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# siRNA selection criteria—Statistical analyses of applicability and significance

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**Abstract**

RNA interference is a powerful tool for gene silencing, which is mediated by introducing siRNA. In the present study, statistical analyses of published siRNA selection criteria, the interpretation of some criteria and systematic searching for new criteria have been carried out for CGB siRNA and siRecords databases. The results of the analyses are as follows: (i) Our study supports the two-state model of the RNA-induced silencing complex (RISC). (ii) Stable 5′-S ends of a siRNA sequence, higher stability of the whole siRNA, and low breaking energy of siRNA duplex occurs in effective siRNA sequences. Also low internal stability of the 5′-AS terminus is preferred. (iii) Secondary structure can be successfully used as an RNAi selection criterion. (iv) Several published sequence criteria have been confirmed and also new criteria have been developed. (v) Also a Target Patterns criterion, which is comparable or better than the best known criteria, has been created.


*Keywords:* RNAi; mRNA; siRNA selection criteria; Target Patterns; mRNA secondary structure; RISC enzyme

RNA interference (RNAi) is a recently discovered process that silences gene expression by the transfection of double-stranded RNA (dsRNA) homologous to the target mRNA segment [1]. The dsRNA is at first cleaved by an RNAase III enzyme (Dicer) into 21–23 nucleotide (nt) segments—short interfering RNA (siRNA) sequences with 2 nucleotide 3′ overhangs [2]. These siRNAs are subsequently incorporated into the RISC [3]. An active RISC complex includes the siRNA antisense strand. The antisense strand can guide the RISC to the target mRNA which has the complementary nucleotide sequence. Finally, the target mRNA is cleaved at a specific site corresponding to the siRNA antisense strand [4].

Although RNAi has been widely used for studying gene functions, the capability of siRNA sequence to trigger RNAi (effectiveness or efficacy of the RNAi sequence) depends greatly on the properties of the siRNA. The known siRNA selection criteria are results of studies of several mRNAs [4–8] or statistical analyses of small (less than 400 members) sets of siRNAs [9]. Larger siRNA database (2182 siRNAs) was used for design of predicted siRNA library BIOPREDsi [10], but not for siRNA selection criteria evaluation. In order to find selection criteria which could provide straightforward way to efficient siRNAs, many papers apply statistical analyses to probe selection criteria (e.g., [11] and references therein).

One of the approaches to shed some light on the processes underlying the RNAi, statistical analyses of published data may give evidence in favour of models that explain the RNAi processes. This empirical method is less conclusive than direct experimental and/or computational

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evidence on the particular system but it is still based on the extensive experimental data (depending on the size of the database). We applied statistical analyses taking advantage of the access to two large freely available siRNA databases: CGB siRNA database [12] and siRecords database [13] and interpret some of the observations. In particular we concentrated on the question whether we can get some significant results about the assembly and function of the RISC and the position of the antisense siRNA strand in it. As discussed in [14], two models—Two-state and Fix-ends—have been introduced. The former assumes a free 3′ end of the siRNA guide strand which hybridizes with the mRNA while the latter assumes both 3′ and 5′ ends of the siRNA being docked to the PIWI and the PAZ domains of the Argonaute protein in the RISC.

Moreover, thermodynamic properties are suitable criteria for siRNA design. Specifically duplex unwinding is critical for the formation of the RISC complex [3]. Also the influence of the internal stability of a complete siRNA duplex ($\Delta G_{\mathrm{cmpl}}$), its 5′-S end (first 4 nucleotides, $\Delta G_{5\mathrm{S}}$), 5′-AS end (last 4 nucleotides, $\Delta G_{5\mathrm{AS}}$) and the middle region ($\Delta G_{\mathrm{mid}}$, 9–15th nucleotide) has been reported [7,15]. Thermodynamic stability of the target mRNA secondary structure (free energy of breaking, $\Delta G_{\mathrm{break}}$) in the location attacked by the RISC complex can also influence RNAi process [16].

Secondary structure of the target mRNA can influence RNAi because it effects binding to the RISC complex. Selection strategies based on modeling of the target mRNA secondary structure have been effective in some cases [17,18] but ineffective in other studies [19]. Therefore, it makes sense to study the influence of secondary structure systematically.

