Improved In Situ Hybridization Efficiency with Locked-Nucleic-Acid-Incorporated DNA Probes

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Low signal intensity due to poor probe hybridization efficiency is one of the major drawbacks of rRNA-targeted in situ hybridization. There are two major factors affecting the hybridization efficiency: probe accessibility and affinity to the targeted rRNA molecules. In this study, we demonstrate remarkable improvement in in situ hybridization efficiency by applying locked-nucleic-acid (LNA)-incorporated oligodeoxynucleotide probes (LNA/DNA probes) without compromising specificity. Fluorescently labeled LNA/DNA probes with two to four LNA substitutions exhibited strong fluorescence intensities equal to or greater than that of probe Eub338, although these probes did not show bright signals when they were synthesized as DNA probes; for example, the fluorescence intensity of probe Eco468 increased by 22-fold after three LNA bases were substituted for DNA bases. Dissociation profiles of the probes revealed that the dissociation temperature was directly related to the number of LNA substitutions and the fluorescence intensity. These results suggest that the introduction of LNA residues in DNA probes will be a useful approach for effectively enhancing probe hybridization efficiency.

The rRNA-targeted fluorescence in situ hybridization (FISH) technique has become a routine molecular tool for analyses of the microbial community (1, 3) since it was first applied by DeLong and coworkers in 1989 (8). However, the technique has limitations that need to be addressed before it can be applied to the detection of a wide range of diverse microbes (3). The major limitation is low fluorescence intensity due to various factors, such as low rRNA content of cells, impermeability of cell walls, and poor probe hybridization efficiency owing to secondary and tertiary structures of the rRNA and effects of ribosomal proteins (3).

Low probe hybridization efficiency is especially problematic. Fuchs et al. mentioned that half of newly designed probes failed to produce detectable signals during in situ hybridization (11). To overcome this problem, Amann and coworkers systematically investigated the accessibility to target sites with hundreds of 16S and 23S rRNA-targeted probes and finally concluded that the hybridization efficiency of probes depended on the accessibility associated with regional variations in the three-dimensional structure of the ribosome, resulting in different hybridization efficiencies within rRNA molecules (5, 10, 11). However, a further study on accessibility using a native ribosome model revealed that the hindrance of target sites by ribosomal proteins was negligible for a paraformaldehyde-fixed sample (4). In addition, rRNA base pairing was more important than rRNA tertiary structure for probe hybridization (4), suggesting that the denaturing of rRNA secondary structure is a crucial factor for probe hybridization. The probes used for the accessibility analyses were designed with theoretical dissociation temperatures (Tds), which were calculated using the formula developed by Suggs et al. (37), of between 48 and 60°C. Hybridization was performed at 46°C. Nonetheless, there were still problems hybridizing with some target sites, especially at stem regions of predicted rRNA secondary structures (5, 10, 11). The theoretical Td-based probe design was not sufficient to guarantee hybridization in situ (42).

Recently, Yilmaz and Noguera proposed a comprehensive model, in which the concept of the affinity of a probe to its folded nucleic acid target was introduced as a complementary idea to that of accessibility (42). In that model, accessibility was regarded as the ability of the probe to access the target site, regardless of whether hybridization will occur. The affinity was defined as the overall Gibbs free energy change ($\Delta G_{\text{overall}}^0$), which was estimated using a model consisting of the DNA-RNA (probe-rRNA hybrid), DNA-DNA (probe self-folding), and RNA-RNA (rRNA self-folding) interactions, and it was considered to be a significant predictor of hybridization efficiency (42). Based on the affinity concept, the probes were elongated to satisfy the $\Delta G_{\text{overall}}^0$ value for efficient hybridization. Consequently, over 90% of rRNA-targeted probes yielded moderate to high brightness after hybridization reached equilibrium (43). However, elongating probes in order to increase affinity may restrict the flexibility of probe design and decrease mismatch discrimination ability.

