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FISHing with locked nucleic acids (LNA): evaluation of different LNA/DNA mixmers

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Abstract

Locked Nucleic Acids (LNA) constitute a novel class of DNA analogues that have an exceptionally high affinity towards complementary DNA and RNA. Using human classical satellite-2 repeat sequence clusters as targets, we demonstrate that LNA/DNA mixmers oligonucleotides are excellent probes for FISH combining high binding affinity with short hybridization time and even with the ability to hybridize without prior thermal denaturation of the template.

Keywords: Locked nucleic acids; LNA; FISH; SATELLITE-2

1. Introduction

The development of molecular probes and image analysis has made fluorescence *in situ* hybridization (FISH) a powerful investigative tool. Although FISH has proved to be a useful technique in many areas, it is a fairly time-consuming procedure with limitations in sensitivity. Probes with higher DNA affinity may potentially improve the sensitivity of the technique. Thus, improvement in hybridization characteristics has been reported for the DNA mimic peptide nucleic acid (PNA) [1].

Locked nucleic acids (LNA) are a novel class of oligonucleotide analogues in which the ribose ring is constrained by a methylene linkage between the 2′-oxygen and the 4′-carbon. The bridge results in a locked 3′-endo conformation, reducing the conformational flexibility of the ribose and increasing the local organization of the phosphate backbone. LNA obeys the Watson–Crick base-pairing rule when hybridizing to complementary DNA and RNA, and LNA bases are linked by the same phosphate backbone as found in native DNA and RNA, allowing synthesis of interspersed LNA/DNA and LNA/RNA mixmers. LNA oligonucleotides are synthesized using conventional phosphoroamidite chemistry, allowing automated synthesis of pure as well as mixed oligonucleotides containing both LNA and DNA monomers. Introduction of LNA monomers into oligonucleotides induces an increase in thermal stability of heteroduplexes between +1 and +2 °C per modification (Table 1). The conformation restriction and increased thermal stability of the heteroduplexes increases the binding affinity of LNA towards complementary DNA and RNA sequences. Furthermore, an important practical advantage is that LNA is soluble in water, which makes its handling and the experiments simple [2,3].

This report describes the development of LNA/DNA mixmer oligonucleotides as probes for FISH on metaphase chromosomes and interphase nuclei. In each experiment a different LNA/DNA mixmer oligonucleotide of the same 23-bp human satellite-2 repeat sequence (attccattcgattccattcgatc) have been used [4]. Oligomers with various LNA content, different labels and hybridization conditions have been used and compared with each other and the optimal conditions have been determined for an efficient LNA–FISH protocol.

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Table 1
DNA/LNA mixmers for human satellite-2 repeat sequence used in this study

Name	LNA/DNA mixmers	LNA monomers	Tm[a]
DNA oligo	attccattcgattccattcgatc	0	60
Dispersed LNA	aTtccatTcgaTtccAttcgaTc	5	71
LNA-3	aTtcCatTcgAtTccAttCgaTc	8	77
LNA Blocks	aTTCcattcgATTccattcGATc	9	73
LNA-2	aTtCcAtTcGaTtCcAtTcGaTc	11	84

LNA modifications are in capital letters
[a] Tm value for each molecule has been calculated using Exiqon Tm Prediction program at http://lna-tm.com/

2. Materials and methods

2.1. Chromosome preparations

Chromosome preparations were made by standard methods from peripheral lymphocyte cultures of two normal males. Slides were prepared 1–4 days prior to an experiment and treated with RNAse (10 μg/μl) at 37 °C for one hour before hybridization.

2.2. Probe preparation

The 23 bp human satellite-2 repeat sequence, attccattcgattccattcgatc, was used for the LNA/DNA mixmers with different content and sequence order of LNA modifications (Table 1). All mixmer-oligonucleotides were synthesized with either Cy3 or biotin at the 5′ end. A DNA oligomer of the same sequence without any LNA modifications was used as a control in each experiment. All the oligonucleotide probes, with or without LNA modification, were kept frozen until used in aliquots of distilled water.

