INTRODUCTION

Antisense oligonucleotide (ASO) therapeutics have been developed extensively over the past several decades and are emerging as the third platform (after small molecules and biologics) for drug development, with more than 100 ASOs currently in clinical development (McClorey & Wood, 2015; Yamamoto, Nakatani, Narukawa, & Obika, 2011). ASOs are synthetic DNA/RNA-like single-stranded oligonucleotides which bind to RNA through sequence-specific Watson–Crick base pairings. A unique mechanism of toxicity for ASOs is hybridization-dependent off-target effects that can potentially occur due to the binding of ASOs to complementary regions of unintended RNAs. To reduce the off-target effects of ASOs, it would be useful to know the approximate number of complementary regions of ASOs, or off-target candidate sites of ASOs, of a given oligonucleotide length and complementarity with their target RNAs. However, the theoretical number of complementary regions with mismatches has not been reported to date. In this study, we estimated the general number of complementary regions of ASOs with mismatches in human mRNA sequences by mathematical calculation and in silico analysis using several thousand hypothetical ASOs. By comparing the theoretical number of complementary regions estimated by mathematical calculation to the actual number obtained by in silico analysis, we found that the number of complementary regions of ASOs could be broadly estimated by the theoretical number calculated mathematically. Our analysis showed that the number of complementary regions increases dramatically as the number of tolerated mismatches increases, highlighting the need for expression analysis of such genes to assess the safety of ASOs.
oligonucleotides were developed that do not induce RNase H1-mediated cleavage of mRNA, but instead act by sterically blocking targeted RNA without inducing its degradation. A representative example of steric blocking ASOs is splice-switching oligonucleotides (SSOs), which modulate pre-mRNA splicing and repair defective RNA to restore the production of functional proteins (Havens & Hastings, 2016). In 2016, two SSOs, eteplirsen (Exondys 51; Sarepta Therapeutics) and nusinersen (Spinraza; Biogen and IONIS Pharmaceuticals), were approved as treatments for Duchenne muscular dystrophy and spinal muscular atrophy, respectively (Aartsma-Rus & Krieg, 2017; Corey, 2017).

However, in common with all drugs, ASOs carry the risk of causing unintended toxicity. ASOs can cause toxicity through two mechanisms: hybridization-independent and hybridization-dependent effects (Frazier, 2015; Lindow et al., 2012). Toxicity via a hybridization-independent mechanism is due to interactions between ASOs and biomolecules such as proteins in a manner similar to the unintended toxicities of small molecules. This type of toxicity is unrelated to Watson–Crick base pairing to RNAs, but rather is caused by the chemical properties of ASOs. However, toxicity via a hybridization-dependent mechanism is characteristic of ASOs and is potentially caused by inadvertent binding of ASOs to unintended RNAs which have a sequence similar to the target RNA. Although there have been few reports of toxicity induced by hybridization-dependent off-target effects in clinical trials of ASOs to date, it was recently shown that the hepatotoxicity of gapmer ASOs is mediated by the RNase H1-dependent reduction in unintended off-target RNAs in mice (Burel et al., 2016; Kamola et al., 2017; Kasuya et al., 2016). This finding suggests that hybridization-dependent off-target effects are the important cause of hepatotoxicity in humans following the administration of gapmer ASOs, similar to those observed in rodents, and indicates the importance of analyzing hybridization-dependent off-target effects for assessing safety.

In light of the above, it would be useful to know the approximate number of complementary regions of ASOs, or off-target candidate sites of ASOs, given the oligonucleotide

![FIGURE 1](image-url) Schematic illustration of the number of complementary regions of antisense oligonucleotide (ASOs)
length and partial complementarity between oligonucleotides and their target RNAs. In general, the number of complementary regions increases as the oligonucleotides become shorter. The number of complementary regions also increases if mismatches between the oligonucleotides and their target RNAs are tolerated. However, the theoretical number of complementary regions with mismatches has not been reported to date. In this study, we mathematically calculated the theoretical number of complementary regions of ASOs with perfect matches or with several mismatches in the total size of human mRNA sequences. We also performed in silico analysis of several thousand hypothetical ASOs designed for three housekeeping genes to obtain the actual number of complementary regions of ASOs in whole human mRNA sequences using GGGenome (http://GGGenome.dbcls.jp/), a fast and sensitive search engine for nucleotide sequence databases.

