Rational Design of siRNAs with the *S*fold Software

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Introduction

In eukaryotic organisms, RNA interference (RNAi) is the sequence-specific gene silencing that is induced by double-stranded RNA (dsRNA) homologous to the silenced gene. In the cytoplasm of mammalian cells, long dsRNAs (>30 nt) can activate the potent interferon and a protein kinase-mediated pathway, which lead to non-sequence-specific effects that can include apoptosis (Kumar and Carmichael, 1998). Elbashir and coworkers (2001a) made the important discovery that small interfering RNAs (siRNAs) of about 21 nt specifically inhibit gene expression, because siRNAs are too short to activate the interferon or protein kinase pathway. The silencing by synthetic siRNAs is transient. This limitation can be overcome by stably expressed short hairpin RNAs (shRNAs), which are processed by Dicer into siRNAs. (Paddison *et al.*, 2002; Brummelkamp *et al.*, 2002). However, it was recently reported that shRNA vectors can induce an interferon response (Bridge *et al.*, 2003).

Because target recognition presumably depends on Watson-Crick base pairing, the RNAi machinery is widely believed to be exquisitely specific. As a reverse genetic tool, RNAi has set the

standard in high throughput functional genomics (Barstead, 2001; Tuschl, 2003). RNAi has also become an important tool in the identification and validation of drug targets in preclinical therapeutic development (Thompson, 2002; Appasani, 2003). Furthermore, RNAi-based human therapeutics are under development.

Initial empirical rules have been established by the Tuschl lab for the design of siRNAs. However, large variation in the potency of siRNAs is commonly observed, and often only a small proportion of the tested siRNAs are effective. Increasingly, emerging experimental evidence suggests that secondary structure and accessibility of target RNA are important factors in determining the potency of siRNAs (Lee *et al.*, 2002; Vickers *et al.*, 2003; Bohula *et al.*, 2003; Far and Sczakiel, 2003).

In this chapter, we first review empirical rules for the design of siRNAs. A novel method is described for improving siRNA design, through combination of empirical rules with prediction of target accessibility. We report a new RNA folding software package, named *S*fold, that offers rational siRNA design tools based on this novel methodology. *S*fold is available through Web servers at http://sfold.wadsworth.org/ and http://www.bioinfo.rpi.edu/applications/sfold/. The issue of specificity and its relationship to potency are discussed. Finally, we propose a computational strategy for maximizing both potency and specificity in high throughput siRNA applications.

Empirical siRNA design rules

Tuschl Rules and expansions

The first set of empirical rules for siRNA design was compiled by Tuschl's group (http://www.rockefeller.edu/labheads/tuschl/sirna.html; Elbashir *et al.*, 2001b). The Tuschl rules can be summarized as follows:

1) siRNA duplexes should be composed of 21-nt sense and 21-nt antisense strands, paired so as to each have a 2-nt 3' dTdT overhang;

2) The targeted region is selected from a given cDNA sequence beginning 50 to 100 nt downstream of the start codon (3' UTRs also have been successfully targeted);

3) The target motif is selected in the following order of preferences: i) NAR(N17)YNN, where N is any nucleotide, R is purine (A or G) and Y is pyrimidine (C or U); ii) AA(N19)TT; iii) NA(N21);

4) Nucleotides 1-19 of the sense siRNA strand correspond to positions 3-21 of the 23-nt target motif;

5) The target sequence is selected to have around 50% GC content; and

6) Selected siRNA sequences should be aligned against EST libraries to ensure that only one gene will be targeted.

The AA(N19)TT motif has a low frequency of occurrence. AA(N19) is a popular motif advocated by two reagent companies, Ambion and Qiagen. It has also been suggested to avoid more than three Gs or three Cs in a row, because polyG and polyC sequences can hyperstack to form agglomerates that may interfere in the siRNA silencing mechanism (http://www.qiagen.com/catalog/auto/cget.asp?p=RNAi_support). Many siRNAs with 60% GC content are effective, but siRNAs with ≥70% GC are not as effective. From the perspective of target accessibility, a possible explanation is that a high GC motif is more likely to occur in a highly structured region, because G•C pairing is energetically stronger than A-U pairing. Examples are provided below to illustrate the rule-based design.

Examples

Example 1. AA(N19) motif.

Targeted gene	Rabbit β-globin
	(GenBank Accession no. V00879; coding region nt 54 - nt 497)
Target position and GC%	418-438; 52.4%
Target sequence	5'-AAUUCACUCCUCAGGUGCAGG-3'
Sense siRNA	5'-UUCACUCCUCAGGUGCAGGTT-3'
Antisense siRNA	5'-CCUGCACCUGAGGAGUGAATT-3'

Example 2. AA(N19)TT motif.

Targeted gene	Enhanced green fluorescent protein (GFP)
	(GenBank Accession no. U55762; nt 583 - nt 1602)
Target position and GC%	571-593; 47.6%
Target sequence	5'-AAGAACGGCAUCAAGGUGAACUU-3'
Sense siRNA	5'-GAACGGCAUCAAGGUGAACTT-3'
Antisense siRNA	5'-GUUCACCUUGAUGCCGUUCTT-3'

Exceptions to the rules

The Tuschl design rules were largely based on studies with *Drosophila melanogaster* embryo lysate (Elbashir *et al.*, 2001b). Exceptions to the rules have been reported. Active siRNAs with a dinucleotide leader other than AA or NA can be found in the literature. siRNAs without specific nucleotide overhangs can be highly efficient (Czauderma *et al.*, 2003). It has been shown that the antisense strand of the siRNA duplex can be almost as effective as the siRNA duplex, and the antisense strand and the double-strand siRNA appear to share the RNAi pathway (Amarzguioui *et al.*, 2003; Holen *et al.*, 2003). Hybrid DNA:RNA molecules were reported to be more effective that RNA:RNA duplexes in both duration and degree of silencing (Lamberton and Christian, 2003).

