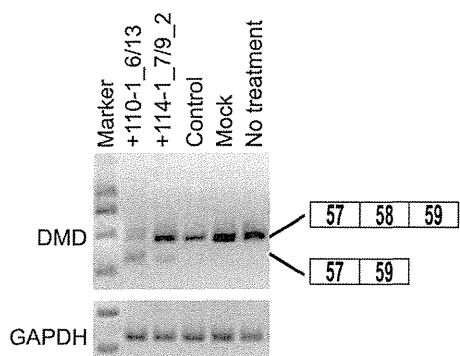


**Figure 7.** Assessment of specificity of LNA SSO. (A and B) Schematic representation of the position of LNA in the 13-mer SSOs (A) and the 9-mer SSOs (B) used in this study. The sequence in the box indicates a mismatch. Capital letter A, G, T: LNA; C: 5-methyl cytosine LNA; lowercase letter: DNA. (C) The reporter cells were transfected with the indicated LNA/DNA mixmer SSOs (30 nM) for 24 h. RT-PCR analyses were performed as described in Figure 3B. (D) The levels of exon 58-skipped mRNA fragments were measured by quantitative real-time RT-PCR (for details see Materials and Methods and Figure 2G). Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments.



**Figure 8.** Exon skipping by LNA SSOs in primary human skeletal muscle cells. HSMC cells were transfected with the indicated LNA/DNA mixmer SSOs (500 nM) for 24 h. RT-PCR analyses show the full-length upper band (575 bp) and the skipped lower band (454 bp). LNA SSO (+10+24), which showed no exon skipping effects, was used as a control. GAPDH was used as an internal control.

the 2'-OMe modification resulted in an increase of only  $\sim 1^\circ\text{C}$  per nucleotide incorporated (47). UV melting experiments performed under low-sodium conditions (10 mM phosphate buffer (pH 7.2) containing 10 mM NaCl) revealed that the  $T_m$  values of LNA SSO (five LNA and ten DNA) and 2'-OMe SSO (five 2'-OMe and ten DNA) were  $69.5^\circ\text{C}$  and  $50.1^\circ\text{C}$ , respectively (Supplementary Table S4). Thus, it seems that a higher melting temperature is associated with higher SSO activity. However, SSOs fully modified with LNA showed low activity despite their high  $T_m$  values. LNA-modified oligonucleotides often form stable self-structures (hairpin or self-dimer) (48). In particular, fully modified LNA SSOs might impair skipping activity because of self-dimerization, which led to decrease of effective SSO concentration for mRNA targeting (data not shown). In addition, we and others previously reported that AONs that possess very high binding affinity exhibit a relatively weak silencing ability (49,50). One of the reasons for this is that because AONs dissociated from the RNase H-dependent cleaved target mRNA could enter a new round of catalysis, LNA AONs with too high affinity might reduce the dissociation rate. Thus, LNA AONs are thought to require optimal binding affinity for efficient turnover activities in antisense reaction (51,52). Indeed, higher antisense effects were obtained by using LNA AONs whose  $T_m$  values were less than  $65^\circ\text{C}$  (10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl) (49,50). In the case of exon skipping, SSO is also thought to be recycled after dissociation from the excised region (53). Thus, the values are different from those obtained for AONs, and there may be an optimum  $T_m$  range to design effective SSOs that incorporate LNA. To date, few studies have shown that LNA can be used in SSOs both *in vivo* and *in vitro* (17,18,19,20,21,22), and no information is available regarding the effects of the position and number of LNAs. Here, we report for the first time that SSOs fully modified with LNA have lower activity than LNA/DNA mixmer SSOs. This result may at least partially be explained by the optimization of  $T_m$  values described above and/or by the kinetics of duplex formation between LNA-based SSOs and RNA (54). Christensen reported that the rate

of association of full-length LNA-based 10-mer oligonucleotides to complementary RNA was lower than that of a LNA/DNA mixmer (five LNA and five DNA) and the association constant of the full-length LNA-based oligonucleotide to complementary RNA was 1.5- to 2-fold less than that of the LNA/DNA mixmer in the presence of magnesium ions. Therefore, SSOs fully modified with LNA may be too rigid to use as splicing modulators, in contrast to LNA/DNA mixmers.

