Supplementary material for

Integrated siRNA design based on surveying of features
associated with high RNAi effectiveness

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Supplementary Discussion

1. Cooperativity between features in their joint effects

Here, we derive how interaction, or cooperativity, between features influences the chances of achieving higher efficacies. Without loss of generality, we consider how the chance of achieving >90% efficacies, $P_{90}$, are affected by a pair of features – $F_1$ and $F_2$ – both of which significantly increase the chance of achieving >90% efficacies. We look at three scenarios: (a) the two features are independent of each other; (b) the two features have positive cooperativity; and (c) the two features have negative cooperativity.

Suppose in the general population of siRNA experiments, the chance of achieving >90% efficacies is $P_{90}^{(0)}$. In the subpopulation of experiments carrying $F_1$, the chance of achieving >90% efficacies is $P_{90}^{(1)}$, and in the subpopulation of experiments carrying $F_2$, the chance of achieving >90% efficacies is $P_{90}^{(2)}$. Let

$$p_1 = P_{90}^{(1)} - P_{90}^{(0)},$$

and

$$p_2 = P_{90}^{(2)} - P_{90}^{(0)}.$$  

The chance of achieving >90% efficacies is boosted by

$$s_1 = \frac{P_{90}^{(1)}}{P_{90}^{(0)}} = \frac{P_{90}^{(0)} + p_1}{P_{90}^{(0)}} = 1 + \frac{p_1}{P_{90}^{(0)}}$$

times by $F_1$, and the chance of achieving >90% efficacies is boosted by

$$s_2 = \frac{P_{90}^{(2)}}{P_{90}^{(0)}} = \frac{P_{90}^{(0)} + p_2}{P_{90}^{(0)}} = 1 + \frac{p_2}{P_{90}^{(0)}}$$

times by $F_2$. If $F_1$ and $F_2$ are independent of each other (having no cooperativity), then the chance of achieving >90% efficacies should be boosted by

$$s_{12} = s_1s_2 = (1 + \frac{p_1}{P_{90}^{(0)}})(1 + \frac{p_2}{P_{90}^{(0)}}) = 1 + \frac{p_1}{P_{90}^{(0)}} + \frac{p_2}{P_{90}^{(0)}} + \frac{p_1p_2}{(P_{90}^{(0)})^2}$$

times. When $p_1 << P_{90}^{(0)}$, $p_2 << P_{90}^{(0)}$ (for our dataset, $P_{90}^{(0)} = 0.34$, and $p_1$, $p_2$ are about 0.02-0.03), the last term $\frac{p_1p_2}{(P_{90}^{(0)})^2}$ can be omitted. Therefore,

$$s_{12} \approx 1 + \frac{p_1}{P_{90}^{(0)}} + \frac{p_2}{P_{90}^{(0)}},$$

and the chance of achieving >90% efficacies is

$$P_{90}^{(12)} = P_{90}^{(0)} \cdot s_{12} \approx P_{90}^{(0)} \left(1 + \frac{p_1}{P_{90}^{(0)}} + \frac{p_2}{P_{90}^{(0)}}\right) = P_{90}^{(0)} + p_1 + p_2.$$

In other words, $F_1$ and $F_2$ are additive in their joint effect in boosting the chance of achieving >90% efficacies.
If $F_1$ and $F_2$ have positive cooperativity, then we have
\[
s_{12} > s_1 s_2 \approx 1 + \frac{p_1}{P_{90}^{(0)}} + \frac{p_2}{P_{90}^{(0)}},
\]
and
\[
P_{90}^{(12)} > (P_{90}^{(0)} + p_1 + p_2).
\]
In other words, the combined effect of the two features exceeds the sum of the individual effects of the two features. In contrast, if $F_1$ and $F_2$ have negative cooperativity, then we have
\[
P_{90}^{(12)} < (P_{90}^{(0)} + p_1 + p_2).
\]
In this case, the combined effect of the two features is less than the sum of the effects of the two individual features.

Note about the sigmoid shape of the ascending curves in the analysis of feature combinations (Figure 3A and B)

Consider that there are $n$ significant features, $F_1, F_2, ..., F_n$. For simplicity, we assume that each of these features leads to equal amount of increase in the chance of achieving >90% efficacy, that is, $p_1 = p_2 = ... = p_n = p$. Following derivations similar to shown above, we can see easily that if these features are independent of each other (showing no cooperativity), then the co-presence of any two features will lead to $2p$ amount of increase in the chance of achieving >90% efficacy; the co-presence of any three features will lead to $3p$ amount of increase in the chance of achieving >90% efficacy; and so on. This will result in a linear relationship between (%records achieving >90% efficacy) and $l$, as illustrated in Supplementary Figure 2 (dotted lines with arrowheads). What we actually see, however, are sigmoid curves (Figure 3A and 4B in the main text, the latter copied to Supplementary Figure 2). At the earlier phase, the curve goes steeper and steeper with increasing $l$, indicating that the features selected had positive cooperativity. At the later phase, the slope decreases and the curve flattens, apparently due to the approaching and reaching of the maximal percentage (100%).

There is an alternative explanation to this observation. Because, in fact, different features did not lead to the same amount of increase in the chance of achieving >90% efficacy when present alone, the increasing slope at the earlier phase of the curve could be due to more features of higher $p$’s being included in the feature combinations. If this is so, we would expect to see that features with higher $p$’s appearing more frequently for higher $l$. However, this is not the case. Features with different $p$ ranges are rather equally spread for different $l$’s (results not shown). Therefore, the increasing slope in the early phase of the sigmoid curve is likely due to positive cooperativity among the features.

2. Survey of features significant associated with high siRNA efficacy

In this section we discuss the significant features found in our survey with the three statistical tests: the Wald test of monotone trend, and the odds ratio permutation tests for two different efficacy levels (>70% and >90%), respectively. We loosely call a feature “significant” if the P value of one of the three tests was below 0.05. The false discovery
rate (FDR)[1] was controlled at slightly different levels for the three tests at this P value threshold: for the Wald test of monotone trend, FDR was controlled at 0.18; for the odds ratio permutation tests for >70% and >90% efficacies, the FDR was controlled at 0.15 and 0.16, respectively. By using a loose P value cut-off, more extensive comparisons could be made between our results and previous findings. The FDR could be controlled much more effectively if a lower P value cut-off level. For instance, at P value threshold 0.01, the FDR for the three tests could be controlled at the level of 0.056, 0.044 and 0.038, respectively. This higher stringency level was forced when we selected features for the “feature combination analyses” for the purposes of finding effective siRNA design rule sets.

**Category 1: direct sequence features**

For the 1st nucleotide of siRNA sequence (throughout this study, we specify nucleotide positions on the sense strand, counting from the 5’ end), Takasaki et al. suggested that nucleotide A and U had a lower probability of occurrence and nucleotide G had a higher probability of occurrence than other nucleotides in functional siRNAs[2]. Amarzguioui and Prydz also suggested that absence of nucleotide U at position 1 was strongly correlated with functionality[3]. In our analysis, there was evidence that both features 1st nucleotide ≠ U and 1st nucleotide=G significantly boosted the chance of achieving >70% efficacies ($P_{70} = 0.0014$ and 0.0024, respectively), and they were associated with significant up-shift of efficacy distribution ($P_{wald} = 0.0495$ and 0.022, respectively). We found no evidence that the absence of nucleotide A at position 1 was associated with significant up-shift of the efficacy distribution ($P_{wald} = 0.32$) or significantly boosted the chances of achieving >70% or >90% efficacies ($P_{70} = 0.12, P_{90} = 0.54$).

For the 3rd nucleotide of siRNA sequence, Reynolds et al. suggested that nucleotide A at this position was correlated with siRNA functionality[4]. However, we found no evidence that 3rd nucleotide=A was associated with significant up-shift of the efficacy distribution ($P_{wald} = 0.18$), or boosted the chances of achieving >70% or >90% efficacies ($P_{70} = 0.32, P_{90} = 0.21$). Our results suggested that the super-feature of this feature 3rd nucleotide ≠ G can significantly boost the chance of achieving >90% efficacy ($P_{90} = 0.018$).

For the 6th nucleotide of siRNA sequence, two groups of researchers suggested that nucleotide A at this position was a positive determinant of siRNA functionality[2, 3]. Hsieh et al. suggested that there was a selection against C at this position for functional siRNA[5]. Our analyses indicated that both features 6th nucleotide ≠ C and 6th nucleotide=A were associated with significant up-shift of the efficacy distribution ($P_{wald} = 0.0052$ and 0.0058, respectively), and they significantly boosted the chances of reaching >70% and >90% efficacies ($P_{70} = 0.00066$ and 0.0024; $P_{90} = 0.0089$ and 0.0058, respectively). Moreover, our results suggested that the feature 6th nucleotide=U, the sub-feature of 6th nucleotide ≠ C, and the feature 6th nucleotide ≠ G, the super-feature of 6th nucleotide=A, were associated with significant up-shift of the efficacy distributions ($P_{wald} = 0.042$ and $P_{wald} = 0.018$ respectively), and boosted the chance of reaching >90% efficacy ($P_{90} = 0.035$ and $P_{90} = 0.018$ respectively). The feature 6th nucleotide ≠ G was also found to boost the chance of achieving >70% efficacy ($P_{70} = 0.048$).
For the 7th nucleotide of siRNA sequence, Takasaki et al. suggested that nucleotide U had a lower probability of occurrence, and nucleotide G had a higher probability of occurrence in functional siRNAs[2]. Our results indicated that 7th nucleotide ≠ U was associated with significant up-shift of efficacy distribution (\(P_{\text{wald}} = 0.0091\)) and elevated the chance of achieving >70% (\(P_{70} = 0.010\)) and >90% (\(P_{90} = 0.0043\)) efficacies. The feature 7th nucleotide=G, though, was not found to be significant (\(P_{70} = 0.069\), \(P_{90} = 0.32\), \(P_{\text{wald}} = 0.17\)). Our result also indicated that the feature 7th nucleotide=A, which is the sub-feature of 7th nucleotide ≠ U, was associated with the up-shift of the efficacy distribution (\(P_{\text{wald}} = 0.016\)), and elevated the chance of reaching >90% efficacy (\(P_{90} = 0.0014\)).

For the 8th nucleotide of siRNA sequence, Takasaki et al. suggested that nucleotide A had a higher probability of occurrence and nucleotide G had a lower probability of occurrence in functional siRNAs[2]. In our analysis, the feature 8th nucleotide=A was not found to be associated with significant up-shift of the efficacy distribution (\(P_{\text{wald}} = 0.27\)) or boosted the chances of achieving higher siRNA efficacies (\(P_{70} = 0.12\), \(P_{90} = 0.77\)). Instead, we found the feature 8th nucleotide=G significantly boosted the chance of achieving >90% efficacy (\(P_{90} = 0.0054\), contrary to the observation by Takasaki et al. Moreover, we found that the feature 8th nucleotide ≠ C, the super-feature of the features 8th nucleotide=A and 8th nucleotide=G, significantly boosted the chance of achieving >90% efficacy (\(P_{90} = 0.023\)).

For the 9th nucleotide of siRNA sequence, Takasaki et al. suggested that nucleotide U has a higher probability and nucleotide G has a lower probability of occurrence in functional siRNA sequences[2]. We found no evidence that the feature 9th nucleotide ≠ G was significant (\(P_{70} = 0.68\), \(P_{90} = 0.36\), \(P_{\text{wald}} = 0.48\)). Instead, the feature 9th nucleotide ≠ U was found to be significant in elevating the chance of achieving >90% efficacy (\(P_{90} = 0.035\)). We also found the feature 9th nucleotide=C, the sub-feature of two above features 9th nucleotide ≠ G and 9th nucleotide ≠ U, was associated with significant up-shift of the efficacy distribution (\(P_{\text{wald}} = 0.00053\)), and was strongly associated with >70% and >90% efficacies (\(P_{70} = 0.0080\) and \(P_{90} = 0.00021\)). Another feature 9th nucleotide ≠ A, was associated with up-shift of efficacy distribution (\(P_{\text{wald}} = 0.021\)), and boosted the chance of reaching >70% efficacy (\(P_{70} = 0.0061\)).

There were disagreements in previous studies about how the occurrence of nucleotide U at position 10 influences siRNA functionality. Reynolds et al. suggested that a U at position 10 was associated with higher efficacies[4], whereas Amarzguioui and Prydz showed that the nucleotide U at position 10 was associated with lack of functionality[3]. We found no evidence that either the feature 10th nucleotide=U or 10th nucleotide ≠ U was associated with significant up-shift of the efficacy distribution, or elevated the chances of achieving higher efficacies (\(P_{70} = 0.20\), \(P_{90} = 0.062\), \(P_{\text{wald}} = 0.13\) for the feature 10th nucleotide=U; \(P_{70} = 0.80\), \(P_{90} = 0.94\), \(P_{\text{wald}} = 0.87\) for the feature 10th nucleotide ≠ U).
For the 11th nucleotide of siRNA sequence, Hsieh et al. suggested that there was a negative selection against nucleotide A and preference for nucleotide C at this position for functional siRNAs[5]. However, in our analyses, there was no evidence that feature 11th nucleotide≠A was associated with significant up-shift of efficacy distribution (P_{\text{wald}} = 0.86) or boosted the efficacies to higher levels (P_{70} = 0.86, P_{90} = 0.86). Moreover, the feature 11th nucleotide≠C was found to slightly boost the chance of achieving >90% efficacies (P_{90} = 0.029).

For the 13th nucleotide of siRNA sequence, Hsieh et al. suggested that there was enrichment for nucleotide A at this position for functional siRNA[5]. Amarzguioui and Prydz suggested that nucleotide U at position 13 was a positive determinant of siRNA functionality[3]. Reynolds et al. suggested that the absence of nucleotide G at position 13 contributed to the siRNA functionality[4]. Our analysis showed that the feature 13th nucleotide=A was associated with significant up-shift of efficacy distribution (P_{\text{wald}} = 0.020) and significantly elevated the chance of achieving >70% efficacy, and slightly boosted the chance of achieving >90% efficacies (P_{70} = 0.008, P_{90} = 0.052). There was no evidence that other two features 13th nucleotide=U and 13th nucleotide≠G was associated with significant up-shift of distribution (P_{\text{wald}} = 0.71 for the feature 13th nucleotide=U; P_{\text{wald}} = 0.31 for the feature 13th nucleotide≠G), or elevated the chances of achieving high efficiencies (P_{70} = 0.84, P_{90} = 0.64 for the feature 13th nucleotide=U; P_{70} = 0.43, P_{90} = 0.29 for the feature 13th nucleotide≠G).