Recently, locked nucleic acid (LNA) has been developed as a novel RNA derivative nucleotide analog, in which the ribose ring is constrained by a methylene linkage between the 2’ oxygen and the 4’ carbon (Fig. 1), resulting in the locked C3’-endo conformation (18, 27). The introduction of LNA residues in oligonucleotides stabilizes the duplex by either pre-organization or increased base stacking (increasing melting temperature [Tm] 1 to 8°C against DNA and 2 to 10°C against
RNA by one LNA substitution in an oligonucleotide (6, 18, 23, 26, 29–31, 40). In addition, LNA is able to hybridize with DNA or RNA according to Watson-Crick base-pairing rules with higher selectivity (13, 14, 18, 24). Due to such advantages, LNA or LNA/DNA mixmers (hereinafter called LNA/DNA) have been used for single-nucleotide polymorphism analysis, real-time PCR, microarray, and other applications (13, 14, 20, 39). In FISH analysis, LNA-incorporated oligodeoxynucleotide probes (LNA/DNA probes) have been successfully applied to human chromosomes, micro-RNAs in zebrafish, mRNA in yeast cells, and so on (16, 25, 34, 35, 38, 41). These studies demonstrated that LNA/DNA probes could bring about high binding affinities with target sequences, resulting in sensitive detection.

From this background of knowledge, we were convinced that the use of such high-affinity LNA/DNA probes could be one solution for overcoming the problem of low hybridization efficiency of probes with RNA in whole cells.

MATERIALS AND METHODS

Bacterial culture and fixation. *Escherichia coli* TOPO10 (Invitrogen) was cultivated in LB medium at 37°C. *Comamonas testosteronei* (NBRC14951) was grown in medium 802 containing polypepton (10 g/liter), yeast extract (2 g/liter), and MgSO4 · 7H2O (1 g/liter) at 30°C. The cells were harvested during exponential growth phase, washed once in phosphate-buffered saline (PBS; 130 mM NaCl, 10.8 mM Na2HPO4, 4.2 mM NaH2PO4 [pH 7.2]), and air dried at 60°C for 10 min. Dehydration was performed through ethanol series (50, 80, and 95% for 5, 1, and 1 min, respectively). Hybridization was carried out in buffer (20 mM Tris-HCl [pH 7.2], 0.01% sodium dodecyl sulfate, 900 mM sodium chloride, 0 to 80% formamide) with 0.5 μM of labeled probe for 3 to 4 h at 46°C in the dark. To remove excess probe, the slide was immersed in the same buffer for 15 min at 48°C.

Microscopic evaluation and quantitative analysis of fluorescence intensity. The samples were covered with SlowFade antifade reagent (Invitrogen) and evaluated on a BX50F microscope (Olympus, Tokyo, Japan) equipped with a 100-W high-pressure mercury burner. For image acquisition, a DP70 color charge-coupled-device camera (Olympus) was used and the exposure time was fixed for all preparations. Fluorescence intensities were quantified by using Quantity One image analysis software (Bio-Rad, Hercules, CA). All experiments were performed in triplicate.

RESULTS

In situ hybridization with DNA probes. First of all, we quantified the fluorescence intensities of DNA probes (Table 1). In this study, the fluorescence intensity was used as an indicator of probe hybridization efficiency. Furthermore, the fluorescence intensity of each probe was expressed as a relative intensity, based on defining the fluorescence intensity of Eub338 at 0% formamide as 1.00. The relative intensities of DNA probes and epifluorescence micrographs of Eub338, Eco468, Eco621, and Eco1068 at 0% formamide are shown in Table 1 and Fig. 2, respectively. Eco227 (class IV), Eco468 (class VI), Eco621 (class VI), Eco836 (class V), and Eco1068 (class V) were reported as dim probes, and Eco252 and Eco681 were classified in the brightest group (class I) by Behrens et al. (5). In this study, Eco468 and Eco621 showed weak signals (relative intensities, 0.05 ± 0.03 and 0.13 ± 0.02, respectively) as reported by Behrens et al. (5), while Eco227, Eco836, and Eco1068 gave relatively strong signals (relative intensities, 0.79 ± 0.07, 0.52 ± 0.06, and 0.44 ± 0.05, respectively). On the other hand, Eco252 and Eco681 (relative intensities, 0.98 ± 0.02 and 1.05 ± 0.03, respectively) employed as bright probes yielded approximately the same intensities as did Eub338, suggesting that the Eub338 probe produced quite strong signals under the experimental conditions of this study. Based on these findings, we selected three probes, Eco468, Eco621, and Eco1068, which gave weaker signals (low hybridization efficiencies), for further investigation.