Third, our study of SSO length indicates that optimal lengths exist for LNA SSOs to modulate splicing. The quantitative real-time RT-PCR results indicated that the 13-mer SSO showed the highest effectiveness for exon skipping, whereas exon skipping activities of the above 15-mer LNA SSOs were decreased with increasing length (Figure 4). In this experiment, we used SSOs in which a LNA monomer was introduced on every other base. Therefore, longer SSOs exhibited higher binding abilities with smaller  $K_d$  values (Supplementary Table S6). These results are in good agreement with the  $T_m$  values. Thus, the decreased exon skipping activity should be brought by the other reason, such as intra- or intermolecular structures due to high number of LNAs in the SSO sequence (see above). On the other hand, in these experiments, all SSOs had a PS backbone in their sequence to provide nuclease resistance (55). Although the PS backbone decreases the  $T_m$  by  $\sim 1^\circ\text{C}$  per substitution (56,57), the PS-LNA oligonucleotides have still high  $T_m$  values due to high affinity of LNA for the complementary RNA (Supplementary Tables S4 and S5) (58). Thus, PS backbone did not influence the binding affinity of PS-LNA SSO toward complementary RNA very much. Taken together, these ideas may explain why shorter LNA SSOs showed high splicing activity, and why 13-mer LNA/DNA SSO mixmers had the highest effectiveness for exon skipping.

Although Ittig *et al.* showed that a 9-mer fully modified LNA SSO has no significant exon skipping effect (20), we here demonstrated, for the first time, that LNA SSOs as short as 7 mers have the potential to modulate splicing in a concentration-dependent manner provided that they are highly modified and display high  $T_m$  values (Figures 5 and 6). Shorter SSOs may have a further advantage in that the production costs of oligonucleotide drugs are higher than that of small molecules; therefore, shorter oligonucleotides may provide a cost-effective solution to the development of oligonucleotide drugs. Although, the activity of the 9-mer SSO (four LNA and five DNA) was weaker than that of the 13-mer SSO, quantitative real-time RT-PCR experiments revealed that the expression of skipped mRNA was similar among 9-, 19-, 21- and 23-mer SSOs (Figure 4). Intriguingly, when we compared two 9-mer LNA/DNA mixmer SSOs containing seven LNA analogues at different positions in each sequence, one of them presented 1.5-fold higher activity than the other despite their similar  $T_m$  values ( $87.1^\circ\text{C}$  and  $83.1^\circ\text{C}$ ) (Supplementary Table S7). Thus, the position of LNA analogues in the SSO sequence may be an important factor for exon skipping. Of note, the 9-mer 2'-OMe SSO (four 2'-OMe and five DNA), which has a low  $T_m$  value ( $40.0^\circ\text{C}$ ), exhibited no exon skipping activity at all (Figure 5).

Kandimalla *et al.* reported that short oligonucleotides (9-mers) bind more specifically than longer oligonucleotides (such as 21-mers), possibly because longer oligonucleotides have a higher chance of binding to various target sequences containing up to two mismatches than do short oligonucleotides, given the sufficient  $T_m$  values of longer oligonucleotides for forming duplexes with mismatched sequences (59). Indeed, Guterstam *et al.* demonstrated that the 18-mer PS LNA/2'-OMe mixmers with four mismatches, including one LNA mismatch, induced exon skipping (17). On the other hand, Obad *et al.* reported that 8-mer LNAs, termed tiny LNAs, inhibit microRNA activity without off-target effects (60). In this study, we evaluated the sequence specificity of LNA/DNA mixmer SSOs by introducing mismatches. The 13-mer LNA SSO (+110-1\_6/13) containing one LNA mismatch was able to induce exon skipping, while the exon skipping activity is abolished when one to three LNA mismatches are introduced in the center of the 9-mer LNA SSO (+114-1\_7/9\_2). Thus, the 9-mer LNA SSO improved mismatch discrimination in comparison with the 13-mer LNA SSO. However, in our *in silico* analysis, the number of target genes that have perfect match with 9-mer LNA SSO (+114-1\_7/9\_2) is far larger than that of the 13-mer LNA SSO (+110-1\_6/13) (914 genes and 8 genes, respectively) (Table 1). Although the ability to discriminate between the matched and mismatched sequences is improved by shorter SSO, these results suggest that it is important to design LNA SSOs in consideration of off target-effects.