2.3. Fluorescence in situ hybridization

FISH was carried out as described previously [5] with the following modifications shown in Table 2. The amount of probe was 6.4, 10, 13.4 and 20 pmoles. Denaturation of the target DNA and the probe were performed at 75 °C for 5 min either separately using 70% formamide or simultaneously under the coverslip in the presence of hybridization mix containing 50% formamide. In addition, effect of no denaturation was also tested. Two alternative hybridization mixtures were used: 50% formamide/2xSSC (pH 7.0) / 10% dextran sulphate or 2xSSC (pH 7.0)/10% dextran sulphate. Hybridization times included 30 min, 1, 2, 3 h and overnight.

Hybridization temperatures included: 37, 55, 60 and 72 °C. Post washing was either as for standard FISH [5], or with 50% formamide/2xSSC at 60 °C, or without formamide. Hybridization signals with biotin labeled LNA/DNA mixmers were visualized indirectly using two layers of fluorescein labeled avidin (Vector Labs) linked by a biotinylated anti-avidin molecule, which amplified the signal 8–64 times. The hybridization of Cy3 labeled molecules, however, was visualized directly after a short washing procedure.

Table 2
Different conditions tried with FISH technique using LNA/DNA mixmers as probes

Probe amount	150 ng (20 pmoles) 100 ng (13.4 pmoles) 75 ng (10 pmoles) 50 ng (6.4 pmoles)
Denaturation	Separate denaturation of slide and probe at 75 °C for 5 min Simultaneous denaturation at 75 °C No denaturation
Hybridization mixture	50% formamide/2xSSC/10% dextran (pH 7.0) 2xSSC/10% dextran sulfate (pH 7.0)
Hybridization time	Overnight 5 h 3 h 1 h 30 min
Hybridization temperature	RT 37 °C 55 °C 60 °C 72 °C
Post wash	Normal FISH wash 60 °C 50% formamide No formamide wash

Slides were mounted in Vectashield (Vector Laboratories) containing 4′-6′-diamidino-2-phenylindole (DAPI). The whole procedure was carried out in the dark. The signals were visualized using a Leica DMRB epifluorescence microscope equipped with a SenSys charge-coupled device camera (Photometrics, Tucson, AZ), and IPLAB Spectrum Quips FISH software (Applied Imaging international Ltd, Newcastle, UK) within 2 days after hybridization. Twenty metaphases were analyzed after each hybridization experiment.

3. Results and discussion

Satellite-II DNA, composed of multiple repeats of 23 and 26 bp sequence, is especially concentrated in the large heterochromatic regions of human chromosomes 1 and 16, but is also found in the heterochromatic regions of chromosomes 9, 15, Y and in other minor sites like the short arms/satellites of the acrocentric chromosomes and some centromeric regions [6]. Satellite-2 sequences are found mainly as three units of 1.3, 1.8, and 2.3 kb on chromosome 1 and as a 6.2 kb block on chromosome 16 [4]. Classical satellite DNA can be visualized by FISH with

traditional genomic and DNA oligonucleotide probes [7,8]. Due to this and the presence of distinct major and minor sites of satellite-2 DNA in the genome, we used the 23-bp satellite-2 repeat sequence, attccattcgattccattcgatc, as a convenient model to test the efficiency of various DNA/LNA mixmers for FISH analysis and the effect of different experimental conditions by recording the number, location and strength of signals on each metaphase. To compare the efficiency of mixmers with different LNA content (Table 1) and to optimize the LNA–FISH protocol, different