For the 15th position of siRNA nucleotide, Takasaki et al. indicated that nucleotide U has a higher probability of occurrence than other nucleotides in this position of functional siRNA sequences[2]. However, in our analysis, there was no evidence that 15th nucleotide=U was associated with significant up-shift of the efficacy distribution (P_{\text{wald}} = 0.10), or boosted the chances of achieving higher efficacies (P_{70} = 0.23, P_{90} = 0.10). Rather, our results suggested that the super-feature 15th nucleotide≠C, and the feature 15th nucleotide=G, both boosted the chances of achieving >70% efficacies (P_{70} = 0.0045 for the feature 15th nucleotide≠C; P_{70} = 0.010 for the feature 15th nucleotide=G). The feature 15th nucleotide≠C was also associated with up-shift of efficacy distribution (P_{\text{wald}} = 0.023).

For the 16th position of siRNA nucleotide, Amarzguioui and Prydz suggested that the presence of nucleotide C at this position was strongly correlated with siRNA functionality[3], and Hsieh et al. suggested that there was enrichment for nucleotide G at this position for functional siRNA[5]. Our analysis indicated that the feature 16th nucleotide=C significantly boosted the chance of achieving the >70% efficacy (P_{70} = 0.026, yet P_{90} = 0.10, P_{\text{wald}} = 0.058). But no evidence was found to support the significance of the feature 16th nucleotide=G (P_{70} = 0.74, P_{90} = 0.71, P_{\text{wald}} = 0.68).

For the 19th position of siRNA nucleotides, Hsieh et al. indicated that there was a strong preference for nucleotide U at position 19 of functional siRNA[5]. Several groups suggested that absence of nucleotide G at this position was strongly correlated with siRNA functionality[2, 3, 5]. Our result agreed well with the widely accepted selection against G at position 19 – the feature 19th nucleotide≠G was associated with a
significant up-shift of efficacy distribution ($P_{wald} = 0.0012$) and strongly boosted the chances of achieving >70% and >90% efficacies ($P_{70} = 0.00049$, $P_{90} = 0.0043$). There was no evidence that the presence of nucleotide U at position 19 was associated with significant up-shift of efficacy distribution ($P_{wald} = 0.086$) or elevated the chances of achieving higher efficacies ($P_{70} = 0.098$, $P_{90} = 0.23$). Moreover, our result suggested that the feature $19th\ nucleotide=A$, the sub-feature of $19th\ nucleotide\neq G$, was associated with significant up-shift of efficacy distribution ($P_{wald} = 0.0014$), and boosted the chance of reaching both >70% and >90% efficacies ($P_{70} = 0.00066$ and $P_{90} = 0.0054$).

Aside from aforementioned features reported in previous studies, the features $2nd\ nucleotide=A$ ($P_{70} = 0.010$, $P_{90} = 0.0026$, $P_{wald} = 0.0019$), $5th\ nucleotide=A$ ($P_{70} = 0.0080$, $P_{90} = 0.0023$, $P_{wald} = 0.010$), $5th\ nucleotide\neq G$ ($P_{70} = 0.032$, $P_{90} = 0.023$, $P_{wald} = 0.035$), $12th\ nucleotide=G$ ($P_{70} = 0.013$, $P_{90} = 0.023$, $P_{wald} = 0.027$) and $18th\ nucleotide\neq C$ ($P_{70} = 0.010$, $P_{90} = 0.00071$, $P_{wald} = 0.0048$) were found to be associated with significant up-shift of the efficacy distribution and significantly boosted the chances of achieving >70% and >90% efficacies. The features $4th\ nucleotide=C$ ($P_{90} = 0.00036$, $P_{wald} = 0.0075$), $14th\ nucleotide\neq C$ ($P_{90} = 0.00053$, $P_{wald} = 0.019$) and $17th\ nucleotide\neq C$ ($P_{90} = 0.012$, $P_{wald} = 0.044$) were found to be associated with significant up-shift of the efficacy distribution and boosted the chance of achieving >90% efficacies. The features $17th\ nucleotide=A$ ($P_{70} = 0.00049$, $P_{wald} = 0.0049$), $17th\ nucleotide\neq G$ ($P_{70} = 0.0018$, $P_{wald} = 0.041$) and $18th\ nucleotide=A$ ($P_{70} = 0.048$, $P_{wald} = 0.041$) were found to be associated with significant up-shift of the efficacy distribution and significantly boosted the chance of achieving >70% efficacies. In addition, the feature $5th\ nucleotide\neq U$ ($P_{70} = 0.026$) significantly boosted the chance of achieving >70% efficacies, and the features $4th\ nucleotide\neq U$ ($P_{90} = 0.043$) and $14th\ nucleotide=U$ ($P_{90} = 0.035$) significantly boosted the chance of achieving >90% efficacies.

**Category 2: sequence derived features**

It was suggested by several groups that G/C was positive determinant of siRNA functionality at this position[3, 6, 7]. In our analysis, we found evidence that the feature $1st\ nucleotide=(G/C)$ was associated with significant up-shift of the efficacy distribution ($P_{wald} = 0.049$) and significantly elevated the chance of achieving >70% efficacy (but not for >90% efficacy) ($P_{70} = 0.0061$, $P_{90} = 0.43$).

For the 10th nucleotide of siRNA sequence, Jagla et al. suggested that A/U at this position was related to siRNA functionality[6]. We found no evidence that $10th\ nucleotide=A/U$ was associated with significant up-shift of the efficacy distribution ($P_{wald} = 0.19$), or elevated the chances of achieving higher efficacies ($P_{70} = 0.54$, $P_{90} = 0.062$).

For the 11th nucleotide of siRNA sequence, Hsieh et al. suggested that there was a strong preference for G/C at this position for functional siRNA[5]. However, we found no evidence that the feature $11th\ nucleotide=(G/C)$ was associated with up-shift of the efficacies distribution ($P_{wald} = 0.92$), or elevated the chances of achieving higher efficacies ($P_{70} = 0.80$, $P_{90} = 0.88$).
For the 19th nucleotide of siRNA sequence, several groups suggested that A/U at this position was associated with siRNA functionality[3, 6, 7]. This observation was confirmed by our analysis. The feature \(19\text{th nucleotide}=\text{(A/U)}\) significantly boosted the chance of achieving >70% and >90% efficacies \(P_{70} = 0.00029, P_{90} = 0.0043\) and was strikingly associated with an up-shift of the efficacy distribution \(P_{\text{wald}} = 0.000058\).

Ui-Tei et al. suggested that in highly effective siRNA, at least five (A/U)s should be contained in the 3’ end one-third of the sense strand[7]. Jagla et al. suggested that more than three (A/U)s between position 13 and 19 was critical for siRNA functionality[6]. Reynolds et al. suggested that the occurrence of three or more (A/U)s in nucleotides 15-19 could be a criterion for selecting functional siRNA[4]. Our results indicated that both features at least three (A/U)s in the seven nucleotides at the 3’ end and at least three (A/U)s in the five nucleotides at the 3’ end were strongly associated with an up-shift of the efficacy distribution \(P_{\text{wald}} = 2.5\text{E}-9\), and 0.000022, respectively), in addition to strongly boosted the chances of achieving both >70% \(P_{70} = 0.00001\), and 0.0018, respectively) and >90% efficacies \(P_{90} = 0.00001\), and 0.00016, respectively). There was evidence that the feature at least five (A/U)s in the seven nucleotides at the 3’ end significantly boosted the chance of achieving >70% efficacy \(P_{70} = 0.026\), but no evidence was found that this feature boosted the chance of >90% efficacy \(P_{90} = 0.26\), or was associated with up-shift of the efficacy distribution \(P_{\text{wald}} = 0.10\).

About long G/C stretches and siRNA functionality, two groups suggested that siRNAs with G/C stretches longer than 9 should be excluded for their lack of functionality[7, 8], and another two groups suggested that no occurrences of G/C stretches of length 7 or longer should not be allowed in siRNA design[9, 10]. Our results indicated that, indeed, the feature no occurrences of G/C stretches of length 7 or longer significantly boosted the chances of achieving >70% and >90% efficacies \(P_{70} = 0.00001, P_{90} = 0.00001\), and were associated with significant up-shifts of the efficacy distribution \(P_{\text{wald}} = 0.000015\). The feature no occurrences of G/C stretches of length 9 or longer was not tested because too few records (<30) carried this feature in our dataset.

About stretches of identical nucleotides and siRNA functionality, several groups suggested that consecutive 3 or 4 identical nucleotides should be avoided in siRNA design to reduce the RNA duplex internal stability[9-13]. In our analysis, both two features no occurrences of three or more identical nucleotides in a row and no occurrences of four or more identical nucleotides in a row were found to be associated with a significant up-shift of the efficacy distribution \(P_{\text{wald}} = 0.0067\), and 0.0014, respectively), in addition to significantly boost the chances of achieving >70% \(P_{70} = 0.0061\), and 0.00001, respectively) and >90% efficacies \(P_{90} = 0.043\), and 0.012, respectively).

About G/C content and siRNA functionality, seven different G/C content ranges reported in previous studies were tested: 30 – 52% [4], 32 – 79% [14], 30 – 70% [15], 35 – 60% [9], 20 – 50% [13], 31.6 – 57.9% [3] and 30 – 79% [11]. Our results indicated that the G/C content ranges 35 – 60%, 31.6 – 57.9% and 30 – 70% were associated with significant up-shifts of the efficacy distribution \(P_{\text{wald}} = 0.00018, 0.00018, \text{and } 0.00028,\)
respectively), and significantly boosted chances of achieving > 70% ($P_{70} = 0.00001$ for all three features) and >90% efficacies ($P_{90} = 0.0019, 0.0019, \text{ and } 0.00001$, respectively). The feature $G/C$ content is between 20 and 50% had a weaker, yet still significant effect in associating with up-shift of the efficacy distribution ($P_{\text{wald}} = 0.037$).

**Category 3: thermodynamic features**

About 5’ end binding energy and siRNA efficacy, Chalk et al. suggested that *sense 5’ binding energy between -9 and-5 Kcal/Mol* was associated with higher siRNA efficacies[16]. We tested this feature but found no evidence that it was associated with an up-shift of the efficacy distribution or boosted the chances of achieving higher efficacies ($P_{70} = 0.71, P_{90} = 0.90, P_{\text{wald}} = 0.87$).

About mid-sequence binding energy, Khvorova et al. suggested that *lower internal energy (N6 – N11)* was strongly associated with higher siRNA functionality[17]. Chalk et al. suggested that the feature *binding energy of N7-N12 > -13 Kcal/Mol* was strongly associated with higher siRNA efficacy[16]. Poliseno et al. made similar observations, and suggested that the energy of N7-N11 was correlated with functional siRNA[18]. We found that the feature *binding energy of N7-N12 ≤ -13 Kcal/Mol* significantly boosted the chance of achieving >70% efficacy ($P_{70} = 0.032$, yet $P_{90} = 0.10, P_{\text{wald}} = 0.054$). However, we did not find any evidence that the features *binding energy of N6-N11 < -13 Kcal/Mol* and *mean of free energy profile of N7-N11 is between 1.97 and -1.65 Kcal/Mol (inclusive)* associated with higher siRNA efficacy distribution, or higher chances of achieving >70% or >90% efficacies ($P_{\text{wald}} = 0.62, P_{70} = 0.46, P_{90} = 0.61$ for the feature *binding energy of N6-N11 < -13 Kcal/Mol*; $P_{\text{wald}} = 0.32, P_{70} = 0.46, P_{90} = 0.43$ for the feature *mean of free energy profile of N7-N11 is between 1.97 and -1.65 Kcal/Mol (inclusive)*).

About 3’ end binding energy and siRNA functionality, Chalk et al. suggested that the feature *binding energy of N16-N19 > -9 Kcal/Mol* was associated with higher siRNA effectiveness[16]. We found that indeed, this feature was associated with a significant up-shift of the efficacy distribution ($P_{\text{wald}} = 0.0025$), and boosted the chance of achieving >70% and >90% efficacies ($P_{70}= 0.010, P_{90} = 0.0026$).

About binding energy difference between the 3’ end and the 5’ end of the siRNA, Khvorova et al. suggested that 5’ terminal of anti-sense strand had enhanced flexibility for functional siRNA[17]. Poliseno et al. suggested that for functional siRNA the five terminal nucleotides of 5’ end of the anti-sense strand had a higher free energy than that of 5’ end of sense strand[18]. Chalk et al. observed that a siRNA was more effective if the free energy of 5’ end of anti-sense strand was higher than that of 5’ side of sense strand, and if their difference was less than 1 KCal/Mol[16]. Our analysis indicated that the feature *binding energy of N16-N19 ≥ binding energy of N1-N4* was associated with a significant up-shift of the efficacy distribution ($P_{\text{wald}} = 0.0043$), and boosted the chances of achieving >70% and >90 efficacies significantly ($P_{70} = 0.013, P_{90} = 0.018$). The feature *binding energy of N16-N19 – binding energy of N1-N4 is between 0 and 1 KCal/Mol* showed even higher levels of significance ($P_{\text{wald}} = 0.010, P_{70} = 0.00036$ and $P_{90} = 0.0078$). These results agreed with the observation by Chalk et al. very well. However, when one more nucleotide was included in the calculations of terminal energy,
the evidence for significance weakened. The feature binding energy of N15-N19 > binding energy of N1-N5 was not found to be associated with an up-shift of the efficacy distribution ($P_{wald} = 0.12$) or higher chances of achieving $>$70% or $>$90% efficacies ($P_{70} = 0.20$, $P_{90} = 0.26$).

About internal folding potential and siRNA functionality, Wang and Mu suggested that functional siRNA sequences should have minimum free energy higher than -5 KCal/Mol[9]. Chalk et al. observed that absolute value of the total hairpin energy need to be less than 1 KCal/Mol for functional siRNAs[16]. Reynolds et al. found there was no functional siRNA with $T_m > 60^\circ C$ in their data set and suggested that $T_m < 20^\circ C$ was a feature associated with higher siRNA efficacies[4]. Our analysis indicated that the feature folding energy of sense strand $\geq -5$ KCal/Mol was associated with a significant up-shift of the efficacy distribution ($P_{wald} = 0.023$), and strongly boosted the chances of achieving $>$70% and $>$90% efficacies ($P_{70} = 0.00004$, $P_{90} = 0.00002$). There was no evidence found to associate the feature absolute value of total hairpin energy $< 1$ KCal/Mol with higher siRNA efficacies ($P_{70} = 0.18$, $P_{90} = 0.23$, $P_{wald} = 0.19$). The feature $T_m < 60^\circ C$ was found to be associated with significant up-shift of efficacy distribution ($P_{wald} = 0.0057$), and significantly boosted chance of achieving $>$70% efficacy ($P_{70} = 0.0014$). However, no evidence was found to associate the feature $T_m < 20^\circ C$ with higher efficacies. Instead, the complementary feature of this feature ($T_m \geq 20^\circ C$) was found to boost the chance of achieving $>$90% efficacy ($P_{90} = 0.0026$). Additionally, our results suggested that the feature $T_m$ is between $20$ and $60^\circ C$ was associated with a significant up-shift of the efficacy distribution ($P_{wald} = 0.003$), and boosted the chance of achieving $>$70% and $>$90% efficacies ($P_{70} = 0.0045$, $P_{90} = 0.023$).

