Are short oligonucleotides less specific *in vivo*?

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RNA Medicines for the 21st Century

Abstract

The challenge of developing effective and safe RNA-targeted therapeutics calls for robust in silico design of target-specific oligonucleotides with minimal effect on unintended targets. In this process, many molecular biologists and statisticians often ask how many perfect match recognition sequences a given oligonucleotide has in the transcriptome. This, in turn, leads to the assumption that the longer a given oligonucleotide, the more specific it will be, whereas short oligonucleotides are often considered unspecific and to have more off-target effects. However, this assumption is only correct if the hybridization stringency can be controlled in such a way that a single mismatch, an insertion, or a deletion will abrogate effective binding to unintended targets. This is indeed the case in most experiments such as PCR, Northern blot analysis, and in situ hybridization. The primary goal is to find experimental settings in which the oligonucleotide preferably hybridizes to the intended target. In the lab, hybridization conditions such as temperature and ionic strength can be controlled to achieve optimal specificity. By comparison, hybridization conditions in live animals or humans cannot be adjusted, instead altering the length, design and chemical modifications of a given oligonucleotide must be utilized to achieve adequate specificity.

Here, we present and compare different oligonucleotide specificity prediction approaches. We show that different methods can produce qualitatively very different results. Algorithms based on minimizing mismatches predict that short oligonucleotides are less specific, whereas methods that maximize the number of basepairs or binding affinity to putative targets predict that longer oligonucleotides are less specific. We assess the aforementioned approaches using expression profiling of mice livers after treatment with either a 13mer or a 16mer LNA phosphorothioate oligonucleotide against ApoB.

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Design objective for therapeutic oligonucleotides

Maximize effect specificity

(maximise effect on intended target transcript while minimizing effects on other transcripts)

Factors governing the effectiveness of an oligo

- Accessibility of oligo to target site on transcript
- Amount of oligo compared to amount of transcript
- Binding affinity between oligo and transcript
- Binding affinity between RNAse H and oligo:target complex

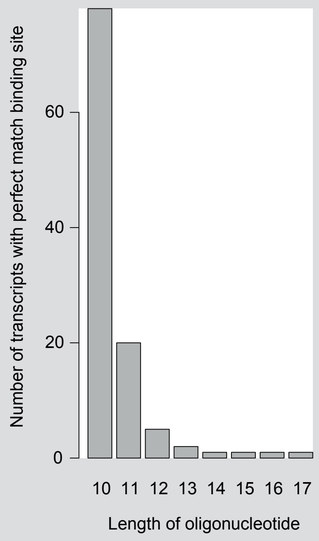
Model LNA oligos used in this study

We designed 8 overlapping oligos with different lengths against ApoB. Bold letters indicate LNA modified nucleotides. None of these oligos were selected for clinical testing.

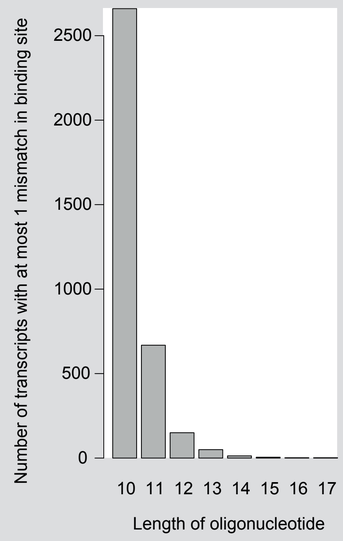
Sequence	length	Sequence	length
CAGCATTGGTATTC**AGT**	17	**GCA**TTGGTAT**TCA**	13
CAGCATTGGTAT**TCA**G	16	**GCA**TTGGTA**TTC**	12
AGCATTGGTAT**TCA**G	15	**CAT**TGGTA**TTC**	11
AGCATTGGTAT**TCA**	14	**CAT**TGGT**ATT**	10

Oligo length dependency when *counting mismatches*

Assume only perfect match binding can recruit RNAse H



Assume perfect match *and one mismatch* binding can recruit RNAse H



When evaluating specificity by counting the number of transcripts besides the intended target with perfect- and 1 mismatch binding: *the longer the oligo, the more specific it is.*

Oligo length dependency when *calculating binding strength*

Binding strength

Simple model for oligo (O) binding to transcript (T) giving the complex (OT)

$$O + T \rightleftharpoons OT$$

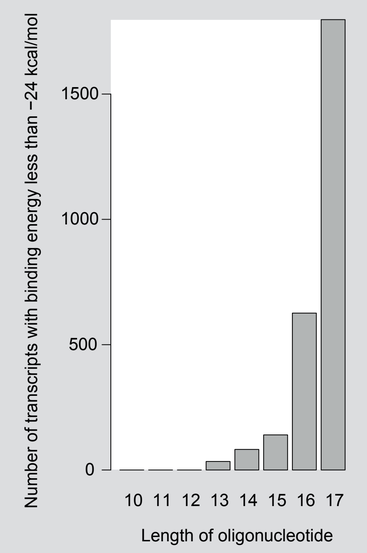
At equilibrium

$$\frac{[OT]}{[O]\,[T]} = K = \exp(-\Delta G/RT)$$

Where R is the ideal gas law constant, T the temperature, and ΔG the free energy of binding.