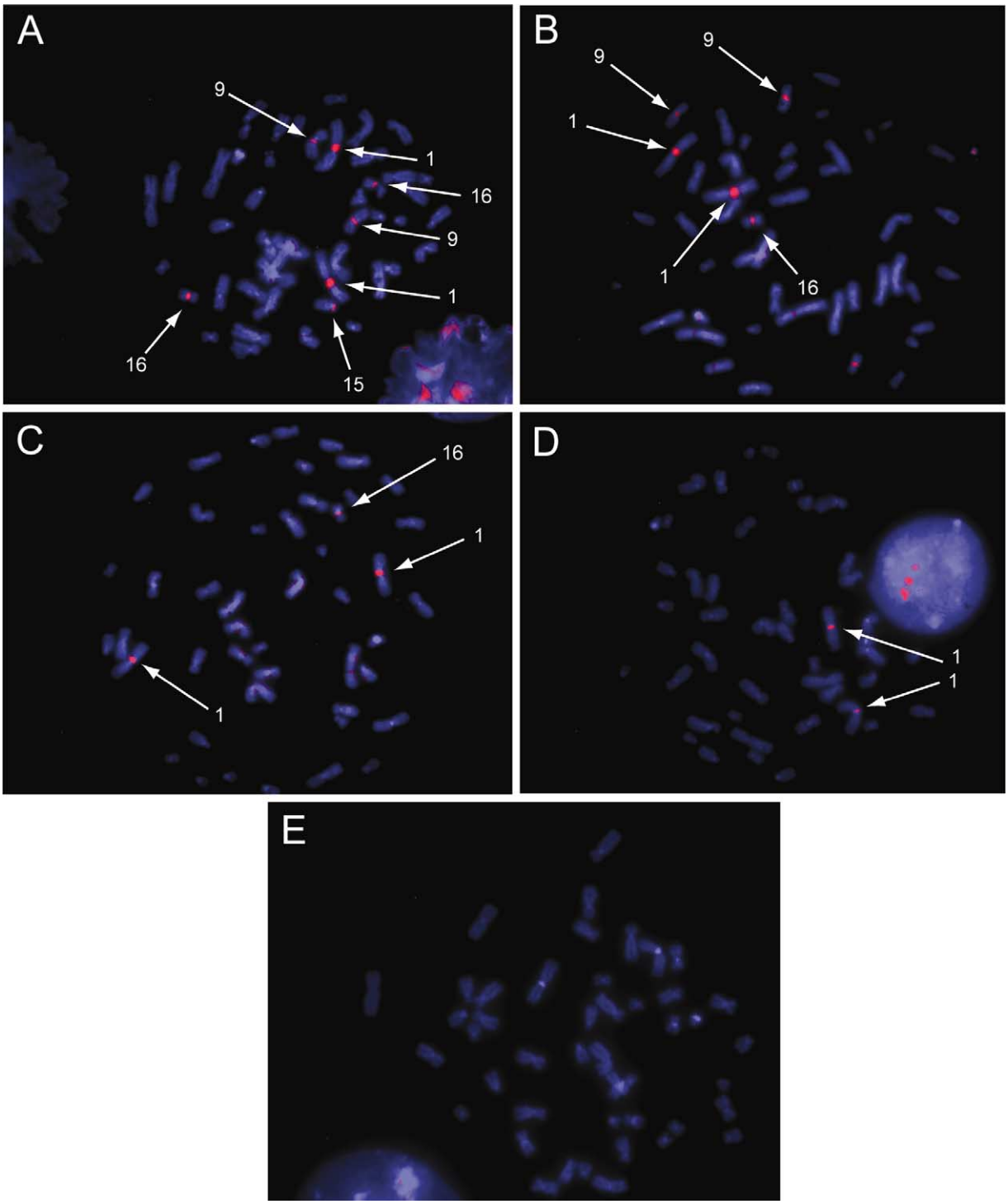

Fig. 1. Comparison of different LNA/DNA mixmer oligonucleotides. Experiment conditions: 6.4 pmoles of Cy3 labeled probe was hybridized for 30 min at 37 °C, after simultaneous denaturation of the target and the probe at 75 °C for 5 min. (A) LNA-2 giving signals on chromosomes 1, 16, 9 and 15, (B) LNA-3 giving bright signals on chromosomes 1, 16 and 9, (C) Dispersed LNA giving signals on chromosomes 1 and 16 only, (D) LNA Block giving smaller signals on chromosomes 1, (E) DNA oligo giving no signals on any of the chromosomes.

conditions were tried at each step of a standard FISH protocol as described in Section 2. Each condition was tried minimum two times on chromosome slides obtained from the same two individuals in order to exclude the individual variation in the heterochromatic regions.

All LNA/DNA mixmer oligonucleotides for human satellite-2 sequence gave very prominent signals when used as FISH probes. In general, the signal on chromosome 1 was always stronger and appeared earlier, followed by signals on chromosomes 16, 9, 15,Y and other acrocentric chromosomes and the paracentromeric regions of other chromosomes like 2 and 10, respectively (Fig. 1). In general, biotin labeled mixmers gave stronger signals with a higher background, whereas Cy3-labeled molecules gave a significantly lower background (Fig. 2).