**Category 4: features defined based on target mRNA sites**

About the location of the siRNA target site on the mRNA, it was suggested that the first 100 nucleotides of CDS, 5’ UTR and 3’UTR should not be targeted by siRNAs since they may contain regulatory protein binding sites[11, 14], an argument agreed with by Wang and Mu, who suggested that only the CDS region be used when designing siRNA experiments[9]. However, Hsieh et al. observed that siRNAs targeting the 3’UTR were equally effective as siRNAs targeting the CDS[5]. In addition, they observed that siRNAs targeting outside of the third quartile of CDS yielded higher knockdown effectiveness. In our analysis, three features target site is not on the 5’UTR, target site is not on the 3’UTR and target site is on CDS were found to quite strongly boost the chances of achieving both $>$70% and $>$90% efficacies ($P_{70} = 0.00001$ and $P_{90} = 0.00001$ for three features), and associate with significant up-shift of efficacy distribution ($P_{wald} = 0.016$, 0.00052, and 0.000055, respectively). These results generally agreed with previous observations[9, 11, 14]. Contrary to the observation made by Hsieh et al., we found the target site on the 4th quartile rather than on the 3rd quartile of CDS had negative effect for siRNA functionality. The feature target site is not on the 4th quartile of CDS quite strongly boosted the chance of achieving $>$70% efficacy ($P_{70} = 0.00015$, yet $P_{90} = 0.088$, $P_{wald} = 0.025$). In addition, we observed that when the target site was on the first three quartiles, RNAi achieved significantly higher efficacies. Indeed, feature target site is on the 3rd quartile of CDS had significant effect in boosting the chance of achieving $>$70% and $>$90% efficacies ($P_{70} = 0.017$, $P_{90} = 0.029$), and associating with up-
shift of efficacy distribution ($P_{\text{wald}} = 0.031$), while feature target site is on the 1st quartile of CDS and target site is on the 2nd quartile of CDS significantly boosted the chance of achieving >70% ($P_{70} = 0.0024$), and >90% ($P_{90} = 0.023$) efficacy, respectively. The odds ratio permutation tests and Wald test did not yield significant determinants for feature target site is on the first 100 nucleotides of CDS ($P_{70} = 0.82$, $P_{90} = 0.57$, $P_{\text{wald}} = 0.59$).

About the features on accessibility of mRNA region targeted by siRNA, Scheer et al. suggested that mRNA region gaining high accessibility score was related to high cleavage efficacy[19]. Ding et al. suggested that the anti-sense siRNA binding energy should be less than -10 KCal/Mol for functional siRNA[12]. Yiu et al. suggested functional siRNA should pass their filtering algorithm that filtered out mRNA target region deemed as inaccessible[20]. Schubert et al. suggested that the siRNA silencing efficacy was positively correlated with local energy of target structure which was measured by LFE[21]. Luo and Chang suggested that H-b index was highly correlated with the gene-silencing efficiency of siRNA[22]. In our analysis, the feature Anti-sense siRNA binding energy $\leq$ -10 KCal/Mol significantly boosted the chance of achieving >70% efficacy ($P_{70} = 0.026$, yet $P_{90} = 0.23$, $P_{\text{wald}} = 0.072$). The feature $LFE_{\text{mss}} \geq -20.9$ KCal/Mol was found to be associated with a weak, yet significant up-shift of efficacy distribution ($P_{\text{wald}} = 0.048$). The feature $H-b$ index $< 28.8$ was found to significantly boost the chance of achieving >70% efficacy, as well as associate with up-shift of efficacy distribution ($P_{70} = 0.032$, $P_{\text{wald}} = 0.012$, yet $P_{90} = 0.12$). But odds ratio permutation tests and Wald test did not yield significant determinations for feature Accessibility score $> 0$ ($P_{70} = 0.57$, $P_{90} = 0.43$, $P_{\text{wald}} = 0.37$). The feature Does not pass Repelling Loop Filter was found to significantly boost the chance of achieving >90% efficacy ($P_{90} = 0.010$), contrary to previous observation. The feature Local folding potential (mean) $\geq -22.72$ KCal/Mol was found to strongly elevate the chance of reaching >70% and >90% efficacies ($P_{70} = 0.00001$, $P_{90} = 0.00001$), and associate with very significant up-shift of efficacy distribution ($P_{\text{wald}} = 9.3E-09$). These observations generally agreed with previous suggestions that the accessibility of mRNA region targeted by siRNA influenced RNAi effectiveness.

**Category 5: features based on experimental settings**

Several groups reported that efficiency of transfection was typically higher for synthetic siRNA than for plasmid DNA[23, 24]. Our survey confirmed the positive effect of synthetic siRNA methods in achieving higher knockdown efficacies. The feature Transfection method = Synthesized oligos was found to significantly boost the chance of achieving >70% efficacy ($P_{70} = 0.026$), and associate with significant up-shift of efficacy distribution ($P_{\text{wald}} = 0.028$).

It is known that cell line types were correlated with siRNA efficacies. Several groups showed that the transfectability of cells is the limiting step in siRNA mediated gene silencing and differs between different cell types[25-27]. For example, HeLa cells were well-known for their ease of transfection[24], and primary cells had lower transfection efficacy than cancer cells[24, 28]. Moreover, there was evidence that the genetic context in each individual tumor cell lines also had affected in RNAi[28, 29]. Our survey indicated that the feature Cell line = HeLa strikingly boosted the chances of achieving
>70% and >90% efficacies ($P_{70} = 0.00001$, $P_{90} = 0.00016$), and was associated with quite significant up-shift of efficacy distribution ($P_{\text{wald}} = 4.0\text{E-09}$). Other four common cell lines HEK293, MCF7, CV-1, and 3T3 had significantly negative effect in achieving higher knockdown percentages ($P_{70} = 0.021$ for feature $\text{Cell line} \neq \text{HEK293}$; $P_{90} = 0.00016$ for feature $\text{Cell line} \neq \text{MCF7}$; $P_{70} = 0.0018$, $P_{90} = 0.00001$; $P_{\text{wald}} = 0.027$ for feature $\text{Cell line} \neq \text{CV-1 and derivatives}$; $P_{70} = 0.026$, $P_{90} = 0.00001$ for feature $\text{Cell line} \neq 3T3$).

It has been reported that the efficacy ratings depended on the test objects (protein or mRNA) and test methods (Western blot, or PCR-related). The turnover of proteins has been implicated in the relationship between knockdown percentage between proteins and mRNAs[30]. In our analysis, there was evidence that when protein levels were tested, the efficacy ratings tended to go significantly higher ($P_{70} = 0.00001$, $P_{90} = 0.00001$, $P_{\text{wald}} = 2.2\text{E-09}$). On other hand, when mRNA levels were measured, the efficacy ratings were significantly lower ($P_{70} = 0.00001$, $P_{90} = 0.00001$, $P_{\text{wald}} = 9.3\text{E-10}$ for feature $\text{Test object} \neq \text{mRNA}$). Similarly, the feature $\text{Test method} = \text{Western blot}$ led to significantly higher efficacy ratings ($P_{70} = 0.00001$, $P_{90} = 0.00001$, $P_{\text{wald}} = 3.8\text{E-14}$). The feature $\text{Test method} = \text{PCR-related}$ led to significantly lower efficacy ratings ($P_{70} = 0.00001$, $P_{90} = 0.00001$, and $P_{\text{wald}} = 2.6\text{E-08}$). The features $\text{Test method} \neq \text{Northern blot}$ and $\text{Test method} \neq \text{Luciferase assay}$ were also associated with a significant up-shift of efficacy distribution ($P_{\text{wald}} = 0.010$ and $4.7\text{E-08}$, respectively), and have very significant chances to achieve >70% and >90% efficacies ($P_{70} = 0.00001$, and $P_{90} = 0.00001$, for both features). The feature $\text{Test method} = \text{bDNA}$ was found to have a strong chance to reach >90% efficacy ($P_{90} = 0.0019$).

### 3. Performance of DRM rule sets in subsets divided by confounding factors

The factors regarding experimental settings, e.g., test method and test object are considered as confounding factors for our purpose of developing siRNA design rules, because although they influence the siRNA efficacy, we do not want to include them in the siRNA design criteria, as that would restrict the applicability of the resulting design rules. Yet, the high level of significance of features concerning these factors ($\text{Test method} = \text{Western blot}$ and $\text{Test object} \neq \text{mRNA}$) prompted us to examine the performance of the DRM rule sets for the subsets of siRNA experiments separated by these features. Supplementary Figure 4 shows how the PPVs of the four subsets of records carrying the features $\text{Test method}=\text{Western blot}$, $\text{Test method} \neq \text{Western blot}$, $\text{Test object}=\text{mRNA}$ and $\text{Test object} \neq \text{mRNA}$, respectively, changed with the stringency level $\alpha$ for the DRM rule sets. It appears that at higher $\alpha$ levels, the DRM rule sets are more effective in selecting good siRNAs for “the Western subset” and “the non-mRNA subset”, and less effective for “the non-Western subset” and “the mRNA subset”. As $\alpha$ decreases, they become less effective for “the Western subset” and “the non-mRNA subset”, but more effective for “the non-Western subset” and “the mRNA subset”. The PPVs for the four subsets become roughly equal at $\alpha < 0.8$.

Due to the small sample size problem, this analysis should not be considered as conclusive. Yet, it suggests that DRM rule sets behave differently for subpopulations of
siRNAs tested under different experimental settings. We will examine this issue further as more data becomes available through the *siRecords* effort. When there is enough data, we will try to develop design rule sets for these different subpopulations of siRNAs separately.

4. Utility of online siRNA design tools

In this section we discuss the issue of relative utility of existing siRNA design tools. A large number of siRNA design tools are now available online. It is interesting to assess which of them are used more frequently than others in the current siRNA design practice. Another reason why we look at this issue is that if the current siRNA design practice is dominated by one or two design tools that are most frequently used, the objectiveness of the performance comparison (shown in Table 3) would be compromised. A straightforward way to analyze the utility of these siRNA design tools is to perform a statistics analysis of those original siRNA studies of what design tools that were used in their siRNA design. However, a large proportion of these original studies (~80%) did not have descriptions about what tools were used in their design. Thus, we seek to develop a method of assessing the utility of the design tools approximately by directly analyzing the *siRecords* data.

We assume that there are $N$ siRNA design tools from which a user can pick to help his/her siRNA design, and that the user picks only one tool to assist the design of any single siRNA experiment (this is a simplifying assumption for the ease of discussion – it is conceivable that the user may seek help from multiple design tools in a real siRNA design task). The design tool chosen is used to make predictions of a pool of candidate siRNA sites, and a proportion of the candidate sites that are predicted to be effective by that design tool are chosen to be tested experimentally. It is not hard to conceive that the higher utility a tool possesses, the better chance that a candidate siRNA predicted to be effective by this tool is picked to be tested. Considering that existing design tools have overlaps in their predictions (that is, some siRNA sites are predicted to be effective by multiple design tools), we focus on the “uniquely predicted effective” (or UPE) sites only, i.e. the candidate siRNA sites that are predicted to be effective by only one tool. Thus, we have

$$\text{Utility}(i) \propto P(T | UPE(i)), \quad i \in [1, N].$$ \hspace{1cm} (4.1)

That is, the utility of a design tool, tool $i$, is proportional to the conditional probability that the UPE sites of this tool are picked to be tested experimentally. By Bayes’ Theorem, we have

$$P(T | UPE(i)) = \frac{P(UPE(i) | T) \cdot P(T)}{P(UPE(i))} \propto \frac{P(UPE(i) | T)}{P(UPE(i))},$$ \hspace{1cm} (4.2)

where $P(T)$, the probability for a candidate siRNA site to be tested, is considered as a constant. From (4.1) and (4.2), we get

$$\text{Utility}(i) \propto \frac{P(UPE(i) | T)}{P(UPE(i))}. \hspace{1cm} (4.3)$$

In other words, the utility of a given design tool is proportional to the ratio of the probability that a tested site is a UPE of this tool, and the probability that any site (tested
or untested) is a UPE of the tool. We will call this ratio the “uniquely positive testing ratio”, or UPTR.

We estimated the UPTR values of the 15 online siRNA design tools. The Set T data involves 774 genes, on which there are 2,453,510 19-mer candidate sites. Among these candidate sites, 1,014 were experimentally tested. We calculated the number of UPE sites, $n_{UPE}^T$, among the 1,014 tested sites for each siRNA design tool. Then, we randomly picked 10,000 sites from the 2,453,510 candidate site pool, and calculated the number UPE sites for among these 10,000 sites, $n_{UPE}$. The UPTR of a design tool was calculated as

$$UPTR = \frac{n_{UPE}^T}{1014} \cdot \frac{n_{UPE}}{10000}.$$  (4.4)

Four online design tools, EMBOSS sirna by Institute Pasteur, SDS/MPI by University of Hong Kong, Ambion siRNA Target Finder by Ambion, Inc. and Jack Lin’s siRNA Sequence Finder by Cold Spring Harbor Laboratory had fairly large coverage of predicted effective sites: among the 1,014 tested sites, 765, 642, 564 and 229 sites were predicted to be effective by these tools respectively. This resulted in 0 UPE site found in the tested set for several of the other design tools. To counter this problem, we loosened the definition of UPE for the remaining 11 tools, in that a site was deemed as a UPE if it was predicted to be effective by this tool, but not predicted to be effective by any of the other lower coverage tools. This compromise was deemed proper because we were only making approximate estimate of these tools’ relative utility. Without making this compromise, this utility comparison of these tools would not be possible.

Supplementary Table 7 shows the $n_{UPE}^T$, $n_{UPE}$ and the UPTR of the 15 siRNA design tools. Five tools, Imgenex sirna Designer by Imgenex Corp., WI siRNA Selection Program by Whitehead Institute, QIAGEN siRNA Design Tool by QIAGEN, Inc., SiMAX by MWG-Biotech, Inc. and IDT RNAi Design by Integrated DNA Technologies, Inc. had the highest levels of utility (with UPTR between 8 and 18). These tools are followed by siDESIGN Center by Dharmacon, Inc., Promega siRNA Target Designer by Promega Corp., BLOCK-iT RNAi Designer by Invitrogen Corp., BIOPREDsi by Novartis Institutes for BioMedical Research and siRNA Target Finder by GenScript Corp. (with UPTR between 2 and 8), then by siSearch by Karolinska Institutet, Ambion siRNA Target Finder by Ambion, Inc., SDS/MPI by University of Hong Kong, EMBOSS sirna by Institute Pasteur and Jack Lin’s siRNA Sequence Finder by Cold Spring Harbor Laboratory (UPTR <2). These results suggest that the current siRNA design practice is not dominated by one or two individual tools; rather, many tools are being used with varied levels of utility.