In situ hybridization with LNA/DNA probes. Relative intensities of LNA/DNA probes at 0% formamide are listed in Table 1, and some examples of epifluorescence photomicrographs are shown in Fig. 2. One-LNA-substituted probes, designated LEco468-1, LEco621-1 and LEco1068-1, showed higher fluorescence intensities than those of the respective DNA probes. The increments of LEco468-1 and LEco621-1 were significant (changes in relative intensity [Δrelative intensity] were 0.47 and 0.63, respectively), whereas that of LEco1068-1 was trivial (Δrelative intensity, 0.13). Two-LNA-substituted probes (LEco468-2, LEco621-2, and LEco1068-2) were applied, and stronger signals were obtained relative to the single-substitution...
TABLE 1. Oligonucleotide probes used in this study

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Sequence (5'-3')</th>
<th>Relative intensity ± SD</th>
<th>FAd (% FA)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eco468</td>
<td>aac gtc aat gag cca aag t</td>
<td>0.05 ± 0.03</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>LECo468-1</td>
<td>aac gtc aat gag cca aGg t</td>
<td>0.52 ± 0.07</td>
<td>13</td>
<td>This study</td>
</tr>
<tr>
<td>LECo468-2</td>
<td>aac gtc aat gag cca aGg t</td>
<td>0.87 ± 0.04</td>
<td>24</td>
<td>This study</td>
</tr>
<tr>
<td>LECo468-2p</td>
<td>aac gtc Aat gag cAa aag t</td>
<td>0.83 ± 0.05</td>
<td>24</td>
<td>This study</td>
</tr>
<tr>
<td>LECo468-3</td>
<td>aac gtc aat gag cca aGg t</td>
<td>1.08 ± 0.02</td>
<td>35</td>
<td>This study</td>
</tr>
<tr>
<td>Eco621</td>
<td>aga tgc agt tcc ggt</td>
<td>0.13 ± 0.02</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>LECo621-1</td>
<td>aga tgc agt tcc cAg gtt</td>
<td>0.76 ± 0.07</td>
<td>30</td>
<td>This study</td>
</tr>
<tr>
<td>LECo621-1p</td>
<td>aga tgc agt Tcc gag ttt</td>
<td>0.82 ± 0.05</td>
<td>27</td>
<td>This study</td>
</tr>
<tr>
<td>LECo621-2</td>
<td>aga tgc agt tcc cAg gtt</td>
<td>1.12 ± 0.02</td>
<td>53</td>
<td>This study</td>
</tr>
<tr>
<td>LECo621-3</td>
<td>aga tgc agt tcc cAg gtt</td>
<td>1.03 ± 0.03</td>
<td>62</td>
<td>This study</td>
</tr>
<tr>
<td>Eco1068</td>
<td>aca ttt cac aac aCg AGc</td>
<td>0.44 ± 0.05</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>LEC1068-1</td>
<td>aca ttt cac aac aCg AGc</td>
<td>0.57 ± 0.07</td>
<td>12</td>
<td>This study</td>
</tr>
<tr>
<td>LEC1068-2</td>
<td>aca ttt cac aac aGg AGc</td>
<td>0.74 ± 0.11</td>
<td>17</td>
<td>This study</td>
</tr>
<tr>
<td>LEC1068-2p</td>
<td>aca ttt cac aac cGg AGc</td>
<td>0.87 ± 0.06</td>
<td>24</td>
<td>This study</td>
</tr>
<tr>
<td>LEC1068-2p2</td>
<td>aca ttt Cac aac aGg AGc</td>
<td>0.80 ± 0.03</td>
<td>23</td>
<td>This study</td>
</tr>
<tr>
<td>LEC1068-3</td>
<td>aca ttt cac aac cGg AGc</td>
<td>0.95 ± 0.05</td>
<td>29</td>
<td>This study</td>
</tr>
<tr>
<td>LEC1068-3p</td>
<td>aca ttt Cac aac cGg AGc</td>
<td>0.97 ± 0.06</td>
<td>32</td>
<td>This study</td>
</tr>
<tr>
<td>LEC1068-4</td>
<td>aca ttt Cac aac cGg AGc</td>
<td>1.00 ± 0.05</td>
<td>42</td>
<td>This study</td>
</tr>
<tr>
<td>Eub338</td>
<td>gct gcc tcc agg AGt</td>
<td>1.00 ± 0.05</td>
<td>46</td>
<td>2</td>
</tr>
<tr>
<td>LEB338-1</td>
<td>gct gcc tcc aGg AGt</td>
<td>1.03 ± 0.02</td>
<td>63</td>
<td>This study</td>
</tr>
<tr>
<td>LEB338-2</td>
<td>gCc gcc tcc aGg AGt</td>
<td>1.07 ± 0.02</td>
<td>82</td>
<td>This study</td>
</tr>
<tr>
<td>LEB338-2v3</td>
<td>gct gcc Tcc agg AGt</td>
<td>1.04 ± 0.02</td>
<td>75</td>
<td>This study</td>
</tr>
<tr>
<td>Eco227</td>
<td>tcc cat ctg ggc aca tc</td>
<td>0.79 ± 0.07</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>Eco252</td>
<td>agc cgt tae ccc ace ta</td>
<td>0.98 ± 0.02</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>Eco681</td>
<td>cat tcc act gcc cta ct</td>
<td>1.05 ± 0.03</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>Eco836</td>
<td>cca cgg ctc aag ggc ac</td>
<td>0.52 ± 0.06</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>GAM42a</td>
<td>gcc ttc cca cAg cgt tt</td>
<td>0.99 ± 0.04</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>LGAM42a-1</td>
<td>gcc ttc cca cAt cgt tt</td>
<td>1.01 ± 0.04</td>
<td>32</td>
<td>This study</td>
</tr>
<tr>
<td>LGAM42a-2</td>
<td>gcc ttc cca cAt cgt tt</td>
<td>1.07 ± 0.04</td>
<td>49</td>
<td>This study</td>
</tr>
<tr>
<td>BET42a</td>
<td>gcc ttc cca cTt cgt tt</td>
<td>ND*</td>
<td>ND</td>
<td>22</td>
</tr>
<tr>
<td>LBET42a-1</td>
<td>gcc ttc cca cTt cgt tt</td>
<td>ND</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>LBET42a-2</td>
<td>gcc tTc cca cTt cgt tt</td>
<td>ND</td>
<td>ND</td>
<td>This study</td>
</tr>
</tbody>
</table>