3.1. Effect of LNA content of the LNA/DNA mixmers

The LNA-2 molecule, which had every other oligonucleotide modified as LNA, (aTtCcAtTcGaTtCcAtTcGaTc) gave the best results in all the experiments performed. The LNA-3 molecule, with every third oligonucleotide modified as LNA, (aTtcCatTcgAtTccAttCgaTc) also gave hybridization signals, but with less efficiency than the LNA-2 probes.

The Dispersed LNA (aTtccatTcgaTtccAttcgaTc), which had 5 dispersed LNA modifications, was much less efficient in short term hybridization, but gave signals on both chromosomes 1 and 16 s after overnight hybridization.

LNA/DNA mixmers with 3 LNA Blocks (aTTCcattcgATTccattcGATc) was inferior as a FISH probe.

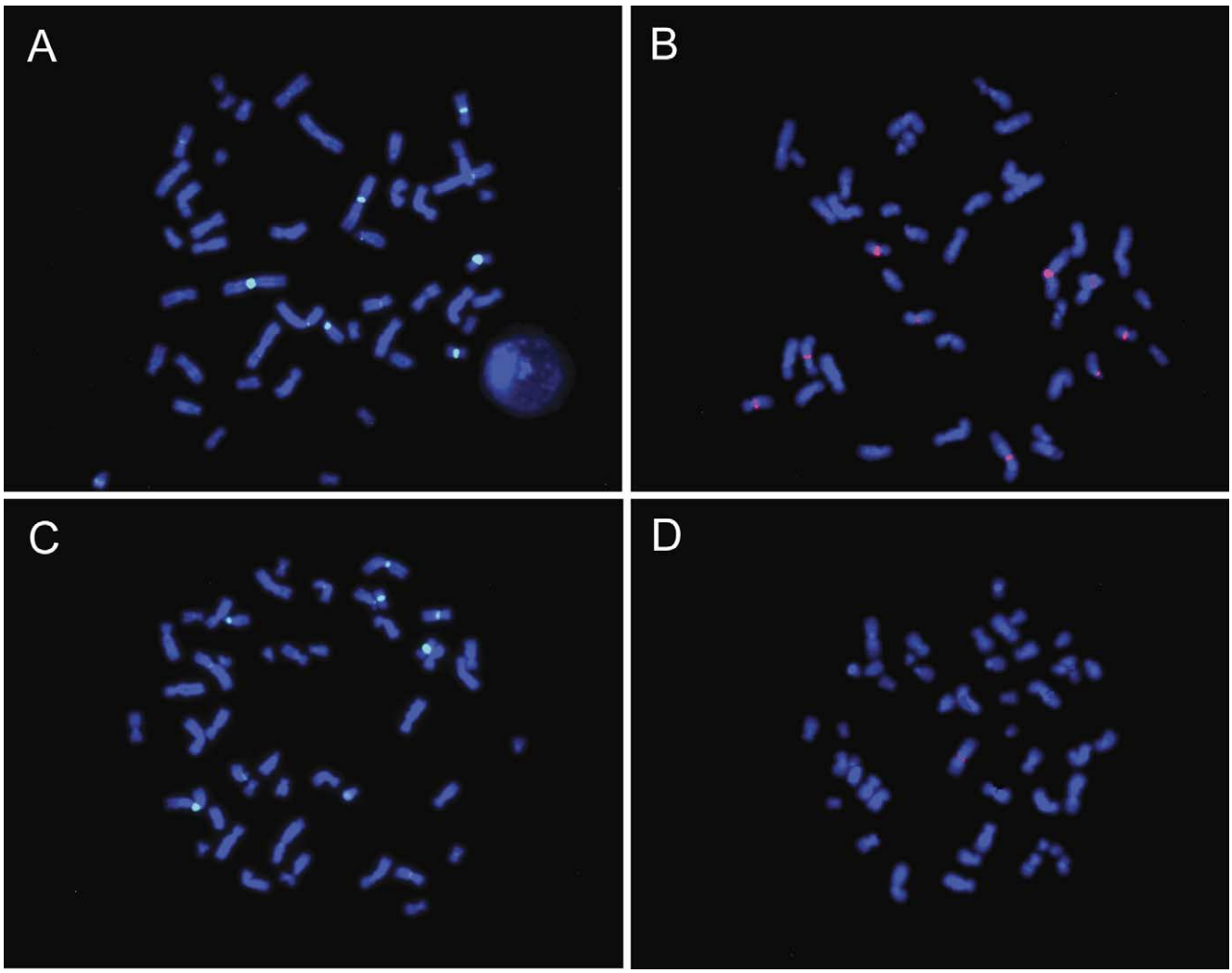

Fig. 2. Comparison of the labeling of the LNA-2 and DNA molecules. (A) Biotin labeled LNA-2 molecule, (B) Cy3-labelled LNA-2 molecule, (C) Biotin labeled DNA molecule, (D) Cy3 labeled DNA molecule. Hybridization was done overnight at 37 °C after simultaneous denaturation for 5 min at 75 °C. No formamide was used in the post hybridization washes. (A,B) Biotin labeled LNA-2 gives more signals than Cy3-labelling. (C,D) Biotin labeled DNA oligonucleotide gives smaller signals on many of the chromosomes while the Cy3-labelled DNA oligo only gives a very weak signal on only one chromosome 1.

3.2. Effect of amount of the LNA/DNA mixmers

The initial experiments performed with 20 pmol of LNA/DNA mixmer resulted in bright and large signals, but with an extremely high background. Thus, lower amounts were tested (13.4, 10, and 6.4 pmol). The amounts giving the optimal signal to noise ratio was found to be 6.4 pmol.

3.3. Effect of denaturation

The signals on the major sites of hybridization (1q, 16q) were equally bright after both types of denaturation. However, smaller and weaker signals were observed on the minor sites with the simultaneous denaturation protocol.

To check the potential 'strand invasion' property of LNA, some of the experiments were performed without a denaturation step. As expected, no signals were obtained by the control DNA oligonucleotide probe. In contrast, hybridization signals on chromosomes 1 and 16 were observed after overnight hybridization with LNA probes, with LNA-2 mixmer giving the best signals. Compared to the signals obtained in experiments involving a denaturation step, the signals were smaller, but prominent and without any background.

3.4. Effect of hybridization time, temperature and post-hybridization washes