In conclusion, we found that the number of LNAs in the SSO sequence, the  $T_m$  of the SSOs and the length of the LNA SSOs are key factors for their activity. We also show for the first time that 7-mer LNA SSOs induce exon skipping. Our findings suggest that LNA SSO-mediated exon skipping may be an attractive therapeutic strategy for genetic diseases.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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[Supplementary Data]

## Design and evaluation of locked nucleic acid-based splice-switching oligonucleotides in vitro

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### SUPPLEMENTARY MATERIAL AND METHODS

#### Thermodynamic analysis of various lengths of LNA/DNA mixmer SSOs duplexes

Gibbs free energy change ( $\Delta G^{\circ}_{25}$ ) during the duplex formation of 9-mer SSO (+114-1\_4/9) and the complementary RNA was calculated by standard thermal UV melting analysis (61). Dissociation constant ( $K_d$ ) for the duplex was determined using the equation,  $K_d = 1/\exp(-\Delta G^{\circ}/298.15R)$ , where  $R$  was gas constant. Because the binding of our LNA/DNA mixmer SSOs to mRNA was too strong to determine the dissociation constant directly from calorimetric or stopped flow experiments,  $\Delta G^{\circ}_{25}$  and dissociation constant at 25°C for the other SSOs were estimated by competition assays.

In our analysis, equal amount of a 5' 6-carboxyfluorescein (6-FAM)-labeled SSO (A strand) and the complementary RNA (B strand) was mixed with various amount of a competitor SSO (X strand) in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl. The initial concentration of each (A and B) strand was  $C_0$  ( $6.67 \times 10^{-8}$  M). To give equal opportunities to the competing strands (A and X) for hybridization with RNA (B strand), B strand was added to the solution in last. The solution was heated to 100°C and annealed to 25°C with cooling rate 0.5°C/min. After separation by 15% non-denaturing PAGE, the gel was analyzed using an ImageQuant LAS4010 (GE Healthcare Bio-sciences AB, Uppsala, Sweden). The fluorescence intensity that corresponds to the amount of a labeled duplex ( $F_{AB}$ ) was measured by using ImageJ software. The decrement of the  $F_{AB}$  with increasing free competitor SSO concentration  $[X]$  was fitted to the following equation with an assumption  $[A] \gg K_{d(AB)}$ , where Max and BG respectively indicated maximum change of  $F_{AB}$  and background fluorescence intensity, and half maximal inhibitory concentration of the competitor SSO ( $IC_{50}$ ) was determined (Supplementary Figure S11).

$$F_{AB} = \text{Max}(1 - [X]/([X] + IC_{50})) + BG$$

In this analysis,  $IC_{50}$  is defined as the concentration of free competitor SSO at which half of the labeled duplex is present at equilibrium. The analysis was carried out at least three times independently and the average  $IC_{50}$  values were adopted. The relative affinities between two competing molecules were calculated as follows.

$$K_{d(BX)}/K_{d(AB)} = 2 \times IC_{50}/C_0$$

$$\Delta\Delta G^{\circ}_{25} = \Delta G^{\circ}_{25(BX)} - \Delta G^{\circ}_{25(AB)} = 298.15R \ln(K_{d(BX)}/K_{d(AB)})$$

Based on the resulting  $\Delta\Delta G^{\circ}_{25}$  and actual measured  $\Delta G^{\circ}_{25}$  value for 9-mer SSO (+114-1\_4/9),  $\Delta G^{\circ}_{25}$  and  $K_d$  values for the other SSOs were estimated.

## SUPPLEMENTARY TABLE AND FIGURES LEGENDS

**Supplementary Table S1.** SSOs used for the first screening. Nine SSOs for dystrophin exon 58 skipping are shown. Sequences are shown from 5' to 3'. Capital letter A, G, T: LNA; C: 5-methyl cytosine LNA; lowercase letter: DNA.

**Supplementary Table S2.** SSOs used for the second screening. Twenty-nine SSOs for dystrophin exon 58 skipping are shown. Sequences are shown from 5' to 3'.