It has been shown, that the sequence of mRNA is a very important determinant of RNAi efficacy [6,21–23]. Specifically, the efficacy is influenced by the presence (or absence) of a nucleotide (or a pair of nucleotides) in defined positions of the siRNA sequence.

## Materials and methods

*Databases*. In this work, two databases were used. The first one was the CGB siRNA database [12], which contained 420 siRNA sequences for 105 mRNA sequences. The second database was the siRecords database [13] with 1220 siRNAs for 564 mRNA sequences. These databases contain the siRNA sequences, their experimental efficacy values (between 0% and 100%) and relevant target mRNAs. Note: The databases are permanently growing. Therefore, our analyses will not be up-to-date relatively quickly. However, trends will remain.

*Thermodynamic calculations*. Internal stability of the siRNA, its 5′-S end, 5′-AS end, and middle region were calculated using the nearest-neighbor method [24] via the Vienna RNA Package [25]. Free energy of breaking of siRNA was also calculated using the Vienna RNA Package. First, the Boltzmann weighted ensemble of suboptimal mRNA secondary structures was created using the RNAsubopt program. The ensemble contained 1000 secondary structures, which were weighted by their free energy. For each secondary structure from the ensemble, its siRNA breaking energy was calculated as a difference between free energy of the original structure and free energy of this structure with an unfolded RISC binding part. The resulting breaking energy was an average of all breaking energies of the ensemble.

*Secondary structure prediction*. We used again the Vienna RNA Package. Specifically, the same ensemble of suboptimal mRNA secondary structures was created as for calculation of breaking energy. Then, accessibility (percentage of unpaired nucleotides) at each position in the mRNA sequence was calculated for this ensemble. Finally, average accessibility of the siRNA sequence was calculated as an average of the positions accessible in the mRNA part corresponding to this siRNA sequence. Similar criterion (sense strand ability to form alternative structures) describes Patzel et al. [26].

*Statistical analyses of energies and average accessibility*. The relation between values of these properties and efficacy for a siRNA sequence has been analyzed statistically in the following way: All siRNA sequences from a database (CGB siRNA or siRecords) were divided into two sets: sequences which have the property value within the defined interval and sequences that do not. For each set, the average value of the siRNA sequence efficacy was calculated. The difference between these two average efficacies ($\Delta^{\mathrm{AVG}}(\mathrm{property})$) was expressed in a percentage value of property average for all siRNA sequences in the database. The significance of this difference was expressed as the Wilcoxon $p$-value and considered for $p < 0.05$. All meaningful intervals of each of the properties have been tested. Analyses were processed independently for both databases.

*Statistical analyses of sequence criteria*. Sequence criteria for siRNAs can be described using regular expressions. Examples of sequence criteria and their regular expressions are listed in supplementary materials. Analyses of sequence criteria were performed in the following way: The siRNA sequences from a database (CGB siRNA or siRecords) were divided into two sets: The first set contained sequences fulfilling the criterion (meaning they matched the regular expression of this criterion) and the second set containing the remaining sequences. The difference in their average efficacies ($\Delta^{\mathrm{AVG}}(\mathrm{property})$) has been calculated and its significance was expressed by means of the Wilcoxon $p$-value. Analyses were again carried out independently for both databases. The analyzed criteria were obtained via systematic generation and from literature [4–6,20–23]. For more details, see supplementary materials. Only criteria which can be expressed via regular expressions were analyzed. We investigated following types of systematically generated regular expressions: single nucleotide ($X$ at position $N$; where $X \in \{\mathrm{A, C, G, U, C\ or\ G, A\ or\ U, No\ A, No\ C, No\ G, No\ U}\}$, $N \in \{1,2,3,\ldots,23\}$) and also nucleotide pairs and triads. Correlation between the number of occurrences of one nucleotide, nucleotide pair and nucleotide triad in a siRNA sequence and efficacy of the sequence was also investigated.