| Item calculated | Theoretical formula |
|-----------------|---------------------|
| Theoretical number of complementary regions of \( n \)-mer ASOs with \( m \)-mer mismatches in the total size of human mRNA sequences | \( X(m) = \frac{3 \times 10^9 \times 0.019 \times \left( \frac{n}{m} \right) \times 3^m}{4^m} \) |
| Probability of existence of complementary regions of \( n \)-mer ASOs with \( m \)-mer mismatches in the total size of human mRNA sequences | \( Q(m) = 1 - \left(1 - \frac{1}{4^m} \left( \frac{n}{m} \right)^3 \times 3^m \right)^{3 \times 10^9 \times 0.019} \) |

ASO, antisense oligonucleotide.

### Table 2

Theoretical number of complementary regions of ASOs in the total size of the human mRNA sequences

| Length of ASO | 0 MM | 1 MM | 2 MMs | 3 MMs | 4 MMs | 5 MMs |
|---------------|------|------|-------|-------|-------|-------|
| 27            | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} |
| 26            | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | 2.0 \times 10^{-1} |
| 25            | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | 6.5 \times 10^{-1} |
| 24            | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | 1.7 \times 10^{-1} | 2.1 |
| 23            | <10^{-1} | <10^{-1} | <10^{-1} | <10^{-1} | 5.8 \times 10^{-1} | 6.6 |
| 22            | <10^{-1} | <10^{-1} | <10^{-1} | 1.3 \times 10^{-1} | 1.9 | 21 |
| 21            | <10^{-1} | <10^{-1} | <10^{-1} | 4.7 \times 10^{-1} | 6.3 | 64 |
| 20            | <10^{-1} | <10^{-1} | <10^{-1} | 1.6 | 20 | 195 |
| 19            | <10^{-1} | <10^{-1} | 3.2 \times 10^{-1} | 5.4 | 65 | 586 |
| 18            | <10^{-1} | <10^{-1} | 1.1 | 18 | 206 | 1,727 |
| 17            | <10^{-1} | 1.7 \times 10^{-1} | 4.1 | 61 | 640 | 4,989 |
| 16            | <10^{-1} | 6.4 \times 10^{-1} | 14 | 201 | 1,956 | >10^4 |
| 15            | <10^{-1} | 2.4 | 50 | 652 | 5,869 | >10^4 |
| 14            | 2.1 \times 10^{-1} | 8.9 | 174 | 2,087 | >10^4 | >10^4 |
| 13            | 8.5 \times 10^{-1} | 33 | 596 | 6,559 | >10^4 | >10^4 |
| 12            | 3.4 | 122 | 2,018 | >10^4 | >10^4 | >10^4 |
| 11            | 14 | 448 | 6,727 | >10^4 | >10^4 | >10^4 |
| 10            | 54 | 1,630 | >10^4 | >10^4 | >10^4 | >10^4 |

ASO, antisense oligonucleotide; MM, mismatch.

### 2.1 RESULTS

#### 2.1 Theoretical number of complementary regions of ASOs in the total size of human mRNA sequences

In general, the number of complementary regions increases as the oligonucleotides become shorter (Figure 1a). The number of complementary regions also increases if mismatches between oligonucleotides and their target RNAs are tolerated (Figure 1b). To estimate the theoretical number of complementary regions of ASOs, we first mathematically calculated the theoretical number of complementary regions with perfect matches or mismatches in the total size of human mRNA sequences. To this end, we hypothesized that the four bases (A, G, C and T) are used randomly in
the three billion ($3 \times 10^9$) base pairs of the human genome and in the protein-coding regions of mRNAs constituting 1.9% of the human genome (International Human Genome Sequencing Consortium, 2004). The calculation formulae and numerical results are shown in Tables 1 and 2, respectively. For complementary sequences with perfect matches, no sites (<1) are found theoretically for oligonucleotides longer than 13-mer, but the number of sites with perfect matches increases when the oligonucleotides are 12-mer or shorter (Table 2, see the column “0 mismatch”; 15-mer: <0.1, 14-mer: 0.21, 13-mer: 0.85, 12-mer: 3.4, 11-mer: 14, 10-mer: 54%). When mismatches are tolerated, the number of complementary sequences increases dramatically as the number of mismatches increases. For example, in the case of 13-mer oligonucleotides, the number of sites with perfect matches is <1 (0.85), that with one mismatch is 33, that with two mismatches is 596, that with three mismatches is 6,559 and that with more than four mismatches is $>10^3$ (Table 2, see the row “Length of ASO = 13”). For 18-mers (the length of nusinersen), the number of sites with zero mismatch or one mismatch is <0.1, and that with two mismatches, three mismatches, four mismatches and five mismatches is 1.1, 18, 206 and 1,727, respectively (Table 2, see the row “Length of ASO = 18”). For 20-mers (the length of mipomersen), the number of sites with zero mismatch, one mismatch or two mismatches is <0.1 and that with three mismatches, four mismatches and five mismatches is 1.6, 20 and 195, respectively (Table 2, see the row “Length of ASO = 20”).