5. Rationale of the DRM procedure

Finally, we discuss the considerations underlying the development of the procedure (what we term the DRM procedure) through which the siRNA design criteria were obtained in this study. In essence, this procedure can be described as follows: First, we construct rules, or conjunctions (combinations) of features that lead to strong boosting of siRNA
efficacy; the positive cooperativity between rules is exploited at this step. Second, we merge the rules, remove redundancy, and formulate rule sets, or disjunctions of rules, the stringency (or specificity) of which is controlled at prescribed levels. All previous studies in siRNA design criteria focused on the first of these two steps, i.e., constructing conjunctive rules from interesting features. What is the advantage of taking the second step of making disjunctive rule sets? The answer is, simply put, by constructing disjunctive rule sets, while maintaining a good level of specificity, we can achieve a higher level of sensitivity, because sensitivities of all rules in the disjunctive rule set add up to produce the sensitivity of the disjunctive rule set.

Suppose we are looking at a single rule, or a conjunction of $l$ features. As $l$ increases, while the specificity of this rule is increasing (given that the features included in the rule are truly helping the selection of effective siRNAs), the sensitivity of the rule will be decreasing exponentially, because whenever a new feature is added into the conjunction, a proportion of the remaining experiments will fail to carry this new feature. This is demonstrated in Supplementary Figure 5, where the sensitivity and specificity of single conjunctive rules are plotted against the number of features $l$ (also see Figure 3C). We need to consider the balance between the gain in specificity and the loss in sensitivity, to determine if the including of the new feature is worthwhile. When we look at a rule set (or a disjunction of $m$ rules), as $m$ increases, the specificity of the rule sets also decreases; meanwhile the sensitivity of the rule set will increase. The simultaneous changes in specificity and sensitivity with $m$ for the disjunctive rule sets are different from the simultaneous changes in specificity and sensitivity with $l$ for a single conjunctive rule, in that the changes for the rule sets are approximately linear (Supplementary Figure 6); and the slope of the rising curve of the sensitivity is greater than the slope of the falling curve of the specificity. Therefore, generally speaking, the higher $m$ is, the better performance the rule sets will achieve, given that the specificity of each of the $m$ rules is well controlled.
**Supplementary Figure 1** A permutation test of odds ratios was used to determine the significance of a feature in its association with higher chances to achieve >70% (and >90%) efficacies. The odds ratio between the feature the 6th nucleotide=A and complementary feature the 6th nucleotide ≠A for >70% efficacies (records with efficacy rating “high” or “very high”) was 1.289, smaller than 243 of 100,000 odds ratios in the null distribution. Thus, the permutation test rendered P= 243/100,000=0.00243 for >70% efficacies for the feature the 6th nucleotide=A.

**Supplementary Figure 2** The sigmoid shape of the ascending curves – (%records achieving >90% or >70% efficacy) vs. l relationships – suggests that there is positive cooperativity between features included in the selected feature combinations. The ascending curves would be straight lines (illustrated by dotted lines with arrowheads) if no cooperativity existed among the features.

**Supplementary Figure 3** The relationships between the number of effective siRNAs predicted and the gene length, for two DRM rule sets, RS0.951 and RS0.845, plotted on log-log scale.

**Supplementary Figure 4** The PPVs for the entire Set T and the four subsets carrying the features Test method=Western blot, Test method ≠ Western blot, Test object=mRNA and Test object ≠ mRNA respectively for 6 DRM rule sets with decreasing α. A siRNA experiment was considered effective if it achieved >70% efficacy (was rated “high” or “very high” efficacy).

**Supplementary Figure 5** Plot of sensitivity and specificity of single rules (conjunctions of l features) vs. l. A siRNA experiment was considered effective if it achieved >70% efficacy (was rated “high” or “very high” efficacy). The sensitivity shows an approximately exponential decay. The features used in conjunctions are the most frequently occurring features included in DRM RS0.951 (Table 2). The rule with l=1 consists of the most frequently occurring feature in RS0.951 (F15); the rule with l=2 consists of the two most frequently occurring features; and so on. In case of ties (e.g., both F2 and F5 occurred 6 times), the tied features are included in the rules separately, and the mean sensitivity and specificity of the resulting rules are used.

**Supplementary Figure 6** Plot of sensitivity and specificity of rule sets (disjunctions of m rules) vs. m. A siRNA experiment was considered effective if it achieved >70% efficacy (was rated “high” or “very high” efficacy). The sensitivity rises in an approximately linear manner with increasing m. The 7 rules included in DRM RS0.951 are used. For a given m, all possible rule sets, or disjunctions of m rules were constructed, and the average sensitivity and specificity of these rule sets (when applied to Set T) are shown. Error bars denote standard errors.
Null distribution of odds ratios

Odds ratio of the feature "6th nucleotide = A"

Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 6

The graph illustrates the relationship between the number of rules included in rule sets (m) and the sensitivity and specificity. As the number of rules increases, sensitivity increases while specificity decreases, indicating a trade-off between the two metrics.
Supplementary Table 1  Direct sequence features examined in this study and their significance in the survey.

