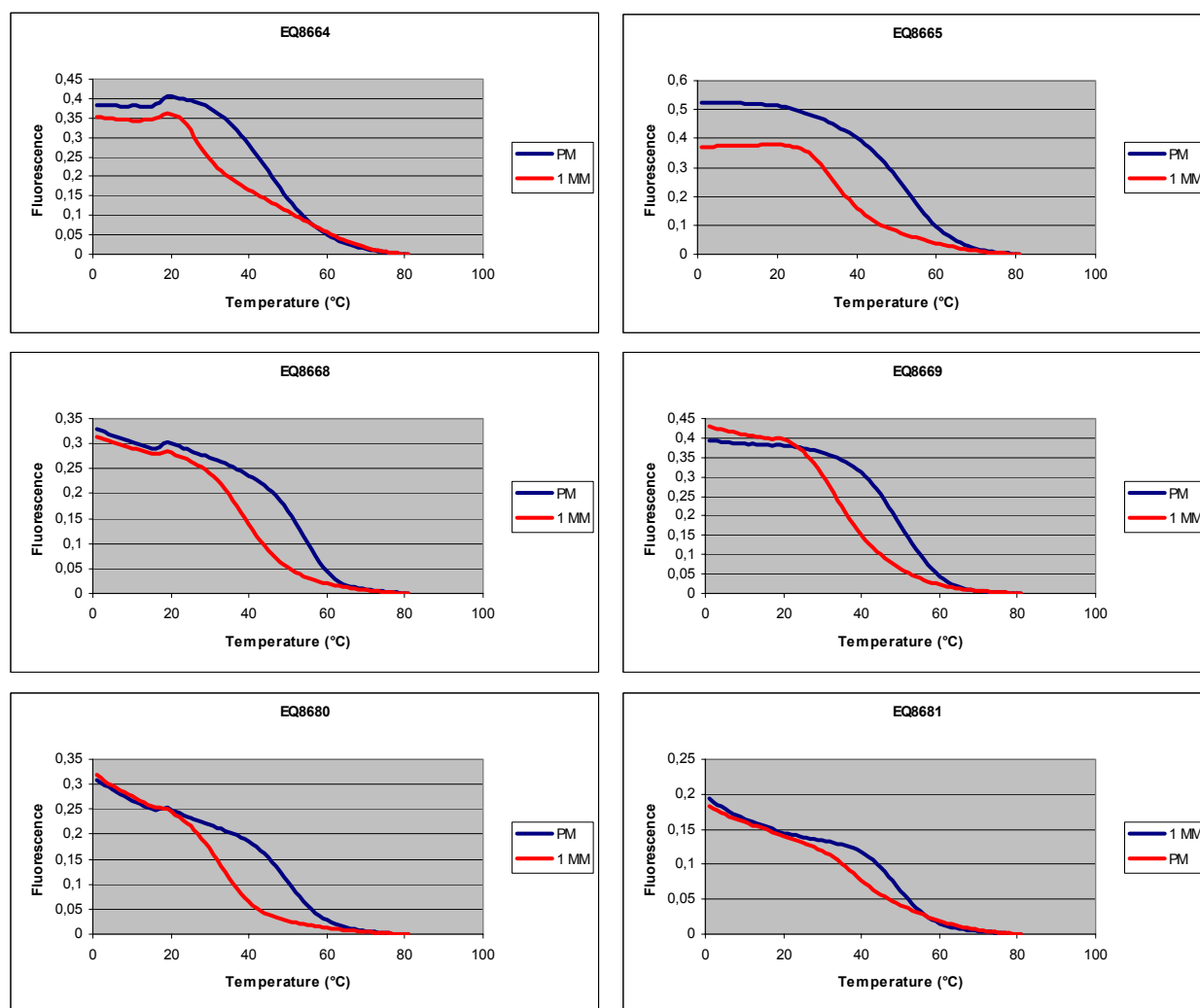


Figure 2: Melting profiles of LNA substituted oligonucleotides. The oligonucleotide sequences for the different panels are listed in Table 1. Blue and red lines indicate perfect match and single mismatch respectively. Melting curves were generated by measuring the fluorescence from an intercalating fluorophore that exclusively bind to double stranded DNA.