### 2.2 Probability of existence of complementary regions of ASOs in the total size of human mRNA sequences

Next, we mathematically calculated the probability of existence of complementary regions of ASOs with perfect matches or mismatches in the total size of human mRNA sequences (Table 3). The calculation formula is shown in Table 1. For complementary sites with perfect matches, the probability that oligonucleotides longer than 16-mer have a match in the human mRNA sequences is less than 5% and that of 13- to 16-mer oligonucleotides ranges from 1.3% to 57% (Table 3, see the column “0 MM”; >18-mer: <0.1%, 17-mer: 0.33%, 16-mer: 1.3%, 15-mer: 5.2%, 14-mer: 19%, 13-mer: 57%). For oligonucleotides shorter than 12-mer, the probability that the oligonucleotides have a perfect match in the human mRNA sequences is more than 97% theoretically (12-mer: 97%, 11-mer>: 100%). When we focus on the complementary sites with mismatches, the probability that oligonucleotides have a match in the human mRNA sequences remarkably increases as the number of tolerated mismatches increases. For example, in the case of 18-mer

**TABLE 3** Probability of existence of complementary regions of ASOs in the total size of the human mRNA sequences

| Length of ASO | 0 MM | 1 MM | 2 MMs | 3 MMs | 4 MMs | 5 MMs |
|---------------|------|------|-------|-------|-------|-------|
| 27            | $<10^{-1}$ | $<10^{-1}$ | $<10^{-1}$ | $<10^{-1}$ | $4.5 \times 10^{-1}$ | 6     |
| 26            | $<10^{-1}$ | $<10^{-1}$ | $<10^{-1}$ | $<10^{-1}$ | 1.5   | 18    |
| 25            | $<10^{-1}$ | $<10^{-1}$ | $<10^{-1}$ | $3.1 \times 10^{-1}$ | 5.1   | 48    |
| 24            | $<10^{-1}$ | $<10^{-1}$ | $<10^{-1}$ | 1.1   | 16    | 88    |
| 23            | $<10^{-1}$ | $<10^{-1}$ | $1.8 \times 10^{-1}$ | 3.8   | 44    | 100   |
| 22            | $<10^{-1}$ | $1.6 \times 10^{-1}$ | 12 | 85    | 100   |       |
| 21            | $<10^{-1}$ | $<10^{-1}$ | 2.4 | 37    | 100   | 100   |
| 20            | $<10^{-1}$ | $3.0 \times 10^{-1}$ | 8.5 | 80    | 100   | 100   |
| 19            | $<10^{-1}$ | 1.2 | 27 | 100   | 100   | 100   |
| 18            | $<10^{-1}$ | 4.4 | 68 | 100   | 100   | 100   |
| 17            | $3.3 \times 10^{-1}$ | 16 | 98 | 100   | 100   | 100   |
| 16            | 1.3 | 47 | 100 | 100   | 100   | 100   |
| 15            | 5.2 | 91 | 100 | 100   | 100   | 100   |
| 14            | 19 | 100 | 100 | 100   | 100   | 100   |
| 13            | 57 | 100 | 100 | 100   | 100   | 100   |
| 12            | 97 | 100 | 100 | 100   | 100   | 100   |
| 11            | 100 | 100 | 100 | 100   | 100   | 100   |
| 10            | 100 | 100 | 100 | 100   | 100   | 100   |

ASO, antisense oligonucleotide; MM, mismatch.
oligonucleotides, the probability of existence of sites with one mismatch, two mismatches and more than three mismatches is 4.4%, 68% and 100%, respectively (Table 3, see the row “Length of ASO = 18”). In the case of 20-mer oligonucleotides, the probability of existence of sites with one mismatch, two mismatches, three mismatches and four mismatches is <0.1%, 8.5%, 80% and 100%, respectively (Table 3, see the row “Length of ASO = 20”).