| Feature name                                               | Ref | % Low | % Medium | % High | % Very high | # Low | # Medium | # High | # Very high | $P_{70}$ | $P_{90}$ | $P_{wald}$ | Significance |
|------------------------------------------------------------|-----|-------|----------|--------|-------------|-------|----------|--------|-------------|---------|---------|-----------|--------------|
| 1st nucleotide of the siRNA sequence=A                    |     | 16.7  | 16.7     | 32.3   | 34.3        | 50    | 50       | 97     | 103         | 0.88    | 0.46    | 0.68      |              |
| 1st nucleotide of the siRNA sequence≠A                     | [2] | 14.6  | 16.3     | 35.0   | 34.1        | 276   | 307      | 659    | 642         | 0.12    | 0.54    | 0.32      |              |
| 1st nucleotide of the siRNA sequence=U                     |     | 21.3  | 15.2     | 30.1   | 33.4        | 63    | 45       | 89     | 99          | 1       | 0.64    | 0.95      |              |
| 1st nucleotide of the siRNA sequence≠U                      | [2, 3]| 13.9  | 16.5     | 35.3   | 34.2        | 263   | 312      | 667    | 646         | 0.0014  | 0.36    | 0.0495    | **           |
| 1st nucleotide of the siRNA sequence=C                     |     | 15.2  | 17.7     | 33.5   | 33.5        | 74    | 86       | 163    | 163         | 0.84    | 0.64    | 0.73      |              |
| 1st nucleotide of the siRNA sequence≠C                      |     | 14.8  | 16.0     | 34.9   | 34.3        | 252   | 271      | 593    | 582         | 0.16    | 0.36    | 0.27      |              |
| 1st nucleotide of the siRNA sequence=G                     | [2] | 12.6  | 16.0     | 36.9   | 34.5        | 139   | 176      | 407    | 380         | 0.0024  | 0.36    | 0.022     | **           |
| 1st nucleotide of the siRNA sequence≠G                      |     | 17.3  | 16.7     | 32.3   | 33.7        | 187   | 181      | 349    | 365         | 1       | 0.64    | 0.98      |              |
| 2nd nucleotide of the siRNA sequence=A                     |     | 12.1  | 16.0     | 33.8   | 38.1        | 77    | 102      | 215    | 243         | 0.010   | 0.0026  | 0.0019    | ***          |
| 2nd nucleotide of the siRNA sequence≠A                      |     | 16.1  | 16.5     | 35.0   | 32.4        | 249   | 255      | 541    | 502         | 0.99    | 1       | 1         |              |
| 2nd nucleotide of the siRNA sequence=U                      |     | 17.4  | 14.0     | 37.2   | 31.4        | 60    | 48       | 128    | 108         | 0.54    | 0.95    | 0.84      |              |
| 2nd nucleotide of the siRNA sequence≠U                      |     | 14.5  | 16.8     | 34.1   | 34.6        | 266   | 309      | 628    | 637         | 0.46    | 0.052   | 0.16      |              |
| 2nd nucleotide of the siRNA sequence=C                      |     | 15.5  | 17.3     | 34.8   | 32.4        | 84    | 94       | 189    | 176         | 0.84    | 0.86    | 0.85      |              |
| 2nd nucleotide of the siRNA sequence≠C                      |     | 14.7  | 16.0     | 34.6   | 34.7        | 242   | 263      | 567    | 569         | 0.16    | 0.14    | 0.15      |              |
| 2nd nucleotide of the siRNA sequence | 15.9 | 17.1 | 33.9 | 33.0 | 105 | 113 | 224 | 218 | 0.90 | 0.77 | 0.86 |
|-------------------------------------|------|------|------|------|-----|-----|-----|-----|------|------|------|
| 2nd nucleotide of the siRNA sequence ≠ G | 14.5 | 16.0 | 34.9 | 34.6 | 221 | 244 | 532 | 527 | 0.098 | 0.23 | 0.14 |
| 3rd nucleotide of the siRNA sequence = A | [4] 13.5 | 17.2 | 34.0 | 35.3 | 94  | 120 | 237 | 246 | 0.32 | 0.21 | 0.18 |
| 3rd nucleotide of the siRNA sequence ≠ A | 15.6 | 15.9 | 34.9 | 33.6 | 232 | 237 | 519 | 499 | 0.68 | 0.79 | 0.82 |
| 3rd nucleotide of the siRNA sequence = U | 14.8 | 15.2 | 34.0 | 36.0 | 72  | 74  | 165 | 175 | 0.20 | 0.12 | 0.19 |
| 3rd nucleotide of the siRNA sequence ≠ U | 15.0 | 16.7 | 34.8 | 33.6 | 254 | 283 | 591 | 570 | 0.80 | 0.88 | 0.81 |
| 3rd nucleotide of the siRNA sequence = C | 15.5 | 17.4 | 33.3 | 33.9 | 75  | 84  | 161 | 164 | 0.84 | 0.57 | 0.70 |
| 3rd nucleotide of the siRNA sequence ≠ C | 14.8 | 16.1 | 35.0 | 34.2 | 251 | 273 | 595 | 581 | 0.16 | 0.43 | 0.30 |
| 3rd nucleotide of the siRNA sequence = G | 16.4 | 15.3 | 37.3 | 30.9 | 85  | 79  | 193 | 160 | 0.61 | 0.98 | 0.92 |
| 3rd nucleotide of the siRNA sequence ≠ G | 14.5 | 16.7 | 33.8 | 35.1 | 241 | 278 | 563 | 585 | 0.39 | 0.018 | 0.085 |
| 4th nucleotide of the siRNA sequence = A | 16.4 | 16.2 | 35.6 | 31.7 | 83  | 82  | 180 | 160 | 0.82 | 0.94 | 0.91 |
| 4th nucleotide of the siRNA sequence ≠ A | 14.5 | 16.4 | 34.3 | 34.8 | 243 | 275 | 576 | 585 | 0.18 | 0.062 | 0.091 |
| 4th nucleotide of the siRNA sequence = U | 14.7 | 16.6 | 37.3 | 31.4 | 70  | 79  | 178 | 150 | 0.50 | 0.96 | 0.79 |
| 4th nucleotide of the siRNA sequence ≠ U | 15.0 | 16.3 | 33.9 | 34.9 | 256 | 278 | 578 | 595 | 0.50 | 0.043 | 0.21 |
| 4th nucleotide of the siRNA sequence = C | 14.1 | 15.4 | 31.5 | 39.0 | 88  | 96  | 197 | 244 | 0.098 | 0.00036 | 0.0075 |
| 4th nucleotide of the siRNA sequence ≠ C | 15.3 | 16.7 | 35.9 | 32.1 | 238 | 261 | 559 | 501 | 0.90 | 1    | 0.99 |
| 4th nucleotide of the siRNA sequence = G | 14.7 | 17.3 | 34.8 | 33.1 | 85  | 100 | 201 | 191 | 0.71 | 0.77 | 0.70 |
| sequence=| 4th nucleotide of the siRNA sequence≠G | 5th nucleotide of the siRNA sequence=| 5th nucleotide of the siRNA sequence≠| 5th nucleotide of the siRNA sequence=| 5th nucleotide of the siRNA sequence≠| 5th nucleotide of the siRNA sequence=| 5th nucleotide of the siRNA sequence≠| 5th nucleotide of the siRNA sequence=| 5th nucleotide of the siRNA sequence≠|
|--------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
|        | 15.0 | 16.0 | 34.5 | 34.5 | 241 | 257 | 555 | 554 | 0.29 | 0.23 | 0.30 | ** |
| 5th nucleotide of the siRNA sequence= | 12.9 | 15.1 | 34.9 | 37.1 | 77 | 90 | 209 | 222 | 0.008 | 0.023 | 0.010 | ** |
| 5th nucleotide of the siRNA sequence≠ | 15.7 | 16.8 | 34.5 | 33.0 | 249 | 267 | 547 | 523 | 0.99 | 0.98 | 0.99  |      |
| 5th nucleotide of the siRNA sequence= | 17.7 | 16.6 | 33.1 | 32.6 | 84 | 79 | 157 | 155 | 0.97 | 0.82 | 0.94  |      |
| 5th nucleotide of the siRNA sequence≠ | 14.2 | 16.3 | 35.0 | 34.5 | 242 | 278 | 599 | 590 | 0.026 | 0.18 | 0.064 | *   |
| 5th nucleotide of the siRNA sequence= | 14.2 | 15.2 | 35.4 | 35.2 | 75 | 80 | 187 | 186 | 0.098 | 0.23 | 0.17  |      |
| 5th nucleotide of the siRNA sequence≠ | 15.2 | 16.7 | 34.4 | 33.8 | 251 | 277 | 569 | 559 | 0.90 | 0.77 | 0.83  |      |
| 5th nucleotide of the siRNA sequence= | 15.4 | 18.5 | 34.8 | 31.2 | 90 | 108 | 203 | 182 | 0.97 | 0.98 | 0.97  |      |
| 5th nucleotide of the siRNA sequence≠ | 14.7 | 15.6 | 34.5 | 35.2 | 236 | 249 | 553 | 563 | 0.032 | 0.023 | 0.035 | ** |
| 6th nucleotide of the siRNA sequence= | [2, 3] | 12.2 | 15.4 | 35.8 | 36.6 | 81 | 102 | 238 | 243 | 0.0024 | 0.043 | 0.0058 | *** |
| 6th nucleotide of the siRNA sequence≠ | 16.1 | 16.8 | 34.1 | 33.0 | 245 | 255 | 518 | 502 | 1 | 0.96 | 0.99  |      |
| 6th nucleotide of the siRNA sequence= | 13.1 | 16.3 | 33.7 | 36.9 | 76 | 94 | 195 | 213 | 0.098 | 0.035 | 0.042 | ** |
| 6th nucleotide of the siRNA sequence≠ | 15.6 | 16.4 | 34.9 | 33.1 | 250 | 263 | 561 | 532 | 0.90 | 0.97 | 0.96  |      |
| 6th nucleotide of the siRNA sequence= | 17.3 | 19.0 | 33.3 | 30.4 | 79 | 87 | 152 | 139 | 1 | 0.99 | 0.99  |      |
| 6th nucleotide of the siRNA sequence≠ | 14.3 | 15.6 | 35.0 | 35.1 | 247 | 270 | 604 | 606 | 0.00066 | 0.0089 | 0.0052 | *** |
| 6th nucleotide of the siRNA sequence= | 18.6 | 15.3 | 35.3 | 30.9 | 90 | 74 | 171 | 150 | 0.95 | 0.98 | 0.98  |      |
| 6th nucleotide of the siRNA sequence ≠ G | 13.9 | 16.7 | 34.4 | 35.0 | 236 | 283 | 585 | 595 | 0.048 | 0.018 | 0.018 | ** |
| 7th nucleotide of the siRNA sequence = A | 14.2 | 15.4 | 31.8 | 38.7 | 82  | 89  | 184 | 224 | 0.12  | 0.0014 | 0.016 | ** |
| 7th nucleotide of the siRNA sequence ≠ A | 15.2 | 16.7 | 35.6 | 32.5 | 244 | 268 | 572 | 521 | 0.88  | 1     | 0.98  |    |
| 7th nucleotide of the siRNA sequence = U | 16.9 | 18.2 | 35.0 | 29.9 | 76  | 82  | 158 | 135 | 0.99  | 1     | 0.99  |    |
| 7th nucleotide of the siRNA sequence ≠ U [2] | 14.4 | 15.9 | 34.5 | 35.2 | 250 | 275 | 598 | 610 | 0.010 | 0.0043 | 0.0091 | *** |
| 7th nucleotide of the siRNA sequence = C | 15.2 | 17.3 | 35.7 | 31.9 | 80  | 91  | 188 | 168 | 0.77  | 0.93  | 0.86  |    |
| 7th nucleotide of the siRNA sequence ≠ C | 14.8 | 16.1 | 34.3 | 34.8 | 246 | 266 | 568 | 577 | 0.23  | 0.074 | 0.14  |    |
| 7th nucleotide of the siRNA sequence = G [2] | 14.0 | 15.2 | 36.0 | 34.8 | 88  | 95  | 226 | 218 | 0.069 | 0.32  | 0.17  |    |
| 7th nucleotide of the siRNA sequence ≠ G | 15.3 | 16.8 | 34.0 | 33.8 | 238 | 262 | 530 | 527 | 0.93  | 0.67  | 0.83  |    |
| 8th nucleotide of the siRNA sequence = A [2] | 11.5 | 18.0 | 37.4 | 33.0 | 66  | 103 | 214 | 189 | 0.12  | 0.77  | 0.27  |    |
| 8th nucleotide of the siRNA sequence ≠ A | 16.1 | 15.8 | 33.6 | 34.5 | 260 | 254 | 542 | 556 | 0.88  | 0.23  | 0.73  |    |
| 8th nucleotide of the siRNA sequence = U | 16.5 | 16.1 | 33.5 | 33.9 | 78  | 76  | 158 | 160 | 0.80  | 0.54  | 0.72  |    |
| 8th nucleotide of the siRNA sequence ≠ U | 14.5 | 16.4 | 34.9 | 34.2 | 248 | 281 | 598 | 585 | 0.20  | 0.46  | 0.28  |    |
| 8th nucleotide of the siRNA sequence = C | 16.5 | 15.0 | 37.5 | 31.1 | 87  | 79  | 198 | 164 | 0.54  | 0.98  | 0.90  |    |
| 8th nucleotide of the siRNA sequence ≠ C | 14.4 | 16.8 | 33.7 | 35.1 | 239 | 278 | 558 | 581 | 0.46  | 0.023 | 0.098 | *  |
| 8th nucleotide of the siRNA sequence = G | 15.5 | 16.2 | 30.4 | 37.9 | 95  | 99  | 186 | 232 | 0.61  | 0.0054 | 0.13  | *  |
| 8th nucleotide of the siRNA sequence ≠ G [2] | 14.7 | 16.4 | 36.3 | 32.6 | 231 | 258 | 570 | 513 | 0.39  | 0.99  | 0.87  |    |
| sequence≠G | 9th nucleotide of the siRNA sequence=A | 9th nucleotide of the siRNA sequence≠A | 9th nucleotide of the siRNA sequence=U [2] | 9th nucleotide of the siRNA sequence≠U | 9th nucleotide of the siRNA sequence=C | 9th nucleotide of the siRNA sequence≠C | 9th nucleotide of the siRNA sequence=G | 9th nucleotide of the siRNA sequence≠G | 10th nucleotide of the siRNA sequence=A | 10th nucleotide of the siRNA sequence≠A | 10th nucleotide of the siRNA sequence=U [4] | 10th nucleotide of the siRNA sequence≠U [3] | 10th nucleotide of the siRNA sequence=C | 10th nucleotide of the siRNA sequence≠C | 10th nucleotide of the siRNA sequence=G | 10th nucleotide of the siRNA sequence≠G |
|-----------|--------------------------------------|--------------------------------------|---------------------------------------|-------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
|           | 17.3 17.6 32.9 32.2 106 108 202 198 0.99 0.90 0.98 | 14.0 15.9 35.3 34.8 220 249 554 547 0.0061 0.10 0.021 | 15.6 15.8 37.3 31.4 87 88 208 175 0.54 0.97 0.86 | 14.7 16.5 33.7 35.1 239 269 548 570 0.46 0.035 0.14 | 11.1 16.6 32.6 39.6 59 88 173 210 0.008 0.00021 0.00053 | 16.1 16.3 35.2 32.3 267 269 583 535 0.99 1 1 | 15.4 15.1 35.9 33.6 74 73 173 162 0.32 0.64 0.52 | 14.8 16.7 34.3 34.3 252 284 583 583 0.68 0.36 0.48 | 15.2 17.7 32.2 34.9 73 85 155 168 0.84 0.29 0.56 | 14.9 16.0 35.3 33.9 253 272 601 577 0.16 0.71 0.44 | 14.9 15.2 33.5 36.4 87 89 196 213 0.20 0.062 0.13 | 14.9 16.8 35.0 33.3 239 268 560 532 0.80 0.94 0.87 | 13.4 16.4 37.3 32.9 76 93 212 187 0.16 0.79 0.41 | 15.5 16.3 33.7 34.5 250 264 544 558 0.84 0.21 0.59 | 16.4 16.4 35.1 32.2 90 90 193 177 0.84 0.90 0.89 | 14.4 16.3 34.5 34.8 236 267 563 568 0.16 0.10 0.11 |
| 11th nucleotide of the siRNA sequence=\(A\) | 14.4 | 15.2 | 34.6 | 35.8 | 81 | 86 | 195 | 202 | 0.13 | 0.14 | 0.14 |
| 11th nucleotide of the siRNA sequence\(\neq\)A | [5] | 15.1 | 16.7 | 34.6 | 33.5 | 245 | 271 | 561 | 543 | 0.86 | 0.86 | 0.86 |
| 11th nucleotide of the siRNA sequence=\(U\) | 12.2 | 19.2 | 33.9 | 34.7 | 58 | 91 | 161 | 165 | 0.54 | 0.36 | 0.29 |
| 11th nucleotide of the siRNA sequence\(\neq\)\(U\) | 15.7 | 15.6 | 34.8 | 33.9 | 268 | 266 | 595 | 580 | 0.46 | 0.64 | 0.71 |
| 11th nucleotide of the siRNA sequence=\(C\) | [5] | 16.3 | 16.3 | 36.3 | 31.2 | 86 | 86 | 192 | 165 | 0.80 | 0.97 | 0.93 |
| 11th nucleotide of the siRNA sequence\(\neq\)\(C\) | 14.5 | 16.4 | 34.1 | 35.0 | 240 | 271 | 564 | 580 | 0.20 | 0.029 | 0.07 |
| 11th nucleotide of the siRNA sequence=\(G\) | 16.4 | 15.3 | 33.8 | 34.6 | 101 | 94 | 208 | 213 | 0.61 | 0.36 | 0.57 |
| 11th nucleotide of the siRNA sequence\(\neq\)\(G\) | 14.3 | 16.8 | 34.9 | 33.9 | 225 | 263 | 548 | 532 | 0.39 | 0.64 | 0.43 |
| 12th nucleotide of the siRNA sequence=\(A\) | 13.1 | 17.8 | 35.7 | 33.3 | 84 | 114 | 228 | 213 | 0.43 | 0.71 | 0.46 |
| 12th nucleotide of the siRNA sequence\(\neq\)\(A\) | 15.7 | 15.7 | 34.2 | 34.4 | 242 | 243 | 528 | 532 | 0.57 | 0.29 | 0.54 |
| 12th nucleotide of the siRNA sequence=\(U\) | 16.3 | 16.7 | 34.9 | 32.1 | 94 | 96 | 201 | 185 | 0.88 | 0.91 | 0.91 |
| 12th nucleotide of the siRNA sequence\(\neq\)\(U\) | 14.4 | 16.2 | 34.5 | 34.8 | 232 | 261 | 555 | 560 | 0.12 | 0.088 | 0.086 |
| 12th nucleotide of the siRNA sequence=\(C\) | 17.0 | 15.8 | 32.7 | 34.5 | 87 | 81 | 168 | 177 | 0.82 | 0.39 | 0.70 |
| 12th nucleotide of the siRNA sequence\(\neq\)\(C\) | 14.3 | 16.5 | 35.2 | 34.0 | 239 | 276 | 588 | 568 | 0.18 | 0.61 | 0.30 |
| 12th nucleotide of the siRNA sequence=\(G\) | 13.4 | 14.5 | 34.9 | 37.3 | 61 | 66 | 159 | 170 | 0.013 | 0.023 | 0.027 |
| 12th nucleotide of the siRNA sequence\(\neq\)\(G\) | 15.3 | 16.8 | 34.5 | 33.3 | 265 | 291 | 597 | 575 | 0.99 | 0.98 | 0.97 |
| 13th nucleotide of the siRNA sequence=\(C\) | [5] | 13.1 | 14.8 | 35.6 | 36.5 | 80 | 90 | 217 | 222 | 0.008 | 0.052 | 0.020 |
| 13th nucleotide of the siRNA sequence\(\neq\)\(C\) | 13.4 | 14.5 | 34.9 | 37.3 | 61 | 66 | 159 | 170 | 0.013 | 0.023 | 0.027 |

**Significant at \(p < 0.05\)**

*Significant at \(p < 0.01\)**
| Sequence | 13th Nucleotide | 14th Nucleotide | 15th Nucleotide |
|----------|-----------------|-----------------|-----------------|
| A        | 15.6 17.0 34.2  | 13.2 16.3 35.9  | 14.8 16.9 35.0  |
| U        | 14.9 18.0 33.5  | 14.6 16.3 32.0  | 14.7 16.1 33.6  |
| C        | 15.7 17.5 34.0  | 14.6 16.3 32.0  | 14.7 16.1 33.6  |
| G        | 16.3 15.3 35.1  | 16.3 15.3 35.1  | 16.4 15.7 32.4  |