*Target Patterns criterion*. A new selection criterion has been developed, based on the results of systematic analysis of regular expressions for sequence criteria. This criterion assigns to each siRNA sequence a Target Patterns value, which can be calculated as follows:

1. The algorithm parameters are configured using training data and a predefined fixed *threshold p-value*:
   - Systematic analysis for single nucleotides, nucleotide pairs and triads is done on a database. For each investigated sequence criteria (represented by regular expression), the efficacy mean values of the matching/not matching targets and the appropriate Wilcoxon $p$-value are stored as *match_mean*(*expression*), *not_match_mean*(*expression*), *p-value*(*expression*).
   - From the whole set of results, just a subset of expressions (*training expressions*) is taken, according to *p-value*(*expression*) < *threshold p-value* are taken.
   - The overall mean efficacy of the database is stored as *overall_mean*.
2. For any target, its Target Pattern value is computed as follows:
   - For each training expression, if the target matches the expression, *match_mean*(*expression*) is remembered.
   - From the set of all remembered *match_mean*(*expression*) values, the most extreme value is taken, i.e., the value having the biggest distance from *overall_mean*:
     $max\{|match\_mean(expression) - overall\_mean|\}$.

The Target Patterns value can be used in a similar way as energies, and other siRNA sequence properties. From the description of the Target Patterns value, it is clear, that this value will be high for effective siRNAs. In this publication, training expressions for the CGB siRNA database were created in the siRecords database and vice versa.

*Processing*. The analyses were processed automatically using software RNAi Analyzer (Svobodova Varekova et al., submitted, http://www.rnaworkbench.com), which has been developed by the authors.

## Results

Table 1 summarizes intervals of properties (free energy of complete siRNA, its 5′-AS end, 5′-S end, and middle region; free energy of breaking; average accessibility), which give highly effective ($\Delta^{AVG}$(property) > 5%) or highly ineffective ($\Delta^{AVG}$(property) < −5%) siRNA sequences. Specifically, we selected the most marked intervals, which means: (a) the largest $\Delta^{AVG}$(property); (b) the Wilcoxon $p$-value less than 0.05.

### *Thermodynamic criteria*

Results summarized in Table 1 demonstrate that the internal stability of the siRNA sequence is a very important criterion for the RNAi design. Specifically, the energy of 5′-AS end, 5′-S end, and the complete siRNA are applicable as selection criteria. The energy of the middle region does not provide statistically significant results.

The siRecords database results concerning the 5′-AS and 5′-S ends are analogical to those published by Khvorova et al. [7]: siRNA sequences with low internal stability of 5′-AS terminus are preferred for RNAi while those with more stable 5′-AS terminus have low efficacy. Moreover, our results show, that stable 5′-S ends are preferred and unstable 5′-S ends occur in less effective siRNA's.

Calculation of $\Delta G_{5S}$, $\Delta G_{5AS}$, and $\Delta G_{mid}$ for siRecords database were performed also by Gong et al. [11]. Gong's results for $\Delta G_{5AS}$ (effective sequences has $\Delta G_{5AS} > -9$ kcal/mol) agree with our results. Our analyses also show this interval as advantageous, but more effective one is $\Delta G_{5AS} > -10.5$ kcal/mol ($\Delta^{AVG} = 8.56$ kcal/mol, $p = 0.03136$) and the best is the one in Table 1. According to Gong, $\Delta G_{5S}$ of effective siRNAs is out of the interval from −9 to −5 kcal/mol, but this criterion is not very significant (Wald $p = 0.1$). From our analyses, siRNAs having $\Delta G_{5S}$ in this interval are also ineffective ($\Delta^{AVG} = -3.01$ kcal/mol) and moreover, this result is statistically significant ($p = 1.03 \times 10^{-04}$). We also found that it is better to use interval from −8 to −5 kcal/mol for ineffective siRNAs. Gong's and our results also agree that energy of middle region cannot be used as a siRNA selection criterion.

The CGB siRNA database provides similar results for 5′-S ends. However, results for 5′-AS ends are different, specifically preferring stable ends (Table 1).