2.3 In silico analysis to obtain the actual number of complementary sequences of ASOs with all of the human mRNA sequences

To validate the theoretical number of complementary sequences of ASOs discussed above, we next performed in silico analysis to obtain the actual number of complementary sites of ASOs with perfect matches or mismatches with all of the human mRNA sequences. We designed several thousand hypothetical ASOs against three human housekeeping genes. The sequence searches allowing nucleotide mismatches were performed using GGGenome (http://GGGenome.dbcls.jp/), developed by the authors’ group, rather than the widely used BLAST software (Altschul, Gish, Miller, Myers, & Lipman, 1990). BLAST might overlook potential complementary regions, as mentioned in our previous work describing siDirect (Naito, Yamada, Ui-Tei, Morishita, & Saigo, 2004) and CRISPRdirect (Naito, Hino, Bono, & Ui-Tei, 2015), which are web servers for designing off-target minimized siRNA and CRISPR guide RNA, respectively. GGGenome quickly searches short nucleotide sequences utilizing suffix arrays and inverse suffix links indexed on solid state drives.

All possible ASO candidates targeting every other position of the three housekeeping genes human GAPDH (glucose-6-phosphate dehydrogenase), HPRT1 (hypoxanthine phosphoribosyltransferase 1) and G6PD (glyceraldehyde-3-phosphate dehydrogenase) were generated, and their potential complementary regions were searched using GGGenome. For example, 1,370 13-mer ASOs targeting every nucleotide position of human GAPDH mRNA were designed (Figure 2) and the complementary regions of each ASO with perfect matches, one mismatch or two mismatches were searched and counted. For 13-mer ASOs designed for GAPDH mRNA, the average number of complementary regions was 3.0 ± 3.7 for zero mismatch (excluding the on-target GAPDH locus), 77 ± 61 for one mismatch and 834 ± 415 for two mismatches (Table 4). In a similar way, we designed all possible 12- to 20-mer ASOs for the three housekeeping genes, and searched and counted their complementary sites to calculate the average number of complementary regions for each oligonucleotide length (Table 5). The average number of complementary regions for each housekeeping gene is shown in Tables S1–S3 (human GAPDH, HPRT1 and G6PD, respectively).

For complementary regions with perfect matches, ASOs shorter than 14-mer hit on average at least one off-target site (Table 5, see the column “0 MM”; 14-mer:1.1 ± 4.8, 13-mer: 3.5 ± 7.7, 12-mer: 11 ± 16). For complementary regions with one mismatch and two mismatches, ASOs shorter than 17-mer and 19-mer hit at least one off-target site (17-mer, one mismatch: 1.0 ± 4.7, 19-mer, two mismatches: 1.5 ± 3.9). When we focus on the complementary regions for each length, the average number of complementary regions increases dramatically as the number of tolerated mismatches increases, in a manner similar to the theoretical number of complementary regions calculated mathematically (Tables 1 and 3). For example, for 13-mer ASOs, the average number of complementary regions with zero mismatch, one mismatch and two mismatches is 3.5 ± 7.7, 82 ± 76 and 856 ± 464, respectively. Similar results were obtained from the analysis of each housekeeping gene (Tables S1–S3).
The size of the human genome \(4.15 \times 10^9 < 4.16\) suggests that ASOs longer than 15-mer typically have a unique target site in the human genome. For example, Stein and Cheng (1993) mentioned that “an oligo of more than 15–17 nucleotides in length would have a unique sequence relative to the entire human genome”. This prediction was based on the premise that ASOs bind complementary regions with perfect matches and did not consider complementary binding with mismatches. In the present study, we estimated the typical number of complementary regions with mismatches, in addition to perfect matches, in all of the human mRNA sequences by mathematical calculation and in silico analysis. Although the actual number of complementary regions obtained from in silico analysis tends to be slightly higher than the theoretical number obtained by mathematical calculation, it falls within one standard deviation of the mean of the theoretical number (Tables 2 and 5). For example, 13-mer oligonucleotides theoretically have 33 complementary regions with one mismatch (Table 2, see the row “Length of ASO = 13”), whereas 13-mer oligonucleotides have on average \(82 \pm 76\) complementary regions with one mismatch as judged from our in silico analysis using several thousand hypothetical ASOs (Table 5, see the row “Length of ASO = 13”). Thus, we conclude that the number of complementary regions of ASOs could be broadly estimated by our theoretical calculation. We attribute the tendency for the actual number of complementary regions to be slightly higher to the existence of direct repeats in the three housekeeping genes. Indeed, when we searched the complementary regions of ASOs that target direct repeats in the three housekeeping genes, we found that these ASOs tend to have a considerable number of complementary regions (data not shown).