Notes:
- [3] [4]
- * p < 0.05
- ** p < 0.01
| 15th nucleotide of the siRNA sequence| 15.0 | 16.1 | 34.4 | 34.5 | 228 | 245 | 524 | 525 | 0.36 | 0.26 | 0.33 |
|-------------------------------------|------|------|------|------|-----|-----|-----|-----|------|------|------|
| ≠ A                                 | [2] 12.6 | 17.6 | 33.8 | 36.0 | 70 | 98 | 188 | 200 | 0.23 | 0.10 | 0.10 |
| ≠ U                                 | 15.7 | 15.9 | 34.9 | 33.5 | 256 | 259 | 568 | 545 | 0.77 | 0.90 | 0.90 |
| ≠ C                                 | 16.9 | 18.4 | 33.1 | 31.6 | 83 | 90 | 162 | 155 | 1 | 0.95 | 0.98 |
| ≠ U                                 | 14.3 | 15.8 | 35.1 | 34.8 | 243 | 267 | 594 | 590 | 0.0045 | 0.052 | 0.023 ** |
| ≠ G                                 | 15.8 | 12.0 | 36.6 | 35.7 | 75 | 57 | 174 | 170 | 0.010 | 0.16 | 0.12 * |
| ≠ G                                 | 14.7 | 17.6 | 34.1 | 33.7 | 251 | 300 | 582 | 575 | 0.99 | 0.84 | 0.88 |
| ≠ A                                 | 14.5 | 16.5 | 35.5 | 33.5 | 80 | 91 | 196 | 185 | 0.43 | 0.64 | 0.52 |
| ≠ A                                 | 15.1 | 16.3 | 34.3 | 34.3 | 246 | 266 | 560 | 560 | 0.57 | 0.36 | 0.48 |
| ≠ U                                 | 16.9 | 16.9 | 32.7 | 33.5 | 84 | 84 | 162 | 166 | 0.95 | 0.67 | 0.86 |
| ≠ U                                 | 14.3 | 16.2 | 35.2 | 34.3 | 242 | 273 | 594 | 579 | 0.048 | 0.32 | 0.14 * |
| ≠ C                                 | [3] 13.8 | 14.7 | 35.6 | 35.9 | 81 | 86 | 209 | 211 | 0.026 | 0.10 | 0.058 * |
| ≠ C                                 | 15.3 | 17.0 | 34.3 | 33.4 | 245 | 271 | 547 | 534 | 0.97 | 0.90 | 0.94 |
| ≠ G                                 | [5] 14.8 | 17.5 | 34.4 | 33.3 | 81 | 96 | 189 | 183 | 0.74 | 0.71 | 0.68 |
| ≠ G                                 | 15.0 | 16.0 | 34.7 | 34.4 | 245 | 261 | 567 | 562 | 0.26 | 0.29 | 0.32 |
| ≠ A                                 | 11.4 | 15.5 | 37.1 | 35.9 | 75 | 102 | 244 | 236 | 0.00049 | 0.10 | 0.0049 *** |
| 17th nucleotide of the siRNA        | 16.4 | 16.7 | 33.5 | 33.3 | 251 | 255 | 512 | 509 | 1 | 0.90 | 1 |
| sequence≠A   | 17th nucleotide of the siRNA sequence=U | 14.8 | 16.3 | 33.1 | 35.9 | 79 | 87 | 177 | 192 | 0.43 | 0.12 | 0.25 |
| sequence≠U  | 17th nucleotide of the siRNA sequence≠U | 15.0 | 16.4 | 35.1 | 33.5 | 247 | 270 | 579 | 553 | 0.57 | 0.88 | 0.75 |
| sequence=C  | 17th nucleotide of the siRNA sequence=C | 16.9 | 15.9 | 36.6 | 30.7 | 83 | 78 | 180 | 151 | 0.82 | 0.99 | 0.96 |
| sequence≠C  | 17th nucleotide of the siRNA sequence≠C | 14.4 | 16.5 | 34.0 | 35.1 | 243 | 279 | 576 | 594 | 0.18 | 0.012 | 0.044 ** |
| sequence=G  | 17th nucleotide of the siRNA sequence=G | 17.8 | 18.0 | 31.0 | 33.2 | 89 | 90 | 155 | 166 | 1 | 0.71 | 0.96 |
| sequence≠G  | 17th nucleotide of the siRNA sequence≠G | 14.1 | 15.9 | 35.7 | 34.4 | 243 | 279 | 576 | 594 | 0.18 | 0.012 | 0.044 ** |
| sequence=A  | 18th nucleotide of the siRNA sequence=A | 13.5 | 15.6 | 34.7 | 36.1 | 96 | 111 | 247 | 257 | 0.048 | 0.074 | 0.041 ** |
| sequence≠A  | 18th nucleotide of the siRNA sequence≠A | 15.6 | 16.7 | 34.6 | 33.1 | 230 | 246 | 509 | 488 | 0.95 | 0.93 | 0.96 |
| sequence=U  | 18th nucleotide of the siRNA sequence=U | 14.3 | 16.2 | 33.4 | 36.0 | 84 | 95 | 196 | 211 | 0.32 | 0.10 | 0.17 |
| sequence≠U  | 18th nucleotide of the siRNA sequence≠U | 15.1 | 16.4 | 35.0 | 33.4 | 242 | 262 | 560 | 534 | 0.68 | 0.90 | 0.83 |
| sequence=C  | 18th nucleotide of the siRNA sequence=C | 17.0 | 18.1 | 35.8 | 29.1 | 74 | 79 | 156 | 127 | 0.99 | 1 | 1 |
| sequence≠C  | 18th nucleotide of the siRNA sequence≠C | 14.4 | 15.9 | 34.3 | 35.4 | 252 | 278 | 600 | 618 | 0.010 | 0.00071 | 0.0048 *** |
| sequence=G  | 18th nucleotide of the siRNA sequence=G | 16.0 | 16.0 | 34.8 | 33.3 | 72 | 72 | 157 | 150 | 0.64 | 0.71 | 0.70 |
| sequence≠G  | 18th nucleotide of the siRNA sequence≠G | 14.7 | 16.4 | 34.6 | 34.3 | 254 | 285 | 599 | 595 | 0.36 | 0.29 | 0.30 |
| sequence=A  | 19th nucleotide of the siRNA sequence=A | 12.7 | 14.2 | 35.3 | 37.8 | 80 | 90 | 223 | 239 | 0.00066 | 0.0054 | 0.0014 *** |
| sequence≠A  | 19th nucleotide of the siRNA sequence≠A | 15.9 | 17.2 | 34.3 | 32.6 | 246 | 267 | 533 | 506 | 1 | 0.99 | 1 |
### Supplementary Table 2  Sequence-derived features examined in this study and their significance in the survey

| Feature name | Ref | % Low | % Medium | % High | % Very high | # Low | # Medium | # High | # Very high | $P_{70}$ | $P_{90}$ | $P_{\text{wald}}$ | Significance |
|--------------|-----|-------|---------|-------|------------|-------|---------|-------|------------|--------|--------|-----------|-------------|
| 1st nucleotide of the siRNA sequence=(G/C) | [3, 6, 7] | 13.4 | 16.5 | 35.9 | 34.2 | 213 | 262 | 570 | 543 | 0.0061 | 0.43 | 0.049 | ** |
| 1st nucleotide of the siRNA sequence≠(G/C) | 19.0 | 15.9 | 31.2 | 33.9 | 113 | 95 | 186 | 202 | 0.99 | 0.57 | 0.95 | |
| 10th nucleotide of the siRNA sequence=(A/U) | [6] | 15.0 | 16.3 | 32.9 | 35.7 | 160 | 174 | 351 | 381 | 0.54 | 0.062 | 0.19 | |
| 10th nucleotide of the siRNA sequence≠(A/U) | 14.8 | 16.4 | 36.2 | 32.6 | 166 | 183 | 405 | 364 | 0.46 | 0.94 | 0.81 | |
| 11th nucleotide of the siRNA sequence=(A/U) | 16.3 | 15.7 | 34.9 | 33.0 | 187 | 180 | 400 | 378 | 0.80 | 0.88 | 0.92 | |

Features marked by *: at least one of the three p-values is less than 0.05.
Features marked by **: at least two of the three p-values are less than 0.05.
Features marked by ***: $(P_{\text{wald}}<0.01)$ and $(P_{70}<0.01$ or $P_{90}<0.01)$.
At $P<0.05$, the FDR is controlled at 0.18, 0.15 and 0.16 for the Wald test of monotone trend, the odds ratio permutation test (>70%), and the odds ratio permutation test (>90%), respectively. At $P<0.01$, the FDR is controlled at 0.056, 0.044 and 0.038 for the three tests, respectively.
| sequence=(G/C) | 11th nucleotide of the siRNA sequence≠(G/C) | 19th nucleotide of the siRNA sequence=(A/U) | 19th nucleotide of the siRNA sequence≠(A/U) | There are at least five (A/U)s in the seven nucleotides at the 3' end | There are four or fewer (A/U)s in the seven nucleotides at the 3' end | There are at least three (A/U)s in the seven nucleotides at the 3' end | There are two or fewer (A/U)s in the seven nucleotides at the 3' end | There are occurrences of three or more identical nucleotides in a row | There are no occurrences of three or more identical nucleotides in a row | There are occurrences of four or more identical nucleotides in a row | There are no occurrences of four or more identical nucleotides in a row | There are at least three (A/U)s in the five nucleotides at the 5' |
|---------------|---------------------------------|---------------------------------|---------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|------------------------------------------------|
| sequence=(G/C) | 13.4 | 17.0 | 34.3 | 35.3 | 139 | 177 | 356 | 367 | 0.20 | 0.12 | 0.078 | | |
| 19th nucleotide of the siRNA sequence≠(G/C) | 12.0 | 16.0 | 35.3 | 36.7 | 135 | 180 | 398 | 413 | 0.00029 | 0.0043 | 0.000058 | *** | |
| There are at least five (A/U)s in the seven nucleotides at the 3' end | 18.1 | 16.7 | 33.8 | 31.4 | 191 | 177 | 358 | 332 | 1 | 1 | 1 | | |
| There are four or fewer (A/U)s in the seven nucleotides at the 3' end | 15.5 | 16.7 | 34.0 | 33.8 | 256 | 275 | 561 | 558 | 0.97 | 0.74 | 0.90 | | |
| There are at least three (A/U)s in the seven nucleotides at the 3' end | [7] 13.1 | 15.4 | 36.5 | 35.0 | 70 | 82 | 195 | 187 | 0.026 | 0.26 | 0.10 | * | |
| There are two or fewer (A/U)s in the seven nucleotides at the 3' end | 13.4 | 16.4 | 33.7 | 36.5 | 246 | 300 | 618 | 668 | 0.00001 | 0.00001 | 2.5e-09 | *** | |
| There are occurrences of three or more identical nucleotides in a row | 16.5 | 17.4 | 33.8 | 32.4 | 173 | 183 | 355 | 340 | 0.99 | 0.96 | 0.99 | | |
| There are no occurrences of three or more identical nucleotides in a row | [9, 13] 13.5 | 15.4 | 35.4 | 35.7 | 153 | 174 | 401 | 405 | 0.0061 | 0.043 | 0.0067 | *** | |
| There are occurrences of four or more identical nucleotides in a row | 22.5 | 21.0 | 26.5 | 30.0 | 45 | 42 | 53 | 60 | 1 | 0.99 | 1 | | |
| There are no occurrences of four or more identical nucleotides in a row | [9-12] 14.2 | 15.9 | 35.4 | 34.5 | 281 | 315 | 703 | 685 | 0.00001 | 0.012 | 0.0014 | *** | |
| There are at least three (A/U)s in the five nucleotides at the 5' | 16.4 | 15.4 | 34.9 | 33.3 | 131 | 123 | 279 | 266 | 0.64 | 0.74 | 0.80 | | |
| Category                                                                 | Value 1   | Value 2   | Value 3   | Value 4   | Value 5   | Value 6   | Value 7   | Value 8   | Significance |
|--------------------------------------------------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|--------------|
| There are two or fewer (A/U)s in the five nucleotides at the 5' end     | 14.1      | 16.9      | 34.4      | 34.6      | 195       | 234       | 477       | 479       | 0.36         | 0.26         | 0.20       |
| There are at least three (A/U)s in the five nucleotides at the 3' end   | [4]       | 12.6      | 16.4      | 34.0      | 37.0      | 164       | 214       | 443       | 483          | 0.0018       | 0.00016    | 0.000022   | ***         |
| There are two or fewer (A/U)s in the five nucleotides at the 3' end     | 18.4      | 16.3      | 35.6      | 29.8      | 162       | 143       | 313       | 262       | 1            | 1            | 1          |           |
| There are occurrences of G/C stretches of length 7 or longer            | [9, 10]   | 42.6      | 14.9      | 23.4      | 19.1      | 20        | 7         | 11        | 9            | 1            | 1          | 1          |
| There are no occurrences of G/C stretches of length 7 or longer         | 14.3      | 16.4      | 34.9      | 34.4      | 306       | 350       | 745       | 736       | 0.00001      | 0.00001      | 0.000015    | ***         |
| G/C content is between 32 and 79%                                       | [14]      | 14.8      | 16.3      | 35.0      | 33.9      | 310       | 340       | 730       | 708          | 0.058        | 0.99       | 0.56       |
| G/C content is not between 32 and 79%                                   | 16.7      | 17.7      | 27.1      | 38.5      | 16        | 17        | 26        | 37        | 0.94         | 0.012        | 0.44       | *          |
| G/C content is between 30 and 70%                                       | [15]      | 14.5      | 16.2      | 34.9      | 34.4      | 307       | 344       | 739       | 728          | 0.00001      | 0.00001    | 0.0028     | ***         |
| G/C content is not between 30 and 70%                                   | 28.8      | 19.7      | 25.8      | 25.8      | 19        | 13        | 17        | 17        | 1            | 1            | 1          | 1          |
| G/C content is between 30 and 52%                                       | [4]       | 13.8      | 16.1      | 34.7      | 35.4      | 146       | 171       | 368       | 375          | 0.082        | 0.12       | 0.056      |
| G/C content is not between 30 and 52%                                   | 16.0      | 16.5      | 34.5      | 32.9      | 180       | 186       | 388       | 370       | 0.92         | 0.88         | 0.94       |
| G/C content is between 35 and 60%                                       | [9]       | 13.3      | 16.7      | 35.1      | 35.0      | 245       | 308       | 649       | 647          | 0.00001      | 0.0019     | 0.00018    | ***         |
| G/C content is not between 35 and 60%                                   | 24.2      | 14.6      | 31.9      | 29.3      | 81        | 49        | 107       | 98        | 1            | 1            | 1          | 1          |
| G/C content is between 20 and 50%                                       | [13]      | 13.8      | 16.2      | 34.4      | 35.7      | 148       | 174       | 370       | 384          | 0.082        | 0.062      | 0.037      | *           |
| G/C content is not between 20                                          | 16.1      | 16.5      | 34.8      | 32.6      | 178       | 183       | 386       | 361       | 0.92         | 0.94         | 0.96       |
G/C content is between 31.6 and 57.9% [3] 13.3 16.7 35.1 35.0 245 308 649 647 0.00001 0.0019 0.00018 ***
G/C content is not between 31.6 and 57.9% 24.2 14.6 31.9 29.3 81 49 107 98 1 1 1

Features marked by *: at least one of the three p-values is less than 0.05.
Features marked by **: at least two of the three p-values are less than 0.05.
Features marked by ***: \((P_{\text{wald}} < 0.01)\) and \((P_{70} < 0.01 \text{ or } P_{90} < 0.01)\).
At \(P < 0.05\), the FDR is controlled at 0.18, 0.15 and 0.16 for the Wald test of monotone trend, the odds ratio permutation test (>70%), and the odds ratio permutation test (>90%), respectively. At \(P < 0.01\), the FDR is controlled at 0.056, 0.044 and 0.038 for the three tests, respectively.