The energy of the complete siRNA is also significant: More stable siRNAs are more effective in both databases, see Table 1. The breaking energy, which reflects the influence of mRNA secondary structures, is also a possible siRNA selection criterion. In spite of the former energy criteria, this provides statistically significant results only for the siRecords database. Specifically, the siRNA sequences that have very stable complementary mRNA parts are markedly less effective for RNAi, while siRNA sequences with unstable mRNA parts are more effective.

### *Secondary structure*

The secondary structure of a siRNA sequence has been described via the average accessibility (see above). From

Table 1
The most marked and statistically significant intervals of energies, average accessibility, and Target Patterns

| Property of siRNA sequence | Database CGB siRNA | | | | Database siRecords | | | |
|---|---|---|---|---|---|---|---|---|
| | Property interval | | $\Delta^{AVG}$ (%) | $p$-value | Property interval | | $\Delta^{AVG}$ (%) | $p$-value |
| | Begin | End | | | Begin | End | | |
| Free energy of complete siRNA (kcal/mol) | −35 | 0 | −31.58 | 0.03119 | −35 | 0 | −10.89 | 0.00024 |
| | −50 | −35 | 23.71 | 0.02988 | −45 | −35 | 12.78 | $4.81 \times 10^{-06}$ |
| Free energy of siRNA 5′-S end (kcal/mol) | −9 | −4 | −20.25 | 0.00585 | −8 | −5 | −9.07 | $8.68 \times 10^{-05}$ |
| | −12 | −9 | 22.94 | 0.00259 | −13 | −8 | 8.79 | 0.00026 |
| Free energy of siRNA 5′-AS end (kcal/mol) | −9 | −5 | −16.24 | 0.01498 | −13.3 | −10.5 | −8.56 | 0.03136 |
| | −13 | −9 | 14.63 | 0.02729 | −10.5 | −4 | 8.71 | 0.02605 |
| Free energy of siRNA middle region (kcal/mol) | No statistically significant correlation | | | | No statistically significant correlation | | | |
| Free energy of breaking (kcal/mol) | No statistically significant correlation | | | | 30 | 37 | −27.36 | 0.03661 |
| | | | | | 0 | 9 | 6.50 | 0.01240 |
| Average accessibility (%) | 25 | 40 | −14.31 | 0.02802 | 15 | 33 | −5.99 | 0.04859 |
| | 40 | 50 | 13.63 | 0.03580 | 33 | 58 | 6.15 | 0.01477 |
| Target Patterns[a] (%) | 60 | 100 | 27.29 | $1.59 \times 10^{-05}$ | 60 | 100 | 13.58 | $1.94 \times 10^{-08}$ |

Advantageous intervals of property are marked white, not advantageous are gray.
[a]Target Patterns selection criterion was used with the threshold $p$-value = 0.01. For calculation of $\Delta^{AVG}$ and $p$-value, siRNA sequences were divided into two sets. The first contained 40% of sequences, having the highest Target Pattern value, and the second contained the remaining sequences.

Table 1, it is visible that average accessibility can be successfully used as an RNAi selection criterion.

Low average accessibilities (meaning that the attacked part of mRNA contains only several unpaired nucleotides) occur in ineffective sequences (i.e., CGB siRNA: 25% < average accessibility < 40%; siRecords: 15% < average accessibility <33%).

*Sequence criteria from literature*

Results of the analyses of sequence criteria from literature are described in supplementary materials. The analyses show that the most succesfull criteria were those which describe nucleotides at the beginning and at the end of a siRNA sequence, specifically first nucleotide (C/G, No U) and nucleotide 19 (A/U, A, No G, No G/C). These criteria are similar in most of the published sets. The selection criteria describing nucleotides in the middle of siRNA sequence are not very general. Some of them are only usable for one database.

*Systematic searching of sequence criteria*

Results of the systematic searching of a single nucleotide sequence criteria are summarized (see Table in supplementary material). From the table, one can see that systematic searching confirms criteria which require nucleotides at the beginning and at the end of a siRNA sequence (positions 1 and 19). Specifically, nucleotides C or G should be at position 1 and nucleotide U must not be in this position. Nucleotides A or U are appropriate at position 19.