**Supplementary Table S3.** SSOs used for the third screening. Ten SSOs for dystrophin exon 58 skipping are shown. Sequences are shown from 5' to 3'.

**Supplementary Table S4.** SSOs targeting 5' splice site used for  $T_m$  and exon skipping analysis. SSOs for each experiment are shown. Sequences are shown from 5' to 3'.  $T_m$  values (low salt: 2  $\mu$ M duplex in 10 mM phosphate buffer (pH 7.2), 10 mM NaCl (n = 4); medium salt: 2  $\mu$ M duplex in 10 mM phosphate buffer (pH 7.2), 100 mM NaCl (n = 3)) were determined ( $\pm$  SD). Capital letter A, G, T: LNA; C: 5-methyl cytosine LNA; capital letter with underline: 2'-OMe RNA; lowercase letter: DNA.

**Supplementary Table S5.** SSOs targeting 3' splice site used for  $T_m$  and exon skipping analysis. SSOs for each experiment are shown. Sequences are shown from 5' to 3'.  $T_m$  values (2  $\mu$ M duplex in 10 mM phosphate buffer (pH 7.2), 10 mM NaCl) were determined in four independent experiments ( $\pm$  SD).

**Supplementary Table S6.** SSOs used for analysis of SSO length. SSOs for each experiment are shown. Sequences are shown from 5' to 3'.  $T_m$  values (2  $\mu$ M duplex in 10 mM phosphate buffer (pH 7.2), 10 mM NaCl) were determined in four independent experiments ( $\pm$  SD). Gibbs free energy change ( $\Delta G^{\circ}_{25}$ ) for SSO (+114-1\_4/9) was calculated by standard thermal UV melting analysis (2  $\mu$ M duplex in 10 mM phosphate buffer (pH 7.2), 100 mM NaCl). Dissociation constant ( $K_d$ ) for SSO (+114-

1\_4/9) was calculated using the equation:  $K_d = 1/\exp(-\Delta G^\circ/298.15R)$ .  $\Delta G^\circ_{25}$  and  $K_d$  for the other SSOs were estimated by competition assays.

**Supplementary Table S7.** SSOs used for analysis of 9-mer SSOs. SSOs for each experiment are shown. Sequences are shown from 5' to 3'.  $T_m$  values (2  $\mu$ M duplex in 10 mM phosphate buffer (pH 7.2), 10 mM NaCl) were determined in four independent experiments ( $\pm$  SD).

**Supplementary Table S8.** SSOs used for analysis of short SSOs. SSOs for each experiment are shown. Sequences are shown from 5' to 3'.  $T_m$  values (2  $\mu$ M duplex in 10 mM phosphate buffer (pH 7.2), 10 mM NaCl) were determined in three or four independent experiments ( $\pm$  SD).

**Supplementary Table S9.** SSOs used for mismatch discrimination. SSOs for each experiment are shown. Sequences are shown from 5' to 3'. Nucleotides of mismatch sites are double underlined.

**Supplementary Table S10.** Primers used for RT-PCR analysis. Sequences of forward (For.) and reverse (Rev.) primer for each target are shown. Sequences are shown from 5' to 3'.

**Supplementary Table S11.** Primers used for quantitative real-time RT-PCR analysis. Sequences of forward (For.) and reverse (Rev.) primer for each target are shown. Sequences are shown from 5' to 3'.

**Supplementary Figure S1.** Schematic representation of the dystrophin reporter minigene and of its splicing pattern. Human dystrophin exons are indicated by open boxes and introns by narrow lines. Solid boxes represent vector sequences. Lines connecting the exons represent the splicing patterns. The expected mRNA structures, indicated below the minigene structure result from inclusion or exclusion of exon 58. Small black arrows and small purple arrows indicate approximate positions of primers used for RT-PCR and quantitative real-time RT-PCR, respectively.

**Supplementary Figure S2.** Screening of LNA SSOs designed to induce dystrophin exon 58 skipping. **(A, B)** Reporter cells were transfected with the indicated SSOs (100 nM) for 24 h. The levels of reporter minigene mRNA fragments were measured by RT-PCR, and the signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. Values represent the mean  $\pm$  standard deviation of triplicate or sixplicate samples. Reproducible results were obtained from two independent experiments. (A) and (B) express the results of the first and the second screening, respectively. Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S3.** Prediction of splice factor binding site in human dystrophin exon 58. The locations of potential binding sites for the splicing factors SRSF1 (SF2/ASF), SRSF1 (IgM-BRCA1), SRSF2 (SC35), SRSF5 (SRp40), and SRSF6 (SRp55) in human dystrophin exon 58 and 50 bases of flanking intronic sequence were predicted by using ESEfinder3.0.