In this study, we clearly showed that the lengths of oligonucleotide sequences significantly alter the number of complementary regions in all of the human mRNA sequences: The shorter the oligonucleotides, the more the number of complementary regions (Tables 2 and 5). Four ASO therapeutics have been approved to date by the FDA for clinical use: formivirsen (Vitravene), mipomersen (Kynamro), eteplirsen (Exondys 51) and nusinersen (Spinraza), and their lengths are 21-mer, 20-mer, 30-mer and 18-mer, respectively (Stein & Castanotto, 2017). The recent development of artificial nucleic acids that bind to their target RNA with high affinity has overall led to shorter ASOs in preclinical and clinical development. For example, IONIS-STAT3-2.5RX/AZD9150 and ISTH0036 are gapmer ASOs under clinical development and are a 16-mer and 14-mer, respectively. Furthermore, Shimo et al. (2014) and Touznik, Maruyama, Hosoki, Echigoya, and Yokota (2017) showed that 13-mer SSOs with LNA modifications efficiently induced exon skipping and exon inclusion at the cellular level, respectively. These 13- to 16-mer ASOs would theoretically have several tens to hundreds of complementary

### Table 4

| 13-mer ASO | Target sequence | Number of complementary regions |
|------------|-----------------|---------------------------------|
|            |                 | 0 MM | 1 MM | 2 MM |
| ASO 0001   | GGCTGGGACTGCG   | 3    | 165  | 1,314 |
| ASO 0002   | GCTGGGACTGCG    | 7    | 128  | 1,123 |
| ASO 0003   | CTGGGACTGCG     | 9    | 183  | 1,606 |
| ASO 1368   | ACCCTGTGCTCAA   | 3    | 70   | 742   |
| ASO 1369   | CCCCTGTGCTGAC   | 4    | 56   | 828   |
| ASO 1370   | CCTGTGCTGAC     | 5    | 64   | 1,001 |
|            | Average         | 3.0 ± 3.7 | 77 ± 61 | 834 ± 415 |

ASO, antisense oligonucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MM, mismatch.

### Table 5

The average number of complementary regions obtained by in silico analysis for hypothetical ASOs designed for three housekeeping genes (human GAPDH, HPRT1 and G6PD). On-target hits are excluded

| Length of ASO | Average number of complementary regions |
|--------------|----------------------------------------|
|              | 0 MM | 1 MM | 2 MM |
| 20           | <10^{-1} | <10^{-1} | 0.52 ± 1.8 |
| 19           | <10^{-1} | 0.11 ± 0.64 | 1.5 ± 3.9 |
| 18           | <10^{-1} | 0.33 ± 1.5 | 4.3 ± 9.8 |
| 17           | <10^{-1} | 1.0 ± 4.7 | 12 ± 21 |
| 16           | 0.14 ± 2.1 | 2.9 ± 8.6 | 36 ± 42 |
| 15           | 0.39 ± 3.2 | 8.6 ± 16 | 104 ± 91 |
| 14           | 1.1 ± 4.8 | 26 ± 33 | 303 ± 208 |
| 13           | 3.5 ± 7.7 | 82 ± 76 | 856 ± 464 |
| 12           | 11 ± 16 | 220 ± 164 | 1,694 ± 721 |

ASO, antisense oligonucleotide; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase 1; MM, mismatch.
regions if one or more mismatches are tolerated (Table 2). However, it has been reported that 16-mer gapmer ASOs with LNA modifications induce hybridization-dependent off-target effects on sections of genes with one or two mismatches, in addition to genes with perfect matches (Kamola et al., 2015). These observations suggest that, at least in the case of gapmer ASOs, genes with mismatches may be influenced by the mechanism of hybridization-dependent off-target effects.

