Technical Note

LNA™ for chromosomal FISH

LNA25/08.2003

Highly efficient fluorescence *in situ* hybridization (FISH) using LNA probes

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Abstract
LNA substituted LNA/DNA mixmer oligonucleotides designed for the human classical satellite-2 repeat sequence and human telomere repeat sequences were shown to be excellent probes for FISH combining their high binding affinity with short hybridization time.

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. This report describes the development of LNA/DNA mixmer oligonucleotides as probes for fluorescence *in situ* hybridization on metaphase chromosomes and interphase nuclei.

Results and discussion

Satellite-II DNA, composed of multiple repeats of a 23-bp and a 26-bp sequence, is especially concentrated in the large heterochromatic regions of human chromosomes 1 and 16 as well as 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. Another area of chromosomes, enriched in repeats, is found in the telomeres, consisting of a 6-bp repeat (ttaggg) sequence. (Jeanpierre, 1994, Moyzis *et al*.,1988).

In the present study LNA substituted oligonucleotides of either the 23-bp human satellite-2 repeat sequence (attccattcgattccattcgatc) or the 24-bp sequence composed of four blocks of the 6-bp telomere repeat (ttaggg) have been used. The different LNA designs of the LNA/DNA mixmer probes for the human satellite–2 repeat used here are listed in Table I. For the telomere specific LNA probe, only the LNA-2 design, with an LNA substitution at every second nucleotide position, was synthesized together with a DNA control. Oligonucleotide FISH probes with different LNA substitution patterns, labels and hybridization conditions were used in a comparative study and subsequently the optimal conditions were determined for an efficient LNA-FISH protocol. All LNA-containing oligonucleotides for human satellite-2 and the telomere repeats gave prominent signals when used as FISH probes. For the human satellite-2 sequence, the LNA-2 design gave the best hybridisation results in the experiments

performed. The LNA-3 probe, with every third oligonucleotide substituted with LNA, also gave hybridisation signals, albeit weaker than those obtained with the LNA-2 probes.

Table I. The LNA/DNA mixmer FISH probes for human satellite-2 repeat sequence used in this study*.

Name	LNA/DNA mixmers	LNA monomers
DNA oligo	attccattcgattccattcgatc	0
Dispersed LNA	aTtccatTcgaTtccAttcgaTc	5
LNA-3	aTtcCatTcgAtTccAttCgaTc	8
LNA Blocks	aTTCcattcgATTccattcGATc	9
LNA-2	ATtCcAtTcaGaTtCcAtTcGaTc	11

*LNA substitutions are depicted in capital letters

The Dispersed LNA probe, substituted with five dispersed LNAs (Table I), was less efficient in short term hybridisation, but gave signals on both chromosomes 1 and 16, respectively, after an overnight hybridisation. The probe with three LNA Blocks was clearly inferior as a FISH probe (Table I). In accordance with the satellite-2 FISH results, the human telomere repeat specific LNA probes were only synthesized with the LNA-2 design together with an unsubstituted DNA control. The human telomere repeat specific LNA oligonucleotide gave prominent signals on almost all telomeres, when used as a FISH probe (Fig. 2).

In general, biotin-labeled LNA probes gave stronger signals with a higher background, whereas Cy3-labeled FISH probes gave a significantly lower background concomitant with a lower, but prominent hybridization signal. The FISH results obtained with the DNA oligonucleotide probes were inferior to LNA substituted probes regardless of their label (Fig. 1).

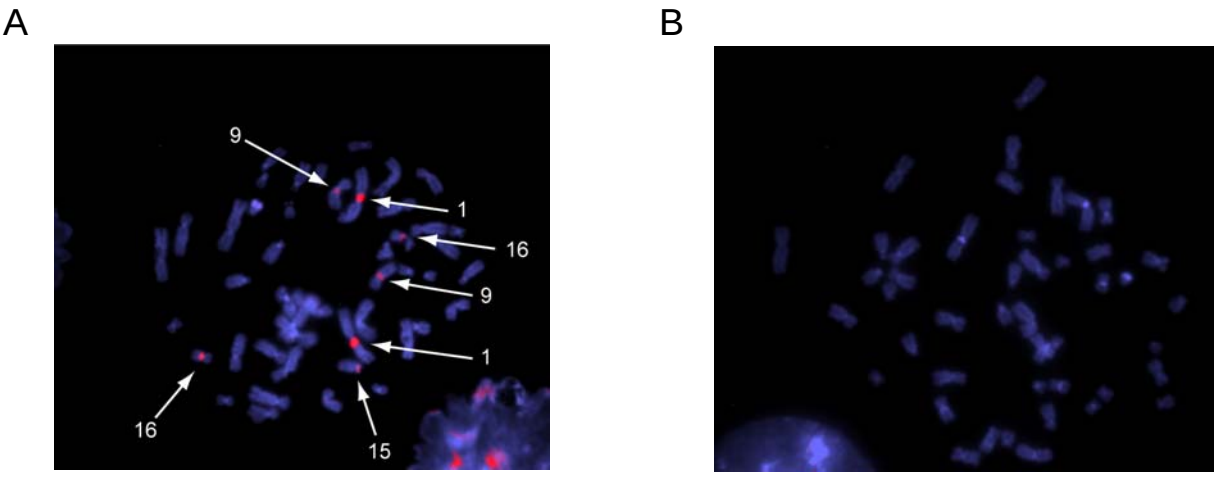

Figure 1. *Comparison of LNA (A) and DNA (B) probes in FISH for the human satellite-2 repeat. The use of Cy3-labelled LNA-2 probe in FISH results in strong hybridization signals on chromosomes 1, 9, 15 and 16, respectively, after hybridization for 30 min at 37℃ (A), whereas no signals can be detected with the DNA FISH probe (B).*