a ND, not determined.

b LNA/DNA probes have “L” at the beginning of the names and the number of LNA is indicated after the E. coli position.

c Bases written in uppercase letters are LNAs, and those in lowercase letters are DNAs. Underlined letters indicate mismatch sequences (Eub338 for A. thermophila and GAM42a for C. testosteroni).

d Except where indicated otherwise, relative intensity indicates the brightness of the probe at 0% formamide relative to the fluorescence intensity of Eub338 at 0% formamide.

e Results show the formamide concentration that yields the 50% probe dissociation.

f Results show the brightness of the probe at 55% formamide relative to the fluorescence intensity of Eub338 at 0% formamide.

LNA/DNA probes. The fluorescence intensity of LECo468-2 surpassed that of Eub338 (Table 1). By substituting three bases, LECo468-3 yielded stronger signals than that of Eub338 (Table 1) and LEC1068-3 was close to Eub338. Finally, LEC1068-4 (four substitutions) yielded the same fluorescence intensity as did Eub338 (Table 1). Ultimately, after the substitution of two to four bases in the probes, the relative intensities of Eco468, Eco621, and Eco1068 increased by 22-, 8.6-, and 2.3-fold, respectively.

Next we investigated the positional effects of LNA substitutions. It is known that substitutions with isolated LNA residues in central positions have a greater effect on Tm than substitutions clustered at termini (7, 19). The following probes were compared: LECo468-2 versus LECo468-2p, LECo621-1 versus LECo621-1p, LEC1068-2 versus LEC1068-2p1 versus LEC1068-2p2, LEC1068-3 versus LEC1068-3p, and LEB338-2 versus LEB338-2v3 (Table 1). It was found that probes having LNA substitutions at different positions yielded different intensities, but the effect was less than that of an additional LNA substitution (Table 1). Interestingly, under low-stringency hybridization conditions, nonspecific hybridization was observed for LEB338-2v3 but not LEB338-2 (Fig. 3). This suggests that specificity might be influenced by the positions of the LNA substitutions. LEB338-2v3 was able to hybridize specifically under high-stringency hybridization conditions (see below).