**Supplementary Figure S4.** Effect of the number of LNA variations on exon skipping activity. **(A, B)** Reporter cells were transfected with the indicated SSOs (30 nM), targeting the 5' (A) or 3' (B) splice site, for 24 h. The levels of reporter minigene mRNA fragments were measured by RT-PCR, and the signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. LNA SSO (+10+24), which showed no exon skipping effects, was used as a control. Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments. The  $T_m$  of each SSO with a complementary RNA under low-sodium conditions is also shown. # indicates that no sigmoidal melting curve was observed, even at higher  $T_m$  values. The data are the mean  $\pm$  standard deviation ( $n = 4$ ). Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S5.** Assessment of the effect of the length of LNA/DNA mixmer SSOs on exon skipping. Reporter cells were transfected with the indicated SSOs (30 nM) for 24 h. The levels of reporter minigene mRNA fragments were measured by RT-PCR, and the signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. LNA SSO (+10+24), which showed no exon skipping effects, was used as a control. Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments. The  $T_m$  of each SSO with a complementary RNA under low-sodium conditions is also shown. # indicates that no sigmoidal melting curve was observed, even at higher  $T_m$  values. The data are the mean  $\pm$  standard deviation ( $n = 4$ ). Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S6.** Exon skipping activity of LNA/DNA mixmer SSOs at various concentrations. **(A, C)** Reporter cells were transfected with the indicated SSOs, targeting the 3' (A) or 5' (C) splice site, at various concentrations for 24 h. The levels of reporter minigene mRNA fragments were measured by RT-PCR, and the signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. LNA SSO (+10+24), which showed no exon skipping effects, was used as a control. **(B)** RT-PCR analysis shows the full-length upper band (587 bp) and the skipped lower band (466 bp). GAPDH was used as an internal control. **(D)** The exon 58 skipping levels were measured by quantitative real-time RT-PCR and normalized to GAPDH mRNA levels, relative to the values in the mock set as 1. Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments. Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S7.** Inducing exon skipping by 9-mer LNA/DNA mixmer SSOs. Reporter cells were transfected with the indicated SSOs (30 nM) for 24 h. The levels of reporter minigene mRNA fragments were measured by RT-PCR and the signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. LNA



SSO (+10+24), which showed no exon skipping effects, was used as a control. Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments. The  $T_m$  of each SSO with a complementary RNA under low-sodium conditions is also shown. The data are the mean  $\pm$  standard deviation ( $n = 4$ ). Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S8.** 9-mer LNA SSOs induce exon skipping in a concentration-dependent manner. **(A-D)** RT-PCR analyses of RNA samples from reporter cells treated with the indicated SSOs at various concentrations for 24 h show the full-length upper band (587 bp) and the skipped lower band (466 bp). LNA SSO (+10+24), which showed no exon skipping effects, was used as a control. GAPDH was used as an internal control. **(E)** The signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments. Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S9.** Exon skipping activity of short (6- to 9-mer) LNA SSOs. RT-PCR analyses of RNA samples from reporter cells treated with the indicated SSOs (30 nM) for 24 h. The signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. LNA SSO (+10+24), which showed no exon skipping effects, was used as a control. GAPDH was used as an internal control. Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments. The  $T_m$  of each SSO with a complementary RNA under low-sodium conditions is also shown. The data are the mean  $\pm$  standard deviation ( $n = 3-4$ ). Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S10.** Effect of mismatches on exon skipping activity and specificity. Reporter cells were transfected with the indicated SSOs (30 nM) for 24 h. The levels of reporter minigene mRNA fragments were measured by RT-PCR, and the signal intensity of each band was normalized according to its nucleotide composition. The exon skipping percentage was calculated as the amount of exon skipped transcript relative to the total amount of exon skipped and full-length transcripts. LNA SSO (+10+24), which showed no exon skipping effects, was used as a control. Values represent the mean  $\pm$  standard deviation of triplicate samples. Reproducible results were obtained from two independent experiments. Mock: treated with Lipofectamine only; no treatment: no transfection.