In this study, we estimated the number of off-target candidate sites by analyzing the number of complementary regions of ASOs in human mRNA. We showed that the number of complementary regions increases dramatically as the number of tolerated mismatches increases. However, it should be noted that the existence of complementary regions does not necessarily cause off-target effects in every situation, as off-target effects are dependent upon several factors such as accessibility to the complementary RNAs, as well as the number of mismatches with the complementary RNAs. The position of mismatches may also affect the off-target effects, although the correlation between the position of mismatches and knockdown efficiencies is currently unclear. Accessibility to the complementary RNAs is assumed to be a critical factor for ASOs function because gapmer ASOs often fail to degrade their target RNAs even if the ASOs bind to the RNAs with perfect complementarity. SSOs appear to be much less affected by off-target effects than gapmer ASOs because SSOs must bind to specific regions of off-target candidate genes, such as exonic splicing enhancers or intronic splicing silencers, to induce off-target effects. For this reason, it is important to conduct actual off-target detection in human cells by expression analysis, in addition to in silico analysis utilizing rigorous off-target search software.

Antisense oligonucleotides are known to act on pre-mRNA and noncoding RNA, as well as on mRNA. Therefore, it is reasonable to search for complementary regions from pre-mRNA and noncoding RNA sequences. However, no comprehensive pre-mRNA and noncoding RNA sequence databases useful in off-target searches are currently available. Furthermore, the physiological and/or pathological roles of noncoding RNA are largely unknown. For this reason, and in light of the safety assessment of ASOs, off-target search in pre-mRNA and noncoding RNA sequences may be a little premature at this time. We expect that standardized databases for pre-mRNA and noncoding RNA will be established in the near future.

4 EXPERIMENTAL PROCEDURES

4.1 In silico analysis

Three housekeeping genes were used: GAPDH (NM_002046.4), HPRT1 (hypoxanthine-guanine phosphoribosyltransferase, NM_000194.2) and G6PD (NM_000402.4). All possible 12- to 20-mer ASO candidates targeting each 12- to 20-mer stretch in each housekeeping gene sequence, starting at the 5′ end and shifting by one nucleotide toward the 3′ end with each successive ASO of a given sequence length, were designed, and their potential complementary regions were searched. Sequence searches were performed using GGGenome (http://GGGenome.dbcls.jp/) for NCBI RefSeq (Haft et al., 2018) release 70 human transcripts (http://GGGenome.dbcls.jp/hs_refseq70/). In Refseq searches, human mRNAs (accession starts with NM_) are selected from search results.

ACKNOWLEDGMENTS

We thank Mr Isamu Muto, BioInformation Technology & Science (BITS) Co., Ltd., for technical assistance with the in silico analyses. We also thank Mr Yasuhiro Takayama for help with the theoretical formulae.

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REFERENCES

Aartsma-Rus, A., & Krieg, A. M. (2017). FDA approves eteplirsen for duchenne muscular dystrophy: The next chapter in the eteplirsen saga. Nucleic Acid Therapeutics, 27, 1–3. https://doi.org/10.1089/ nat.2016.0657

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215, 403–410. https://doi.org/10.1016/ S0022-2836(05)80360-2

Burel, S. A., Hart, C. E., Cauntay, P., Hsiao, J., Machemer, T., Katz, M., … Henry, S. A. (2016). Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. Nucleic Acids Research, 44, 2093–2109. https://doi.org/10.1093/nar/gkv1210

Corey, D. R. (2017). Nusinersen, an antisense oligonucleotide drug for spinal muscular atrophy. Nature Neuroscience, 20, 497–499. https://doi.org/10.1038/nn.4508

Frazier, K. S. (2015). Antisense oligonucleotide therapies: The promise and the challenges from a toxicologic pathologist’s perspective. Toxicologic Pathology, 43, 78–89. https://doi.org/10.1177/01926233155181840

Haft, D. H., DiCuccio, M., Badredin, A., Brover, V., Chetvernin, V., O’Neill, K., … Pruitt, K. D. (2018). RefSeq: An update on prokaryotic genome annotation and curation. Nucleic Acids Research, 46, D851–D860. https://doi.org/10.1093/nar/gkx1068

Havens, M. A., & Hastings, M. L. (2016). Splice-switching antisense oligonucleotides as therapeutic drugs. Nucleic Acids Research, 44, 6549–6563. https://doi.org/10.1093/nar/gkw533

International Human Genome Sequencing Consortium (2004). Finishing the euchromatic sequence of the human genome. Nature, 431, 931–945.
Kamola, P. J., Kitson, J. D., Turner, G., Maratou, K., Eriksson, S., Panjwani, A., … Parry, J. D. (2015). In silico and in vitro evaluation of exonic and intronic off-target effects form a critical element of therapeutic ASO gapmer optimization. *Nucleic Acids Research*, 43, 8638–8650. https://doi.org/10.1093/nar/gkv857