The analyses also provided statistically significant results for the first nucleotide in the 3′ overhang (position −2). Nucleotides A or U are suitable at this position. It is in agreement with the beginning of the first Tuschl rule (i.e., AA(N19)UU).

A very interesting and useful result is that significant criteria have been found also for positions in the middle of a siRNA sequence (nucleotide 9 (not U) and nucleotide 15 (C or G)). These findings are new, from middle positions, only 8 (Hohjoh [21]), 10 (Reynolds [20]) and 16 (Hsieh [22]) were reported as important for siRNA design.

In supplementary materials, we present: (a) results of systematic searching for single/pair/triad nucleotide criteria and nucleotide contents criteria; (b) sanity check, which demonstrates that the found criteria are not random.

*Target Patterns criterion*

The Target Patterns criterion was tested for both databases and for threshold $p$-values of 0.01 and 0.05. As regular expressions, we used all various combinations of all possible single, pair and triad nucleotide criteria. The analyses show, that utilization of the threshold $p$-value 0.01 and the training set containing all single, pair and triad nucleotide criteria provides the best results. These results are summarized in Table 1 and demonstrate that the Target Patterns criterion provides very high $\Delta^{AVG}$ with great significance. For comparison, these results are better than those for the second best criterion (free energy of complete siRNA) and comparable with the best criterion (CG content [4,6,12,20]). Therefore, it is useful to apply Target Patterns in algorithms for siRNA design.

## Discussion

420 siRNAs for 105 mRNA sequences from the CGB siRNA database and 1220 siRNAs for 564 mRNA sequences from siRecords database have been studied using our automatic tool (RNAi Analyzer) for the statistical analyses of siRNA selection criteria. Analyses of published selection criteria and systematical searching of new criteria revealed several new facts that may aid in prediction of higher efficacy siRNAs.

The thermodynamic criteria of the siRNA sequence are very important for RNAi design, specifically: Stable 5′-S ends of siRNA sequence, higher stability of whole siRNA and low breaking energy of siRNA duplex are very important properties. Also, the low internal stability of the 5′-AS terminus is preferred. Middle region internal stability does not impede knockdown efficacy. Our results agree with results of Gong et al. [11]. Moreover, our analysing methodology (inverse to the Gong's methodology) was more sensitive to finding significant intervals. Specifically, Gong divided siRNAs into four subsets according efficacies and compared average energy (or other property) between them by Wald test. We divided siRNAs into two subsets (energy in or out of defined interval) and compared average efficacies of them by Wilcoxon test.

We found that secondary structure (described via average accessibility) can be successfully used as an RNAi selection criterion. Effective siRNA sequences are shown in Table 1.

Sequence criteria are very important for siRNA design. The testing of published criteria sets shows, that the most successful criteria describe nucleotides at the beginning and at the end of a siRNA sequence, specifically for first nucleotide (C/G, No U) and for nucleotide 19 (A/U, A, No G, No G/C). Via a systematical search, we confirmed criteria for nucleotide 1 and 19, found new criteria for middle region (nucleotide 9 (No U), nucleotide 15 (G, C/G, No U)) and also for the 3′ overhang (first nucleotide in overhang alias nucleotide −2 (A, A/U, No C)). These criteria are statistically significant in both databases and can be very useful for siRNA design.

The systematic analysis of the sequence criteria for single nucleotides, pairs and triads is shown in supplementary materials. We should stress out that from all combinations only those are shown in the tables that have significant difference between the average efficacies as measured by the Wilcoxon $p$-value being smaller than 0.05. All three tables give us rather asymmetric results as to the nucleotide positions in the siRNAs. While changes at the beginning of the strand (position −2) give us large differencies in the average

efficacies, we could not find any changes for the position larger than 20. This would strongly indicate that those nucleotides [20,21] do not take part in the hybridization with the mRNA. Instead they are responsible for the bonding to the PIWI domain in the RISC (according to [14]).

A new selection criterion Target Patterns was created based on the results of systematic analysis of regular expressions for sequence criteria. The analyses show, that this criterion is comparable with or better than the best known selection criteria. Therefore, it is useful to apply Target Patterns in algorithms for siRNA design.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.056.

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