**Supplementary Figure S11.** Quantitative analysis of competitive inhibition of 5' 6-FAM-labeled SSO duplex formation to the complementary RNA. Equal amount of a 5' 6-FAM-labeled SSO (A strand) and the complementary RNA (B strand) was mixed with various amount of an unlabeled competitor SSO (X strand) and annealed in 10 mM phosphate buffer (pH 7.2) containing 100 mM NaCl. After separation by 15% non-denaturing PAGE, the decrement of the fluorescence intensity that

corresponds to the amount of a labeled duplex (AB signal) with increasing free competitor SSO concentration [X] was curve fitted and half maximal inhibitory concentration of the competitor SSO ( $IC_{50}$ ) was determined.

**Supplementary Table S1.**

Entry	ID	Sequence
1	-5+10	tCt tGa aGg cCt gTg
2	+10+24	aGt tTt cAa tTc cCt
3	+25+39	gAt tAc aGg tTc tTt
4	+40+54	cTc aAg aGt aCt cAt
5	+55+69	aAa tAt tCg tAc aGt
6	+70+84	aGg cTg cTc tGt cAg
7	+85+99	cTc tAg tCc tTc cAa
8	+100+114	cTc cTg gTa gAg tTt
9	+115-8	tCa aTt aCc tCt gGg

**Supplementary Table S2.**

Entry	ID	Sequence
1-1	-20-6	aAa tGa gAt gAa aAg
1-2	-17-3	gTg aAa tGa gAt gAa
1-3	-14+1	cCt gTg aAa tGa gAt
1-4	-11+4	aGg cCt gTg aAa tGa
1-5	-8+7	tGa aGg cCt gTg aAa
1	-5+10	tCt tGa aGg cCt gTg
1-6	-2+13	cCc tCt tGa aGg cCt
1-7	+2+16	aTt cCc tCt tGa aGg
1-8	+5+19	tCa aTt cCc tCt tGa
1-9	+8+22	tTt tCa aTt cCc tCt
6-1	+58+72	cAg aAa tAt tCg tAc
6-2	+61+75	tGt cAg aAa tAt tCg
6-3	+64+78	cTc tGt cAg aAa tAt
6-4	+67+81	cTg cTc tGt cAg aAa
6	+70+84	aGg cTg cTc tGt cAg
6-5	+73+87	cAa aGg cTg cTc tGt
6-6	+76+90	tTc cAa aGg cTg cTc
6-7	+79+93	tCc tTc cAa aGg cTg
6-8	+82+96	tAg tCc tTc cAa aGg
9-1	+103+117	gGg cTc cTg gTa gAg
9-2	+106+120	tCt gGg cTc cTg gTa
9-3	+109-2	aCc tCt gGg cTc cTg
9-4	+112-5	aTt aCc tCt gGg cTc
9	+115-8	tCa aTt aCc tCt gGg
9-5	+118-11	cAt tCa aTt aCc tCt
9-6	+121-14	cCa cAt tCa aTt aCc
9-7	-3-17	gTt cCa cAt tCa aTt
9-8	-6-20	aTa gTt cCa cAt tCa
9-9	-9-23	aTt aTa gTt cCa cAt

**Supplementary Table S3.**

Entry	ID	Sequence
1-6-1	-4+11	ctC ttG aaG gcC tgT
1-6-2	-3+12	Cct Ctt Gaa Ggc Ctg
1-6	-2+13	cCc tCt tGa aGg cCt
1-6-3	-1+14	tcC ctC ttG aaG gcC
1-6-4	+1+15	Ttc Cct Ctt Gaa Ggc
9-2-1	+104+118	tgG gcT ccT ggT agA
9-2-2	+105+119	Ctg Ggc Tcc Tgg Tag
9-2	+106+120	tCt gGg cTc cTg gTa
9-2-3	+107+121	ctC tgG gcT ccT ggT
9-2-4	+108-1	Cct Ctg Ggc Tcc Tgg