Kamola, P. J., Maratou, K., Wilson, P. A., Rush, K., Mullaney, T., McKevitt, T., … Parry, J. D. (2017). Strategies for in vivo screening and mitigation of hepatotoxicity associated with antisense drugs. *Molecular Therapy. Nucleic Acids*, 8, 383–394. https://doi.org/10.1016/j.omtn.2017.07.003

Kasuya, T., Hori, S., Watanabe, A., Nakajima, M., Gahara, Y., Rokushima, M., … Kugimiya, A. (2016). Ribonuclease H1-dependent hepatotoxicity caused by locked nucleic acid-modified gapmer antisense oligonucleotides. *Scientific Reports*, 6, 30377. https://doi.org/10.1038/srep30377

Kole, R., Krainer, A. R., & Altman, S. (2012). RNA therapeutics: Beyond RNA interference and antisense oligonucleotides. *Nature Reviews. Drug Discovery*, 11, 125–140. https://doi.org/10.1038/nrd3625

Lindow, M., Vornlocher, H. P., Riley, D., Kornbrust, D. J., Burchard, J., Whiteley, L. O., … Levin, A. A. (2012). Assessing unintended hybridization-induced biological effects of oligonucleotides. *Nature Biotechnology*, 30, 920–923. https://doi.org/10.1038/nbt.2376

McClorely, G., & Wood, M. J. (2015). An overview of the clinical application of antisense oligonucleotides for RNA-targeting therapies. *Current Opinion in Pharmacology*, 24, 52–58. https://doi.org/10.1016/j.coph.2015.07.005

Naito, Y., Hino, K., Bono, H., & Ui-Tei, K. (2015). CRISPRdirect: Software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics*, 31, 1120–1123. https://doi.org/10.1093/bioinformatics/btu743

Naito, Y., Yamada, T., Ui-Tei, K., Morishita, S., & Saigo, K. (2004). siDirect: Highly effective, target-specific siRNA design software for mammalian RNA interference. *Nucleic Acids Research*, 32, W124–W129. https://doi.org/10.1093/nar/gkh442

Shimo, T., Tachibana, K., Saito, K., Yoshida, T., Tomita, E., Waki, R., … Obika, S. (2014). Design and evaluation of locked nucleic acid-based splice-switching oligonucleotides in vitro. *Nucleic Acids Research*, 42, 8274–8287.

Stein, C. A., & Castanotto, D. (2017). FDA-approved oligonucleotide therapies in 2017. *Molecular Therapy*, 25, 1069–1075. https://doi.org/10.1016/j.ymthe.2017.03.023

Stein, C. A., & Cheng, Y. C. (1993). Antisense oligonucleotides as therapeutic agents—Is the bullet really magical? *Science*, 261, 1004–1012. https://doi.org/10.1126/science.8351515

Thomas, G. S., Cromwell, W. C., Ali, S., Chin, W., Flaim, J. D., & Davidson, M. (2013). Mipomersen, an apolipoprotein B synthesis inhibitor, reduces atherogenic lipoproteins in patients with severe hypercholesterolemia at high cardiovascular risk: A randomized, double-blind, placebo-controlled trial. *Journal of the American College of Cardiology*, 62, 2178–2184. https://doi.org/10.1016/j.jacc.2013.07.081

Touznik, A., Maruyama, R., Hosoki, K., Echigoya, Y., & Yokota, T. (2017). LNA/DNA mixmer-based antisense oligonucleotides correct alternative splicing of the SMN2 gene and restore SMN protein expression in type 1 SMA fibroblasts. *Scientific Reports*, 7, 3672. https://doi.org/10.1038/s41598-017-03850-2

Yamamoto, T., Nakatani, M., Narukawa, K., & Obika, S. (2011). Antisense drug discovery and development. *Future Medicinal Chemistry*, 3, 339–365. https://doi.org/10.4155/fmc.11.2

**SUPPORTING INFORMATION**

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**How to cite this article:** Yoshida T, Naito Y, Sasaki K, et al. Estimated number of off-target candidate sites for antisense oligonucleotides in human mRNA sequences. *Genes Cells*. 2018;23:448–455. [https://doi.org/10.1111/gtc.12587](https://doi.org/10.1111/gtc.12587)