The minimum free energy of binding betweem oligo and transcript is calculated by a nearest-neighbour type method as implemented in RNAhybrid (Rehmsmeier et al., RNA, 2004).

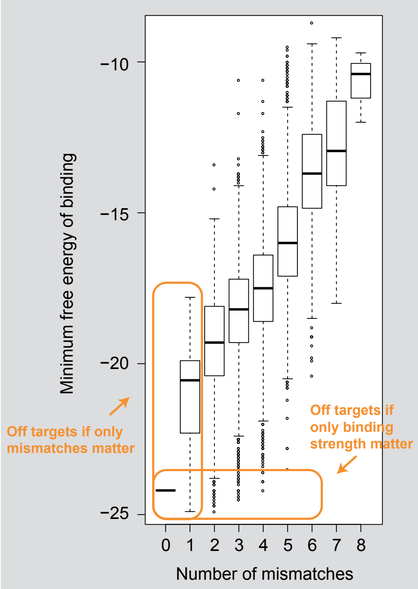
Assume binding stronger than -24 kcal/mol can recruit RNAse H



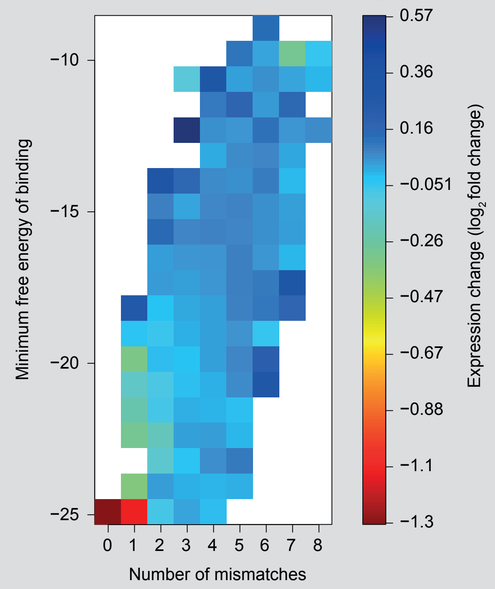
When evaluating specificity by counting the number of transcripts besides the intended target with stronger than -24 kcal/mol binding: *the shorter the oligo, the more specific it is.*

Relating mm counting, binding strength and effectiveness

Theory

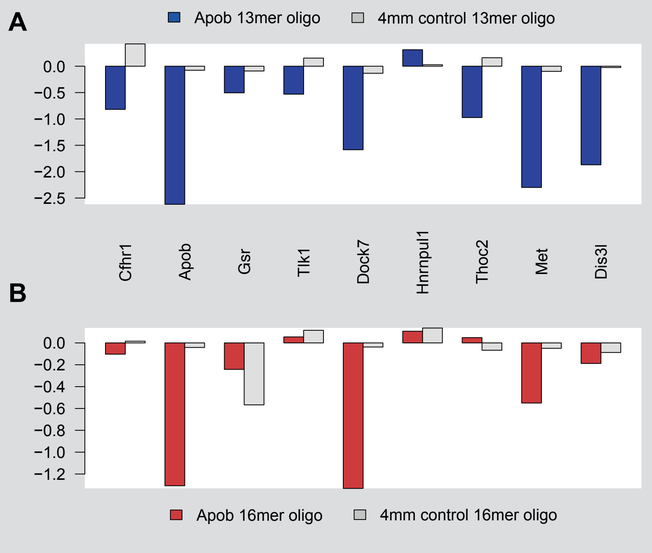


Experimental (ApoB 13mer oligo)



Using microarrays, we measured gene expression changes in mouse liver when treating with a 13mer oligo targeting ApoB compared to saline. For each transcript, we also counted number of mismatches and binding strength to the oligo.

Identification of possible off-targets for ApoB 13mer oligo



For genes with binding strength < -21 kcal/mol and only 2 or fewer mismatches to oligo, we (**A**) identified those that had highly significant expression change ($P < 0.0005$ by t-test) after treatment with 13mer oligo. Eight out of nine show decreased expression which are more than expected by chance ($P < 0.01$) by binomial test). In (**B**) we show the expression changes when treating with a 16mer ApoB targeting oligo. In (**C**) we show target sites.

Discussion

Using transcript profiles from a 13mer and a (overlapping) 16mer oligo targeting ApoB, we have shown that both a low number of mismatches, and a strong binding of oligo to a transcript, can result in RNAse H recruitment and resulting reduction of transcript amount. Indeed, in the case of Met (Hepatocyte growth factor receptor), as many as four mismatches to the 16mer oligo still seems to allow recruitment of RNAse H when the overall binding strength is as low as ~ -24 kcal/mol. Therefore, short oligos are not necessarily less specific than longer oligos. Rather, the longer the oligo, the more nucleotides there are to get a strong binding, even when there are mismatches. Both mismatch counting, and binding strength evaluation, however, are only moderately effective in identifying off-targeting as here evaluated by gene expression.
More experiments are needed to establish generally predictive models for oligo specificity.

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