**Supplementary Table S4.**

Entry	ID	Sequence	$T_m$ (°C)	
			Low salt	Medium salt
1	-1+14_15/15	TCC CTC TTG AAG GCC	>95.0	–
2	-1+14_8/15	TcC cTc TtG aAg GcC	82.6 ± 2.3	–
3	-1+14_7/15	tCc CtC tTg AaG gCc	84.6 ± 2.7	–
4	-1+14_5/15	tcC ctC ttG aaG gcC	69.5 ± 0.5	79.1 ± 0.2
5	-1+14_4/15	tcC ctC ttG aaG gcC	68.8 ± 2.6	74.8 ± 1.2
6	-1+14_3/15	tcC ctC ttG aaG gcC	62.1 ± 1.7	70.2 ± 1.2
7	-1+14_2/15	tcC ctC ttG aaG gcC	57.7 ± 2.1	66.1 ± 1.0
8	-1+14_1/15	tcC ctC ttG aaG gcC	55.1 ± 1.5	63.3 ± 0.2
9	-1+14_OMe	tcC ctC ttG aaG gcC	50.1 ± 1.8	58.4 ± 0.4
10	-1+14_DNA	tcc ctC ttg aag gcc	39.9 ± 1.3	52.2 ± 1.1

**Supplementary Table S5.**

Entry	ID	Sequence	$T_m$ (°C)
1	+108-1_15/15	<u>C</u> C <u>T</u> <u>C</u> T <u>G</u> <u>G</u> G <u>C</u> <u>T</u> C <u>C</u> <u>T</u> G <u>G</u>	>95.0
2	+108-1_8/15	<u>C</u> c <u>T</u> <u>c</u> T <u>g</u> <u>G</u> g <u>C</u> <u>t</u> C <u>c</u> <u>T</u> g <u>G</u>	>88.0
3	+108-1_7/15	<u>c</u> C <u>t</u> <u>C</u> t <u>G</u> <u>g</u> G <u>c</u> <u>T</u> c <u>C</u> <u>t</u> G <u>g</u>	87.8 ± 0.6
4	+108-1_5/15	<u>C</u> c <u>t</u> <u>C</u> t <u>g</u> <u>G</u> g <u>c</u> <u>T</u> c <u>c</u> <u>T</u> g <u>g</u>	79.0 ± 1.9
5	+108-1_4/15	<u>C</u> c <u>t</u> <u>C</u> t <u>g</u> <u>G</u> g <u>c</u> <u>T</u> c <u>c</u> <u>T</u> g <u>g</u>	73.8 ± 0.8
6	+108-1_3/15	<u>C</u> c <u>t</u> <u>C</u> t <u>g</u> <u>G</u> g <u>c</u> <u>U</u> c <u>c</u> <u>T</u> g <u>g</u>	66.8 ± 0.4
7	+108-1_2/15	<u>C</u> c <u>t</u> <u>C</u> t <u>g</u> <u>G</u> g <u>c</u> <u>U</u> c <u>c</u> <u>T</u> g <u>g</u>	63.8 ± 1.0
8	+108-1_1/15	<u>C</u> c <u>t</u> <u>C</u> t <u>g</u> <u>G</u> g <u>c</u> <u>U</u> c <u>c</u> <u>U</u> g <u>g</u>	61.7 ± 0.2
9	+108-1_OMe	<u>C</u> c <u>t</u> <u>C</u> t <u>g</u> <u>G</u> g <u>c</u> <u>U</u> c <u>c</u> <u>U</u> g <u>g</u>	57.4 ± 0.8
10	+108-1_DNA	<u>c</u> c <u>t</u> <u>c</u> t <u>g</u> <u>g</u> g <u>c</u> <u>t</u> c <u>c</u> <u>t</u> g <u>g</u>	53.0 ± 1.0