Effects of LNA substitutions on probe affinity. Next, we investigated whether probe affinities were enhanced by substituting with LNA. Since affinity directly correlates with Tm, changes in affinity can be estimated by examining the Tm shift. Nevertheless, hybridization at high and various temperatures is not recommended because of the adverse effects of high temperatures on the samples and the technical difficulty of setting up such an experiment. Therefore, in this study, we changed the formamide concentration in the hybridization buffer and determined the formamide concentration that yields 50% probe dissociation (FAd; the FAd is equivalent to the Td of the
probe). Determined values of FAd for all probes are listed in Table 1, and dissociation curves of the two representative sequences (Eub338 and Eco621) are shown in Fig. 3. The augmentation of the FAd of each probe was observed with an increasing number of LNA substitutions. The increment was 5 to 20% for any single substitution. These results confirmed that the affinities of probes were improved by LNA substitutions, resulting in enhanced fluorescence. Although the FAd increased with substituted LNA number in Eub338, little enhancement in intensity was observed (Table 1 and Fig. 3). In addition, the series of GAM42a and LEco621-2 and LEco621-3 showed behavior similar to that of Eub338 (Table 1).

Effects of LNA substitutions on probe specificity. Since substitutions of LNA bases for DNA bases brought about significant improvements in in situ hybridization efficiency, the effects of LNA substitutions on probe specificity were investigated. First, to examine the discrimination ability of LNA/DNA probes against single-mismatch sequences, the GAM42a probe, which is specific for the *Gammaproteobacteria* (*E. coli*) and has a single mismatch relative to *Bacterioproteobacteria* (*C. testosteroni*), was employed (22). As previously indicated, without the competitor probe BET42a, it was difficult for the GAM42a probe to differentiate *E. coli* from *C. testosteroni* under hybridization conditions that did not result in a reduction of fluorescence intensity (22). The LNA/DNA probes LGAM42a-1 and LGAM42a-2 have LNA substitutions at the mismatch position (Table 1). Likewise, we could not distinguish between *E. coli* and *C. testosteroni* with the LNA/DNA probes without a loss of signal intensity. Hybridization with the competitor probes LBET42a-1 for LGAM42a-1 and LBET42a-2 for LGAM42a-2 successfully differentiated *E. coli* from *C. testosteroni* without compromising signal intensity.

Next, the Eub338 probe, which is specific for most of the domain *Bacteria* but has two mismatches relative to *A. thermophila*, a member of the *Chloroflexi* subphylum group I (33), was employed. As was previously described elsewhere (33), Eub338 did not hybridize to *A. thermophila*. likewise, the LNA/DNA probes LEub338-1, LEub338-2, and LEub338-2v3 resulted in no signals from *A. thermophila* under the optimized hybridization conditions for each probe.
DISCUSSION

Improvement of in situ hybridization efficiency. The results presented here demonstrate that the introduction of LNA nucleotides into DNA probes is useful for improving the efficiency of hybridization with rRNA targets. Three probe sequences, Eco468, Eco621, and Eco1068, evaluated in this study showed dramatic signal enhancements after substituting two to four LNA bases for DNA bases. The difference was attributable to increased probe affinity for the target sequence, which was further verified by elevation of the FAd.

One significant finding in this study is that the number of LNA substitutions in the oligonucleotide is the most important factor for improving hybridization efficiency. In each tested probe sequence, the number of LNA nucleotides correlated with the fluorescence intensity before the signal reached a plateau (Fig. 4). In addition, the number of LNA nucleotides correlated with the FAd (Table 1). From these results, we observed that the fluorescence intensity correlated with the FAd and reached a plateau when the FAd was higher than approximately 35% (Fig. 4). In other words, the hybridization efficiency of probes having a FAd of over 35% formamide must be high. This observation is in agreement with the study of Yilmaz and Noguera (42), in which elongated probes were found to improve hybridization efficiency but generally increase FAd. On the other hand, it was difficult to predict the hybridization efficiency of LNA/DNA probes according to the concept of $\Delta G_{\text{overall}}$ proposed by Yilmaz and Noguera (42) because the thermodynamic values of LNA substitutions are available to date for only the LNA/DNA-DNA hybrid (23) (data not shown). If additional information, such as the thermodynamic effects of LNA substitutions on LNA/DNA-RNA hybrid, were available, the theoretical prediction of hybridization efficiency using thermodynamic parameters might be possible, which could be helpful for determining the number of LNA substitutions in oligonucleotides.