The hybridization signals faded away in most of the slides within two days. When hybridized with directly labeled LNA, the whole slide showed staining with Cy3 after three days. Thus, slides had to be analyzed within 48 hours after hybridization. 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 an overnight hybridization with LNA probes, with LNA-2 mixmer resulting in the strongest signals. Compared to the signals obtained in experiments involving a denaturation step, the signals were weaker, but still prominent and without any background.

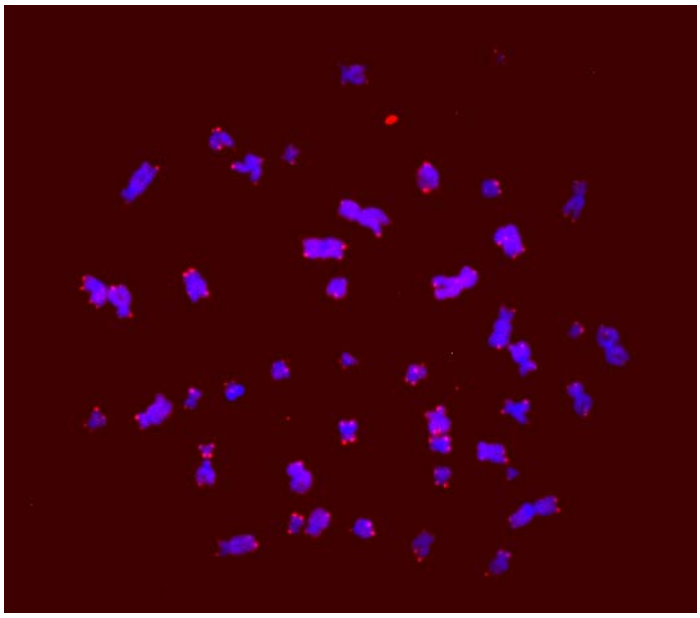

***Figure 2**. Hybridisation of the Cy3-labelled human telomere repeat specific, LNA-2 substituted oligonucleotide probe on human metaphase chromosomes results in prominent signals on almost all telomeres.*

The experiments described here demonstrate that LNA substituted oligonucleotides are very efficient FISH probes. LNA/DNA mixmers gave strong signals after only one hour 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. 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^{o}C for 5 minutes, and hybridized for one hour then followed by a short post wash without any formamide (3x 5 minutes 0.1xSSC at 60^{o}C; 2x 5minutes 4XSSC/0.05% Tween at 37^{o}C; 5 minute PBS). The FISH experiments indicate that LNA containing probes would be highly valuable for the detection of a variety of other repetitive elements. The superior hybridization characteristics of LNA substituted oligonucleotides could even enable detection of base pair differences between repetitive sequences. Further studies should be performed to evaluate the potential use of LNA-FISH in the detection of low copy repeats and single copy target sequences.

Experimental procedures

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 (10µg/µl) at 37^{o}C for one hour before hybridization.

Probe preparation
The 23-bp human satellite-2 repeat sequence, attccattcgattccattcgatc, or a 24-bp telomere sequence (ttagggttagggttagggttaggg) representing 4 blocks of 6-bp telomere repeat (ttaggg) were used for the LNA/DNA mixmers with different LNA substitution patterns (Table I). All mixmers oligonucleotides were both synthesized either with a Cy3 or a biotin group at the 5' end. An unsubstituted DNA oligonucleotide probe was used as a control in each experiment. All the oligonucleotide probes were kept frozen until used in aliquots of distilled water.

Fluorescence *in situ* hybridization
FISH was carried out as described previously (Silahtaroglu *et al*., 1998) with the following modifications. 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^{o}C for 5 minutes either separately using 70% formamide or simultaneously under the coverslip in the presence of hybridization mixture containing 50% formamide. In addition, the effect of 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 hr, 2 hrs, 3 hrs and overnight. Hybridization temperatures included: 37°C, 55°C, 60°C and 72°C. Post-washing was either as for standard FISH, 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 Laboratories, USA) linked by a biotinylated anti-avidin molecule, which amplified the signal 8-64 times. The hybridization of Cy3-labeled molecules was visualized directly after a short washing procedure. Slides were mounted in Vectashield (Vector Laboratories, USA) 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, USA), and IPLAB Spectrum Quips FISH software (Applied Imaging international Ltd., Newcastle, UK) within two days after hybridization. 20 metaphases were analyzed after each hybridization experiment.

Acknowledgments
This project is supported by Danish Research Agency (Project no: 2013-01-0033). Wilhelm Johannsen Centre for Functional Genome Research is established by the Danish National Research Foundation. Alla Jakobsen and Mette Bjoern are acknowledged for expert technical assistance.

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Latest revision August 12, 2003