**Supplementary Table S6.**

Entry	ID	Sequence	$T_m$ (°C)	$\Delta G_{25}^\circ$ (kcal/mol)	$K_d$ (M)
1	+114-1_4/9	cCt CtG gGc	$66.7 \pm 0.4$	$-20.7 \pm 0.2$	$6.9 \times 10^{-16}$
2	+112-1_5/11	cCt CtG gGc Tc	$75.8 \pm 0.7$	-23.9	$3.2 \times 10^{-18}$
3	+110-1_6/13	cCt CtG gGc TcC t	$83.3 \pm 0.7$	-27.2	$1.2 \times 10^{-20}$
4	+108-1_7/15	cCt CtG gGc TcC tGg	$87.8 \pm 0.6$	-29.2	$3.7 \times 10^{-22}$
5	+106-1_8/17	cCt CtG gGc TcC tGg Ta	>88.0	-29.4	$2.6 \times 10^{-22}$
6	+104-1_9/19	cCt CtG gGc TcC tGg TaG a	>88.0	-29.7	$1.8 \times 10^{-22}$
7	+102-1_10/21	cCt CtG gGc TcC tGg TaG aGt	>88.0	-29.5	$2.3 \times 10^{-22}$
8	+100-1_11/23	cCt CtG gGc TcC tGg TaG aGt Tt	>88.0	-30.0	$1.1 \times 10^{-22}$



**Supplementary Table S7.**

Entry	ID	Sequence	$T_m$ (°C)
1	+114-1_4/9	cCt CtG gGc	66.7 ± 0.4
2	+114-1_7/9_1	cCT CTG GGc	87.1 ± 1.9
3	+114-1_7/9_2	CCT CTG gGc	83.1 ± 2.7
4	+114-1_OMe	cCt CtG gGc	40.0 ± 1.0

**Supplementary Table S8.**

Entry	ID	Sequence	$T_m$ (°C)
1	+114-1_9/9	CCT CTG GGC	87.0 ± 2.2
2	+114-1_7/9_2	CCT CTG gGc	83.1 ± 2.7
3	+114-1_4/9	cCt CtG gGc	66.7 ± 0.4
4	+115-1_8/8	CCT CTG GG	76.3 ± 1.5
5	+115-1_6/8	CCT CTg Gg	72.1 ± 0.4
6	+115-1_4/8	cCt CtG gG	61.0 ± 1.6
7	+116-1_7/7	CCT CTG G	72.8 ± 1.4
8	+116-1_5/7	CCT CtG g	57.7 ± 2.3
9	+116-1_3/7	cCt CtG g	47.9 ± 0.7
10	+117-1_6/6	CCT CTG	53.4 ± 2.8
11	+117-1_4/6	CCT cTg	45.2 ± 2.3
12	+117-1_3/6	cCt CtG	37.1 ± 2.1

Supplementary Table S9.

Entry	ID	Sequence
1	+110-1_6/13	cCt CtG gGc TcC t
2	+110-1_G117A	cCt Ct <u>A</u> gGc TcC t
3	+110-1_G117C	cCt Ct <u>C</u> gGc TcC t
4	+110-1_G117T	cCt Ct <u>T</u> gGc TcC t
5	+110-1_G115C/G117C	cCt Ct <u>C</u> g <u>C</u> c TcC t
6	+110-1_G115C/g116c/G117C	cCt Ct <u>C</u> <u>c</u> Cc TcC t
7	+114-1_7/9_2	CCT CTG gGc
8	+114-1_T118A	CCT C <u>A</u> G gGc
9	+114-1_T118C	CCT C <u>C</u> G gGc
10	+114-1_T118G	CCT C <u>G</u> G gGc
11	+114-1_G117C/C119G	CCT <u>G</u> T <u>C</u> gGc
12	+114-1_G117C/T118C/C119G	CCT <u>G</u> C <u>C</u> gGc

**Supplementary Table S10.**

Gene		Sequence	Size (bp)
Dystrophin reporter minigene	For. primer:	AACGGTACCAACGCTGCTGTTCTTTTCA	587 (Exon 58 inclusion)
	Rev. primer:	CTTGGAGCCGTAAGGAACT	466 (Exon 58 skipping)
DMD	For. primer:	AAACAATGGCAAGACCTCCA	
	Rev. primer:	CTGGACAGACGTGGAAAGAAA	
	Nested For. primer:	GTCTCTCAACATTAGGTCCCATT	575 (Exon 58 inclusion)
	Nested Rev. primer:	CTCCTCGAAGTGCCTTGACT	454 (Exon 58 skipping)
GAPDH	For. primer:	ACCACAGTCCATGCCATCAC	452
	Rev. primer:	TCCACCACCCTGTTGCTGTA	