Example 3:

On-Chip measurement of melting properties of short immobilized LNA substituted oligonucleotide capture probes

Capture probes used on microarrays often exhibit melting properties that deviate from the properties observed in solution. The cause of these differences is unknown, but it is likely that the influence of the surface on the physical chemistry of the attached capture probes is of importance. A detailed understanding of the thermal stability of capture probes immobilized on DNA arrays is important to properly design experiments and interpret data from DNA array experiments. We have therefore measured the melting temperature of surface attached oligonucleotides at Exiqon. In these experiments, a fluorescently labeled oligonucleotide target is hybridized to a capture probe that has been covalently attached to a solid support (i.e. a microarray). Upon heating, the labeled target oligonucleotide dissociate, and the resulting reduction in fluorescence intensity can be quantified with a fluorescence

microscope setup (Figure 3). An example of this type of measurement is shown in Figure 4, where a 12-mer LNA substituted capture probe has been hybridized to 30-mer Cy5 labeled targets with either a perfect match or a single nucleotide mismatch. As frequently observed for hybridization experiments in solution, the ΔT_m for G-T mismatches is low. However, LNA substitutions in the probe sequence may increase the ΔT_m and thus lead to an improved discrimination.

Method for generating on-chip melting curves:

The microarrays were spotted on Immobilizer™ Microarray Slides (Exiqon, Denmark) with a spot to spot distance of 200 μm using a Packard BCA I non-contact spotter. The oligonucleotide probes were spotted with a concentration of 10 μM using the Spotting Solution from Exiqon. The microarray slides were hybridized for 2 hours at room temperature with the respective Cy5 labeled 30-mer targets at a concentration of 0.01 (M in 0.3x standard sodium citrate (SSC) and 0.1 % Tween 20. The slides were washed briefly with 0.3x SSCT at room temperature and dried by