Although signals could be observed after only 30 min of hybridization, the optimal hybridization conditions for LNA-2, which gave the best signals, were 1 h at 37 °C with hybridization buffer containing 50% formamide.

A 3 × 5-min wash with 0.1xSSC/60 °C and 4XSSC/0.05% Tween/37 °C, respectively, followed by a 5 min PBS wash was found to be sufficient for washing the slides after hybridization with DNA–LNA mixmers.

The signals faded away in most of the slides within 2 days. When hybridized with directly labeled LNA, the whole slide was stained with Cy3 after 3 days. Thus, slides had to be analyzed within 48 h after hybridization.

4. Conclusion

The experiments have demonstrated that LNA/DNA mixmer oligonucleotides are very efficient FISH probes. LNA/DNA mixmers gave strong signals after only 1 h of hybridization, and it was possible to omit the use of formamide both from the denaturation step and from the post hybridization washing step and still obtain a very good signal to noise ratio. The ability of LNA to hybridize without prior denaturation could be due to a strand invasion property of LNA, but this needs to be investigated further with other LNA/DNA probes and at different conditions. Based on the combined results of these experiments, the optimal LNA–FISH procedure was defined as follows: 6.4 pmoles of Cy-3 labeled LNA-2 probe was denatured together with the target at 75 °C for 5 min, and hybridized for one hour then followed by a short post wash without any formamide (3 × 5 min 0.1Xssc at 60 °C; 2 × 5 min 4XSSC/0.05% Tween at 37 °C; 5 min PBS). The FISH experiments indicate that LNA containing probes should be valuable for the detection of a variety of other repetitive elements, such as centromeric α-repeats and telomeric repeats. Other high affinity backbone designs such as PNAs have been used as a FISH probe for the detection of copy numbers of telomeric repeat sequences [9]. LNA probes work at least equally well or better with FISH technique and have the advantage of being water soluble and behaving like DNA with a much improved melting temperature. The superior hybridization characteristics of LNA containing oligonucleotides could lead to detection of base pair differences between repetitive sequences. Further studies should be performed to evaluate the potential use of LNA–FISH for detection of low copy repeats and single copy targets.

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