**Supplementary Table 3** Thermodynamic features examined in this study and their significance in the survey

| Feature name | Ref | % Low | % Medium | % High | % Very high | # Low | # Medium | # High | # Very high | \(P_{70}\) | \(P_{90}\) | \(P_{\text{wald}}\) | Significance |
|--------------|-----|-------|----------|--------|-------------|-------|----------|--------|-------------|-------|-------|-------------|-------------|
| \(T_m \geq 60^\circ C\) | | 19.5 | 16.2 | 32.3 | 31.9 | 107 | 89 | 177 | 175 | 1 | 0.93 | 0.99 | *** |
| \(T_m < 60^\circ C\) [4] | 13.4 | 16.0 | 35.4 | 34.8 | 219 | 268 | 579 | 570 | 0.0014 | 0.074 | 0.0057 | *** |
| \(T_m\) is between 20 and 60°C | 13.2 | 16.5 | 35.0 | 35.3 | 200 | 249 | 529 | 534 | 0.0045 | 0.023 | 0.003 | *** |
| \(T_m\) is not between 20 and 60°C | 18.8 | 16.1 | 33.8 | 31.4 | 126 | 108 | 227 | 211 | 1 | 0.98 | 1 | * |
| \(T_m < 20^\circ C\) [4] | 15.3 | 15.3 | 40.3 | 29.0 | 19 | 19 | 50 | 36 | 0.36 | 1 | 0.76 | * |
| \(T_m \geq 20^\circ C\) | 14.9 | 16.4 | 34.3 | 34.4 | 307 | 338 | 706 | 709 | 0.64 | 0.0026 | 0.24 | * |
| Binding energy of N16-N19 > -9 KCal/Mol [16] | 11.8 | 17.1 | 34.0 | 37.1 | 124 | 179 | 357 | 389 | 0.010 | 0.0026 | 0.00025 | *** |
| Binding energy of N16-N19 ≤ -9 KCal/Mol | 17.8 | 15.7 | 35.2 | 31.4 | 202 | 178 | 399 | 356 | 0.99 | 1 | 1 | * |
| Binding energy of N6-N11 ≥ -13 KCal/Mol | 14.1 | 17.3 | 34.3 | 34.4 | 157 | 192 | 381 | 382 | 0.54 | 0.39 | 0.38 | * |
| Binding energy of N6-N11 < -13 KCal/Mol [17] | 15.8 | 15.4 | 35.0 | 33.9 | 169 | 165 | 375 | 363 | 0.46 | 0.61 | 0.62 | * |
| Binding energy of N7-N12 > -13 KCal/Mol | 15.3 | 17.9 | 34.0 | 32.8 | 158 | 185 | 352 | 339 | 0.97 | 0.90 | 0.95 | * |
| Condition                                                                 | [16] | 14.6 | 15.0 | 35.1 | 35.3 | 168 | 172 | 404 | 406 | 0.032 | 0.10 | 0.054 | *  |
|--------------------------------------------------------------------------|------|------|------|------|------|-----|-----|-----|-----|-------|------|-------|    |
| Binding energy of N7-N12 ≤ -13 KCal/Mol                                   |      |      |      |      |      |     |     |     |     |       |      |       |    |
| Mean of free energy profile of N7-N11 is between -1.97 and -1.65 KCal/Mol (inclusive) | [18] | 12.7 | 18.5 | 34.4 | 34.4 | 66  | 96  | 179 | 179 | 0.46  | 0.43  | 0.32  |    |
| Mean of free energy profile of N7-N11 is not between -1.97 and -1.65 KCal/Mol (inclusive) |      |      |      |      |      |     |     |     |     |       |      |       |    |
| Binding energy of N1-N4 is between -9 and -5 KCal/Mol (exclusive)        | [16] | 16.4 | 15.7 | 35.6 | 32.3 | 99  | 95  | 215 | 195 | 0.71  | 0.90  | 0.87  |    |
| Binding energy of N1-N4 is not between -9 and -5 KCal/Mol (exclusive)   |      | 14.4 | 16.6 | 34.2 | 34.8 | 227 | 262 | 541 | 550 | 0.29  | 0.10  | 0.13  |    |
| Binding energy of N16-N19 ≥ binding energy of N1-N4                      | [16, 17] | 13.4 | 16.3 | 34.7 | 35.6 | 191 | 233 | 495 | 507 | 0.013 | 0.018 | 0.0043 | ** |
| Binding energy of N16-N19 < binding energy of N1-N4                      |      | 17.8 | 16.4 | 34.4 | 31.4 | 135 | 124 | 261 | 238 | 0.99  | 0.98  | 1     |    |
| Binding energy of N16-N19 – binding energy of N1-N4 is between 0 and 1 KCal/Mol | [16] | 12.6 | 14.9 | 32.5 | 39.9 | 44  | 52  | 113 | 139 | 0.010 | 0.00036 | 0.0078 | *** |
| Binding energy of N16-N19 – binding energy of N1-N4 is not between 0 and 1 KCal/Mol |      | 15.4 | 16.6 | 35.0 | 33.0 | 282 | 305 | 643 | 606 | 0.99  | 1     | 0.99  |    |
| Binding energy of N15-N19 > binding energy of N1-N5                      | [18] | 13.5 | 17.1 | 34.8 | 34.6 | 181 | 229 | 465 | 463 | 0.20  | 0.26  | 0.12  |    |
| Binding energy of N15-N19 ≤ binding energy of N1-N5                      |      | 17.1 | 15.1 | 34.4 | 33.3 | 145 | 128 | 291 | 282 | 0.80  | 0.74  | 0.88  |    |
| Absolute value of total hairpin energy < 1 KCal/Mol                     | [16] | 12.7 | 17.0 | 35.0 | 35.3 | 48  | 64  | 132 | 133 | 0.18  | 0.23  | 0.19  |    |
| Absolute value of total hairpin energy ≥ 1 KCal/Mol                     |      | 15.4 | 16.2 | 34.5 | 33.9 | 278 | 293 | 624 | 612 | 0.82  | 0.77  | 0.81  |    |
| Folding energy of sense strand ≥ | [9] | 14.6 | 16.4 | 34.6 | 34.4 | 307 | 345 | 729 | 724 | 0.00004 | 0.00002 | 0.023 | ** |
### Supplementary Table 4  Features defined based on target mRNA sites examined in this study and their significance in the survey

| Feature name | Ref | % Low | % Medium | % High | % Very high | # Low | # Medium | # High | # Very high | $P_{70}$ | $P_{90}$ | $P_{\text{wald}}$ | Significance |
|--------------|-----|-------|----------|--------|-------------|-------|----------|--------|-------------|--------|--------|---------------|--------------|
| Local folding potential (mean) ≥ -22.72 KCal/Mol | [31] | 12.0 | 14.6 | 34.7 | 38.7 | 132 | 161 | 382 | 426 | 0.00001 | 0.00001 | 9.3e-09 | *** |
| Local folding potential (mean) < -22.72 KCal/Mol | | 17.9 | 18.1 | 34.5 | 29.5 | 194 | 196 | 374 | 319 | 1 | 1 | 1 | |
| Anti-sense siRNA binding energy > -10 KCal/Mol | [12] | 15.8 | 16.3 | 34.1 | 33.8 | 269 | 279 | 582 | 577 | 0.97 | 0.77 | 0.93 | |
| Anti-sense siRNA binding energy ≤ -10 KCal/Mol | | 11.9 | 16.4 | 36.5 | 35.2 | 57 | 78 | 174 | 168 | 0.026 | 0.23 | 0.072 | * |
| Accessibility score = 0 | | 15.5 | 15.7 | 34.8 | 34.0 | 277 | 282 | 623 | 610 | 0.43 | 0.57 | 0.63 | |
| Accessibility score > 0 | [19] | 12.5 | 19.1 | 33.9 | 34.4 | 49 | 75 | 133 | 135 | 0.57 | 0.43 | 0.37 | |
| H-b index ≥ 28.8 | | 17.6 | 15.5 | 34.0 | 32.9 | 197 | 174 | 381 | 369 | 0.97 | 0.88 | 0.99 | |
| H-b index < 28.8 | [22] | 12.1 | 17.2 | 35.3 | 35.4 | 129 | 183 | 375 | 376 | 0.032 | 0.12 | 0.012 | ** |
| Does not pass Repelling Loop Filter | | 13.2 | 16.7 | 31.7 | 38.4 | 37 | 47 | 89 | 108 | 0.20 | 0.0089 | 0.087 | * |
| Passes Repelling Loop Filter | [20] | 15.2 | 16.3 | 35.0 | 33.5 | 289 | 310 | 667 | 637 | 0.80 | 0.99 | 0.91 | |
| Local free energy of the most stable structure ≥ -20.9 KCal/Mol | [21] | 14.2 | 15.9 | 34.1 | 35.8 | 156 | 174 | 373 | 392 | 0.13 | 0.052 | 0.048 | * |

Features marked by *: at least one of the three p-values is less than 0.05.
Features marked by **: at least two of the three p-values are less than 0.05.
Features marked by ***: ($P_{\text{wald}}<0.01$) and ($P_{70}<0.01$ or $P_{90}<0.01$).
At $P<0.05$, the FDR is controlled at 0.18, 0.15 and 0.16 for the Wald test of monotone trend, the odds ratio permutation test (>70%), and the odds ratio permutation test (>90%), respectively. At $P<0.01$, the FDR is controlled at 0.056, 0.044 and 0.038 for the three tests, respectively.
| Local free energy of the most stable structure $< -20.9$ KCal/Mol | 15.6 | 16.8 | 35.2 | 32.4 | 170 | 183 | 383 | 353 | 0.86 | 0.95 | 0.95 |
| Average local free energy of the ten most stable structures $\geq -20.85$ KCal/Mol | [21] 14.4 | 15.9 | 34.9 | 34.8 | 157 | 173 | 381 | 380 | 0.16 | 0.23 | 0.16 |
| Average local free energy of the ten most stable structures $< -20.85$ KCal/Mol | 15.5 | 16.8 | 34.3 | 33.4 | 169 | 184 | 375 | 365 | 0.84 | 0.77 | 0.84 |
| Target site is on the 1st quartile of CDS | 12.8 | 14.9 | 38.0 | 34.3 | 93 | 108 | 276 | 249 | 0.0024 | 0.43 | 0.056 | * |
| Target site is not on the 1st quartile of CDS | 16.0 | 17.1 | 32.9 | 34.0 | 233 | 249 | 480 | 496 | 1 | 0.57 | 0.94 |
| Target site is on the 2nd quartile of CDS | 15.8 | 15.6 | 31.4 | 37.2 | 78 | 77 | 155 | 184 | 0.54 | 0.023 | 0.21 | * |
| Target site is not on the 2nd quartile of CDS | 14.7 | 16.6 | 35.6 | 33.2 | 248 | 280 | 601 | 561 | 0.46 | 0.98 | 0.79 |
| Target site is on the 3rd quartile of CDS | 12.5 | 15.3 | 34.8 | 37.3 | 49 | 60 | 136 | 146 | 0.017 | 0.029 | 0.031 | ** |
| Target site is not on the 3rd quartile of CDS | [5] 15.4 | 16.6 | 34.6 | 33.4 | 277 | 297 | 620 | 599 | 0.98 | 0.97 | 0.97 |
| Target site is on the 4th quartile of CDS | 18.7 | 19.1 | 30.4 | 31.8 | 53 | 54 | 86 | 90 | 1 | 0.91 | 0.98 |
| Target site is not on the 4th quartile of CDS | 14.4 | 15.9 | 35.2 | 34.5 | 273 | 303 | 670 | 655 | 0.00015 | 0.088 | 0.025 | ** |
| Target site is on the 5't UTR | 25.6 | 15.4 | 38.5 | 20.5 | 10 | 6 | 15 | 8 | 1 | 1 | 0.98 |
| Target site is not on the 5't UTR | [11, 14] 14.7 | 16.4 | 34.5 | 34.4 | 316 | 351 | 741 | 737 | 0.00001 | 0.00001 | 0.016 | ** |
| Target site is on the 3't UTR | 20.6 | 19.1 | 38.2 | 22.1 | 27 | 25 | 50 | 29 | 1 | 1 | 1 |
| Target site is not on the 3't UTR | [11, 14] 14.6 | 16.2 | 34.4 | 34.9 | 299 | 332 | 706 | 716 | 0.00001 | 0.00001 | 0.00052 | *** |
| Target site is on the first 100 nucleotides of CDS | 15.2 | 14.5 | 35.9 | 34.5 | 22 | 21 | 52 | 50 | 0.18 | 0.43 | 0.41 |
| Target site is not on the first 100 nucleotides of CDS | [11, 14] 14.9 | 16.5 | 34.5 | 34.1 | 304 | 336 | 704 | 695 | 0.82 | 0.57 | 0.59 |
Target site is on CDS [9] 14.4 16.2 34.3 35.2 288 324 688 705 0.00001 0.00001 0.000055 ***
Target site is not on CDS 21.2 18.4 38.0 22.3 38 33 68 40 1 1 1

Features marked by *: at least one of the three p-values is less than 0.05.
Features marked by **: at least two of the three p-values are less than 0.05.
Features marked by ***: (P_{wald} <0.01) and (P_{70}<0.01 or P_{90}<0.01).
At P<0.05, the FDR is controlled at 0.18, 0.15 and 0.16 for the Wald test of monotone trend, the odds ratio permutation test (>70%), and the odds ratio permutation test (>90%), respectively. At P<0.01, the FDR is controlled at 0.056, 0.044 and 0.038 for the three tests, respectively.

**Supplementary Table 5** Features based on experimental settings examined in this study

| Feature name                              | Ref | % Low | % Medium | % High | % Very high | # Low | # Medium | # High | # Very high | P_{70} | P_{90} | P_{wald} | Significance |
|-------------------------------------------|-----|-------|----------|--------|-------------|-------|----------|--------|-------------|--------|--------|---------|--------------|
| Cell line = HeLa                          | 7.9 | 10.6  | 41.2     | 40.3   | 33          | 44    | 172      | 168    | 0.00001     | 0.00001 | 4e-09  | ***     |
| Cell line ≠ HeLa                          | 16.6| 17.7  | 33.1     | 32.7   | 293         | 313   | 584      | 577    | 1           | 1      | 1      | ***     |
| Cell line = HEK293                        | 17.1| 17.9  | 28.5     | 36.5   | 45          | 47    | 75       | 96     | 0.98        | 0.088  | 0.61   | *       |
| Cell line ≠ HEK293                        | 14.6| 16.1  | 35.5     | 33.8   | 281         | 310   | 681      | 649    | 0.021       | 0.91   | 0.39   | *       |
| Cell line = MCF7                          | 12.9| 21.0  | 38.7     | 27.4   | 8           | 13    | 24       | 17     | 0.90        | 1      | 0.79   | *       |
| Cell line ≠ MCF7                          | 15.0| 16.2  | 34.5     | 34.3   | 318         | 344   | 732      | 728    | 0.098       | 0.00016| 0.21   | *       |
| Cell line = CV-1 and derivatives          | 35.2| 1.9   | 37.0     | 25.9   | 19          | 1     | 20       | 14     | 1           | 1      | 0.97   | **      |
| Cell line ≠ CV-1 and derivatives          | 14.4| 16.7  | 34.6     | 34.3   | 307         | 356   | 736      | 731    | 0.0018      | 0.00001| 0.027  | **      |
| Cell line = 3T3                           | 16.7| 18.5  | 40.7     | 24.1   | 9           | 10    | 22       | 13     | 0.97        | 1      | 0.92   | *       |
| Cell line ≠ 3T3                           | 14.9| 16.3  | 34.5     | 34.4   | 317         | 347   | 734      | 732    | 0.026       | 0.0001 | 0.085  | **      |
| Test method = Western blot                | 10.8| 15.5  | 34.8     | 38.9   | 138         | 198   | 445      | 498    | 0.00001     | 0.00001| 3.8e-14| ***     |
| Test method ≠ Western blot                | 20.8| 17.6  | 34.4     | 27.3   | 188         | 159   | 311      | 247    | 1           | 1      | 1      | 1       |
| Test method = PCR-related                 | 17.7| 21.8  | 34.2     | 26.2   | 100         | 123   | 193      | 148    | 1           | 1      | 1      | 1       |
| Test method ≠ PCR-related                 | 14.0| 14.4  | 34.8     | 36.9   | 226         | 234   | 563      | 597    | 0.00001     | 0.00001| 2.6e-08| ***     |
| Test method = bDNA                        | 22.2| 11.1  | 26.7     | 40.0   | 10          | 5     | 12       | 18     | 0.84        | 0.0019 | 0.47   | *       |
| Test method ≠ bDNA | 14.8 | 16.5 | 34.8 | 34.0 | 316 | 352 | 744 | 727 | 0.16 | 1 | 0.53 |
|-------------------|-------|-------|-------|-------|------|------|------|------|-------|---|-------|
| Test method = Northern blot | 24.0 | 15.6 | 34.4 | 26.0 | 23  | 15  | 33  | 25  | 1    | 1 | 0.99 |
| Test method ≠ Northern blot | 14.5 | 16.4 | 34.6 | 34.5 | 303 | 342 | 723 | 720 | 0.00001 | 0.00001 | 0.010 ** |
| Test method = Luciferase assay | 44.7 | 2.1  | 44.7 | 8.5  | 21  | 1   | 21  | 4   | 1    | 1 | 1 |
| Test method ≠ Luciferase assay | 14.3 | 16.7 | 34.4 | 34.7 | 305 | 356 | 735 | 741 | 0.00001 | 0.00001 | 4.7e-08 *** |
| Transfection method = Synthesized oligos | 14.1 | 16.0 | 34.9 | 35.0 | 213 | 241 | 527 | 529 | 0.026 | 0.074 | 0.028 ** |
| Transfection method = Vector-based | 16.8 | 17.2 | 34.0 | 32.0 | 113 | 116 | 229 | 216 | 0.97 | 0.93 | 0.97 |
| Test object = mRNA | 18.6 | 20.0 | 34.2 | 27.1 | 136 | 146 | 250 | 198 | 1 | 1 | 1 |
| Test object ≠ mRNA | 13.1 | 14.5 | 34.8 | 37.6 | 190 | 211 | 506 | 547 | 0.00001 | 0.00001 | 9.3e-10 *** |
| Test object = protein | 12.7 | 14.5 | 35.0 | 37.8 | 182 | 209 | 503 | 543 | 0.00001 | 0.00001 | 2.2e-09 *** |
| Test object ≠ protein | 19.3 | 19.8 | 33.9 | 27.0 | 144 | 148 | 253 | 202 | 1 | 1 | 1 |