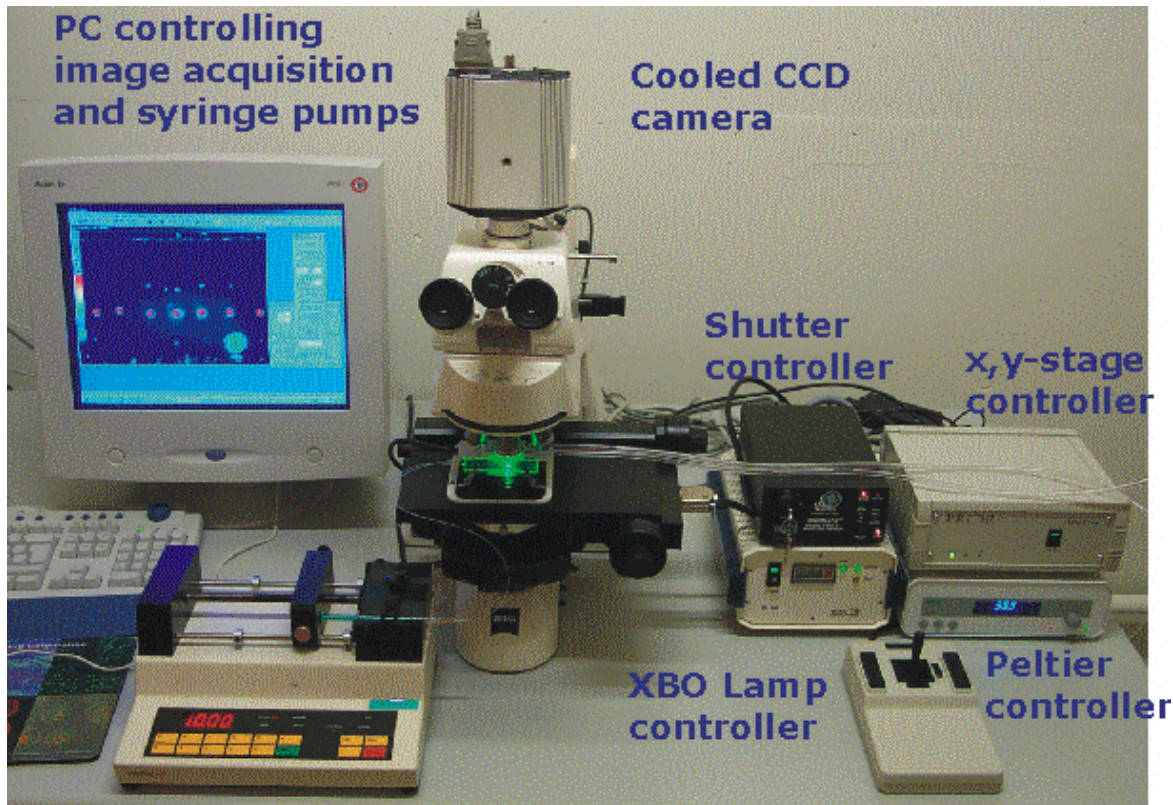


Figure 3: Fluorescence microscope setup for generating melting curves of surface attached oligonucleotides. The temperature of the stage was controlled with a peltier element and the temperature on the microarrays was measured with a thermo coupler.

centrifugation. 50 μ L of 0.3x degassed SSCT were added to the slide, a coverslip was mounted and sealed with nail polish. The spots in the microarray were visualized with a fluorescence microscope (Zeiss Axioskop 2) equipped with an XBO75 lamp, an automatic Uniblitz shutter (Vincent Associates, NY, USA), a cooled Photometrics Coolsnap cf CCD camera (Roper Scientific, NJ, USA)

and a stage with automated temperature control by a Peltier element (Linkam Scientific, UK). The temperature of the stage was increased linearly with 2.5°C per minute and fluorescence images were acquired every minute. The images were analyzed with ArrayVision software. The correlation between the temperature of the microscope stage and the temperature within the washing solution on the slide was subsequently determined using a thermo coupler with a diameter of 50 μ m, (produced by Unisense, DK) allowing direct measurement in the washing buffer between the slide and coverslip i.e. directly on the microarray.

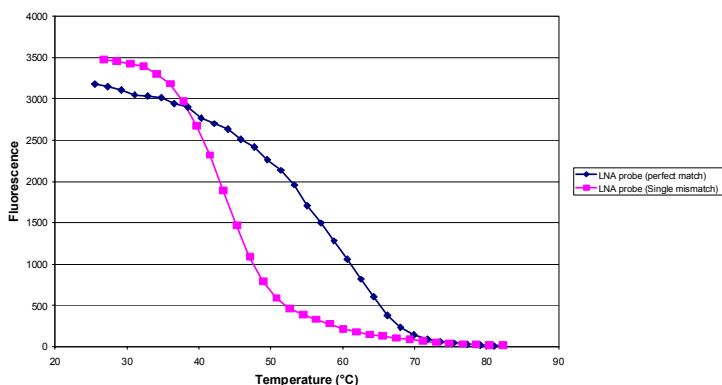


Figure 4: An example of on-chip melting profiles of surface attached LNA-substituted 12-mer capture probes targeting the HNF1-369 mutation (hybridized with a 30-mer target with either perfect or a single mismatch). The sequence of the oligonucleotides were 5'-gagacCCAcgAG-3' for the capture probe. This probe was hybridized with 5'gcttccctcgtgggtctcagcagctgggg3' (perfect match target) or with 5'gcttccctcgtagggtctcagcagctgggg3' (single mismatch target).

References:

Bonner T.I., Brenner D.J., Neufeld B.R. and Britten R.J. (1973). Reduction in the rate of DNA reassociation by sequence divergence. *J. Mol. Biol.* 81: 123-135.
 Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Smith J.A., Seidman J.G. and Struhl K. (1987). *Current Protocols in Molecular Biology*. New York: Wiley.

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