Besides the LNA used in this study, attempts have been made to improve hybridization efficiency by using artificial nucleotide analogs, which have higher binding affinity than DNA. Peptide nucleic acid (PNA) (Fig. 1) is well known for its applications to the FISH technique (28, 36). Because of the uncharged backbone of PNA, PNA probes show higher affinities than do DNA probes, with increased sequence selectivity (9). However, PNA probe synthesis is limited in terms of probe length and sequence. Therefore, it is sometimes difficult to design PNA probes with sufficient specificities (28). On the other hand, although the number of substitutable LNA nucleotides in a probe is limited, there are no other restrictions for designing LNA/DNA probes. Hence, LNA/DNA probes offer added design flexibility relative to PNA probes.

The nucleotide analog 2'-O-methyl RNA (Fig. 1) can also be used for probe synthesis. 2'-O-methyl RNA probes show improved hybridization efficiency for extracted rRNA (21). However, LNA has a greater effect on Tm than does 2'-O-methyl RNA (15, 19), indicating an advantage of LNA substitutions for significant improvement in probe affinity.

In addition to utilizing artificial nucleotide analogs, Yilmaz and Noguera reported an improvement of hybridization efficiency by probe elongation (42). However, LNA/DNA probes have several advantages over elongated probes. One advantage is the added flexibility of probe design. The elongation may restrict the available sequences for which a probe could be designed. Another advantage of LNA/DNA probes is accessibility. Yilmaz and Noguera showed that elongated probes hybridized insufficiently after short hybridization times, possibly due to limited accessibility to target sites (42). On the other hand, hybridization for 3 h was sufficient without formamide for the LNA/DNA probes used in this study.

Specificity of LNA/DNA probes. The specificity of oligonucleotide probes was investigated by using the GAM42a and Eub338 probes. In this study, LNA substitutions were carried out at the mismatch positions (Table 1). Practically, LNA/DNA probes can be readily used for in situ hybridization as hybridization with competitor probes allowed easy discrimination for single mismatches without a loss of signal intensity. Furthermore, two mismatches can easily be discriminated without competitor probes.

The effects of LNA substitutions on mismatch discrimination have been investigated. The sequence selectivity of the LNA/DNA oligonucleotides is, if anything, improved compared to that of the corresponding DNA oligonucleotides (18,
24). Furthermore, LNA oligonucleotides show increased ΔTms between the fully complementary sequence and the one-mismatch sequence compared to those of the DNA oligonucleotides (13, 14, 24). Thus, substituting multiple LNA residues might result in higher specificities. However, LNA/DNA probes in which LNA and DNA nucleotides alternate showed strong nonspecific binding due to greatly increased affinity and it was difficult to determine the FAd and the optimum hybridization conditions for these probes (data not shown). The number of LNA substitutions needs to be controlled in order to maximize both the hybridization efficiency and the specificity.

Conclusion and future perspectives. From these results, we can recommend an approach to probe design that will enhance hybridization efficiency. The oligonucleotide should have a dissociation point higher than 35% formamide, which can normally be achieved by one to four LNA substitutions in a probe of typical length (i.e., 18 to 25 bases). The positions of the LNA substitutions within the oligonucleotide and the continuity of LNA substitutions (7, 19) may be important in some cases. Additional aspects of LNA/DNA probe design, such as the effects of LNA substitutions on probe secondary structure, will require further study. It is, however, recommended that LNA substitutions at self-complementary regions should be avoided due to strong base pairing in LNA-LNA hybrids (17, 32).

Our results also suggest that LNA/DNA probes will be applicable for the identification of microorganisms in natural environments. LNA/DNA probes targeting variable regions of 16S rRNA, where hybridization efficiency is often low (11), would be designable without compromising specificity. Furthermore, the introduction of LNA residues in oligonucleotides probably improves hybridization efficiency for not only rRNA but also mRNA and DNA. LNA/DNA probes would be useful for the detection of mRNA and genes on chromosomes with oligonucleotide probes.

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