Features marked by *: at least one of the three p-values is less than 0.05.  
Features marked by **: at least two of the three p-values are less than 0.05.  
Features marked by ***: ($P_{wald} < 0.01$) and ($P_{70} < 0.01$ or $P_{90} < 0.01$).  
At P<0.05, the FDR is controlled at 0.18, 0.15 and 0.16 for the Wald test of monotone trend, the odds ratio permutation test (>70%), and the odds ratio permutation test (>90%), respectively. At P<0.01, the FDR is controlled at 0.056, 0.044 and 0.038 for the three tests, respectively.
**Supplementary Table 6** Non-redundant rule sets for four α levels (denoted as $RS_\alpha$) following the DRM procedure. Listing of $F_1$-$F_{17}$ is at the end of the table.

$RS_{0.951}$

| Feature | $F_1$ | $F_2$ | $F_3$ | $F_4$ | $F_5$ | $F_6$ | $F_7$ | $F_8$ | $F_9$ | $F_{10}$ | $F_{11}$ | $F_{12}$ | $F_{13}$ | $F_{14}$ | $F_{15}$ | $F_{16}$ | $F_{17}$ |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|---------|---------|---------|---------|---------|---------|---------|
| Rule 1  | √     |       |       |       |       |       |       |       |       | √       |         |         |         |         |         |         |         |         |
| Rule 2  |       | √     |       |       |       |       |       |       |       |         | √       |         |         |         |         |         |         |         |
| Rule 3  | √     |       |       | √     |       |       |       |       |       |         |         | √       |         |         |         |         |         |         |
| Rule 4  |       |       | √     |       | √     |       |       |       |       |         |         |         | √       |         |         |         |         |         |
| Rule 5  |       |       |       |       | √     |       |       |       |       |         |         |         |         | √       |         |         |         |         |
| Rule 6  |       |       |       |       |       |       | √     | √     | √     |         |         |         |         |         | √       |         |         |         |
| Rule 7  |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         | √       |         |         |

$RS_{0.895}$

| Feature | $F_1$ | $F_2$ | $F_3$ | $F_4$ | $F_5$ | $F_6$ | $F_7$ | $F_8$ | $F_9$ | $F_{10}$ | $F_{11}$ | $F_{12}$ | $F_{13}$ | $F_{14}$ | $F_{15}$ | $F_{16}$ | $F_{17}$ |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|---------|---------|---------|---------|---------|---------|---------|
| Rule 1  | √     |       |       |       |       |       |       |       |       | √       |         |         |         |         |         |         |         |         |
| Rule 2  |       | √     |       |       |       |       |       |       |       |         | √       |         |         |         |         |         |         |         |
| Rule 3  | √     |       |       | √     |       |       |       |       |       |         |         | √       |         |         |         |         |         |         |
| Rule 4  |       |       | √     |       | √     |       |       |       |       |         |         |         | √       |         |         |         |         |         |
| Rule 5  |       |       |       |       | √     |       |       |       |       |         |         |         |         | √       |         |         |         |         |
| Rule 6  |       |       |       |       |       |       | √     | √     | √     |         |         |         |         | √       |         |         |         |         |
| Rule 7  |       |       |       |       |       |       |       |       |       |         |         |         |         |         | √       |         |         |         |
| Rule 8  |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         | √       |         |         |
| Rule 9  |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         | √       |         |

$RS_{0.845}$

| Feature | $F_1$ | $F_2$ | $F_3$ | $F_4$ | $F_5$ | $F_6$ | $F_7$ | $F_8$ | $F_9$ | $F_{10}$ | $F_{11}$ | $F_{12}$ | $F_{13}$ | $F_{14}$ | $F_{15}$ | $F_{16}$ | $F_{17}$ |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------|---------|---------|---------|---------|---------|---------|---------|
| Rule 1  | √     |       |       |       |       |       |       |       |       | √       |         |         |         |         |         |         |         |         |
| Rule 2  |       | √     |       |       |       |       |       |       |       |         | √       |         |         |         |         |         |         |         |
| Rule 3  | √     |       |       | √     |       |       |       |       |       |         |         | √       |         |         |         |         |         |         |
| Rule 4  |       |       | √     |       | √     |       |       |       |       |         |         |         | √       |         |         |         |         |         |
| Rule 5  |       |       |       |       | √     |       |       |       |       |         |         |         |         | √       |         |         |         |         |
| Rule 6  |       |       |       |       |       |       | √     | √     | √     |         |         |         |         | √       |         |         |         |         |
| Rule 7  |       |       |       |       |       |       |       |       |       |         |         |         |         |         | √       |         |         |         |
| Rule 8  |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         | √       |         |         |
| Rule 9  |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         | √       |         |
| Rule 10 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 11 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         | √       |         |
| Rule 12 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 13 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 14 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 15 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 16 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 17 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 18 |       |       |       |       |       |       |       |       |       |         |         |         |         |         |         |         |         |         |
| Rule 19 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 20 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

$RS_{0.827}$

| Feature | $F_1$ | $F_2$ | $F_3$ | $F_4$ | $F_5$ | $F_6$ | $F_7$ | $F_8$ | $F_9$ | $F_{10}$ | $F_{11}$ | $F_{12}$ | $F_{13}$ | $F_{14}$ | $F_{15}$ | $F_{16}$ | $F_{17}$ |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Rule 1  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 2  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 3  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 4  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 5  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 6  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 7  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 8  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 9  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 10 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 11 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 12 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 13 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 14 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 15 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 16 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 17 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 18 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 19 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 20 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 21 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 22 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 23 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 24 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 25 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 26 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 27 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 28 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 29 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 30 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 31 | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |

$RS_{0.796}$

| Feature | $F_1$ | $F_2$ | $F_3$ | $F_4$ | $F_5$ | $F_6$ | $F_7$ | $F_8$ | $F_9$ | $F_{10}$ | $F_{11}$ | $F_{12}$ | $F_{13}$ | $F_{14}$ | $F_{15}$ | $F_{16}$ | $F_{17}$ |
|---------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Rule 1  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 2  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 3  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 4  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 5  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |
| Rule 6  | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     | ✓     |


| Rule | F₁ | F₂ | F₃ | F₄ | F₅ | F₆ | F₇ | F₈ | F₉ | F₁₀ | F₁₁ | F₁₂ | F₁₃ | F₁₄ | F₁₅ | F₁₆ | F₁₇ |
|------|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|
| Rule 7 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 8 | √  | √  | √  | √  |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 9 | √  | √  | √  |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 10 | √  | √  |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 11 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 12 | √  |    | √  |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 13 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 14 | √  |    |    |    | √  |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 15 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 16 | √  |    |    | √  |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 17 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 18 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 19 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 20 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 21 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 22 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 23 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 24 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 25 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 26 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 27 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 28 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 29 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 30 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 31 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 32 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 33 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 34 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 35 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 36 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 37 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 38 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 39 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 40 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |
| Rule 41 | √  |    |    |    |    |    |    |    |    |     |     |     |     |     |     |     |     |

$R_{S_{0.784}}$
| Rule 8  | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 9  | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 10 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 11 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 12 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 13 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 14 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 15 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 16 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 17 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 18 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 19 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 20 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 21 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 22 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 23 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 24 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 25 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 26 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 27 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 28 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 29 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 30 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 31 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 32 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 33 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 34 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 35 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 36 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 37 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Rule 38 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

List of features:

| Feature Index | Feature Names |
|---------------|---------------|
| F_1           | 2nd nucleotide=A |
| F_2           | 4th nucleotide=C |
| F_3           | 6th nucleotide≠C |
| F_4           | 7th nucleotide=U |
| F_5           | 9th nucleotide=C |
| F_6           | 17th nucleotide=A |
| F_7           | 18th nucleotide≠C |
| F_8           | 19th nucleotide=(A/U) |
| F_9           | At least three (A/U)s in the seven nucleotides at the 3’ end |
| F_{10}        | No occurrences of four or more identical nucleotides in a row |
| F_{11}        | No occurrences of G/C stretches of length 7 or longer |
| Design Program         | Institution/Company            | $n^T$ | $n^T_{UPE}$ | $n_{UPE}$ | UPTR   |
|------------------------|--------------------------------|-------|-------------|-----------|--------|
| siDESIGN Center        | Dharmacon, Inc.                | 33    | 8           | 19        | 4.152  |
| siRNA Target Finder    | GenScript Corp.                | 25    | 10          | 46        | 2.144  |
| Imgenex sirna Designer | Imgenex Corp.                  | 115   | 27          | 15        | 17.751 |
| IDT RNAi Design (SciTools) | Integrated DNA Technologies, Inc. | 27    | 6           | 7         | 8.453  |
| BLOCK-iT RNAi Designer | Invitrogen Corp.               | 21    | 9           | 26        | 3.414  |
| siSearch               | Karolinska Institutet          | 16    | 7           | 47        | 1.469  |
| SiMAX                  | MWG-Biotech, Inc.              | 162   | 71          | 66        | 10.609 |
| BIOPREDsi              | Novartis Institutes for BioMedical Research | 13    | 6           | 21        | 2.818  |
| Promega siRNA Target Designer | Promega Corp.                | 72    | 41          | 106       | 3.815  |
| QIAGEN siRNA Design Tool | QIAGEN, Inc.                  | 149   | 78          | 64        | 12.019 |
| WI siRNA Selection Program | Whitehead Institute     | 87    | 38          | 24        | 15.615 |
| Ambion siRNA Target Finder* | Ambion, Inc.                 | 564   | 7           | 77        | 0.897  |
| Jack Lin's siRNA Sequence Finder* | Cold Spring Harbor Laboratory | 229   | 1           | 91        | 0.108  |
| Considerations in [32] | Pertinent features | Significance |
|-------------------------|--------------------|--------------|
| Sequence symmetry of siRNA duplexes | 17th nucleotide ≠ C | ** |
| | 17th nucleotide ≠ G | ** |
| | 18th nucleotide = A | ** |
| | 18th nucleotide ≠ C | *** |
| | 19th nucleotide = A | *** |
| | 19th nucleotide ≠ C | *** |
| | 19th nucleotide ≠ G | *** |
| | 19th nucleotide = (A/U) | *** |
| | At least five (A/U)s in the seven nucleotides at the 3’ end | * |
| | At least three (A/U)s in the seven nucleotides at the 3’ end | *** |
| | At least three (A/U)s in the five nucleotides at the 3’ end | *** |
| | The 5’ end of guide strand is (A/U) enriched | |
| | The 5’ end of guide strand is less stable | |
| | G/C content range between 30% and 52% | |
| | G/C content is between 30 and 52% | |
| | Devoid of internal repeats or palindromes | |
| | Folding energy of sense strand ≥ -5 KCal/Mol | ** |
| | $T_m$ < 60°C | *** |
| | $T_m$ is between 20 and 60°C | *** |
| | The center of the duplex have low internal stability | |
| | Binding energy of N6-N11 ≥ -13 KCal/Mol | |
| | Anti-sense siRNA binding energy > -10 KCal/Mol | |
| | $H$-b index < 28.8 | * |
| | Passes the repelling loop filter | |
| | Local free energy of the most stable structure ≥ -20.9 KCal/Mol | * |
| | Sequence characteristics | U or A at position N19 | |
| | 19th nucleotide = A | *** |
| | 19th nucleotide ≠ G | *** |
| | 19th nucleotide = (A/U) | *** |
| Feature Description                                                                 | Conditions                                                                 |
|-----------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| G or C at position N1                                                              | 1st nucleotide ≠ U                                                         |
|                                    | 1st nucleotide = G                                                         |
|                                    | 1st nucleotide = (G/C)                                                     |
| A+U richness between position N13 and N19                                           | At least five (A/U)s in the seven nucleotides at the 3’ end                |
|                                    | At least three (A/U)s in the seven nucleotides at the 3’ end                |
| A or U at position N10                                                             | 10th nucleotide = (A/U)                                                    |
| U at position 3                                                                    | 3rd nucleotide ≠ U                                                         |
| Devoid of extended runs of altering G/C pairs or three G’s                        | No occurrences of G/C stretches of length 7 or longer                      |
|                                    | No occurrences of three or more identical nucleotides in a row             |

Features are shown in italic if they are pertinent to the considerations proposed in [32], but not specified explicitly.
Features with no marks: All three p-values are greater than 0.05.
Features marked by *: at least one of the three p-values is less than 0.05.
Features marked by **: at least two of the three p-values are less than 0.05.
Features marked by ***: \( P_{\text{wald}} < 0.01 \) and \( P_{70} < 0.01 \) or \( P_{90} < 0.01 \).
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