Target secondary structure and accessibility

RNAs form stable secondary structures through Watson-Crick and wobble G-U base pairing. Structural elements in the secondary structure include both helices and single-stranded loop regions. The single-stranded regions are likely to be accessible for RNA-targeting nucleic acids through base pairing interactions. Target accessibility has long been established as an important factor for the potency of antisense oligonucleotides and *trans*-cleaving ribozymes. For siRNAs, the empirical rules do not take into account the target secondary structure and accessibility. This lack of consideration is perhaps because siRNAs are more potent than antisense oligonucleotides. However, based on the large variation in the potency of siRNAs synthesized against different sites on the same target mRNA, it has been speculated that the low activity of the majority of siRNAs may be the result of limited accessibility of the target sequence due to secondary structure of target mRNA (Holen *et al.*, 2002). Recently, increasing experimental evidence based on a number of experimental approaches has emerged to support the importance of target secondary structure and accessibility. Lee and coworkers (2002) demonstrated that the potency of siRNAs is determined by the target accessibility, with accessibility assessed by an oligo library. Bohula and coworkers (2003), using an oligo array method, and Vickers and coworkers (2003) both reported that the target secondary structure has an important effect on the potency of siRNAs. More recently, based on computational predictions and validation by antisense oligonucleotides for accessibility, Far and Sczakiel (2003) demonstrated that target accessibility determined by local RNA structure is important for the potency of both antisense oligonucleotides and siRNAs.

***S*fold software for rational siRNA design**

It has been widely assessed that usually one out of four or five siRNAs designed by empirical rules

is effective. For example, in a study that screened 356 siRNAs against 64 genes, the average number of siRNAs screened, in order to find one that induced >80% knockdown on the mRNA level, was 4.2 (Liszewski 2003). Because synthetic siRNA duplexes are expensive, methods to improve siRNA design are clearly needed.

We have developed a novel method for prediction of target accessibility, based on a probability profiling approach for predicting single-stranded regions in RNA secondary structure (Ding and Lawrence, 2001). It is believed that an mRNA can exist in a population of structures (Christoffersen *et al.*, 1994). High probability regions in the profile reveal target sites that are *commonly accessible* for a large number of statistically representative structures for the target RNA (see Fig. 1). Through assignment of statistical confidence in predictions, this novel approach bypasses the long-standing difficulty of selecting a single structure for accessibility evaluation.

We have employed the algorithm to develop a software package, *S*fold, for the rational design of RNA-targeting nucleic acids. An application module, *S*irna, is included in the software. *S*irna aims to provide tools for improved siRNA design, by combining secondary structure and accessibility prediction with the established empirical design rules. *S*fold is available through Web servers at http://sfold.wadsworth.org/ and http://www.bioinfo.rpi.edu/applications/sfold/. Documentation, frequently asked questions (FAQs), and a summary of promising preliminary validation data are available on the Web sites. The basic steps in using *S*irna for siRNA design are: 1) selection of accessible sites from the probability profile of the target RNA; 2) for such selected accessible sites, choice of siRNAs that meet the requirements of the empirical rules, as well as the requirement of favorable binding energy between the antisense siRNA strand and its target sequence. Stronger binding is indicated by lower binding energy (stacking energies are negative

valued). For example, an antisense siRNA with a binding energy of −20 kcal/mol is more effective than an antisense siRNA with a binding energy of −10 kcal/mol. The module provides both graphical files and text files to facilitate the design process. *S*irna provides output files for both the popular AA(N19) target motif and other Tuschl motifs. Based on the empirical rules, both filtered output and complete output without filtering are available. Portions of two output files for rabbit β-globin are shown below.

Sample output

File *aan19_f.out* gives filtered output for siRNAs targeting AA(N19) motifs:

Line 1: ID of AA(N19) motif target position target sequence

GC content antisense siRNA binding energy (kcal/mol)

Line 2: sense siRNA (5' → 3') antisense siRNA (5' → 3')

Filter criteria:

A) 40% ≤ GC % ≤ 60%;

B) Antisense siRNA binding energy ≤ −15 kcal/mol;

C) Exclusion of target sequence with at least one of AAAA, CCCC, GGGG, or UUUU.

4	43- 63	AAACAGACAGAAUGGUGCAUC	42.9% -16.9
		ACAGACAGAAUGGUGCAUCTT	GAUGCACCAUUCUGUCUGUTT
5	44- 64	AACAGACAGAAUGGUGCAUCU	42.9% -16.0
		CAGACAGAAUGGUGCAUCUTT	AGAUGCACCAUUCUGUCUGTT
6	53- 73	AAUGGUGCAUCUGUCCAGUGA	47.6% -16.2
		UGGUGCAUCUGUCCAGUGATT	UCACUGGACAGAUGCACCATT
18	249- 269	AAGAAGGUGCUGGCUGCCUUC	57.1% -15.8
		GAAGGUGCUGGCUGCCUUCTT	GAAGGCAGCCAGCACCUUCTT

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File *sirna_f.out* gives filtered output of siRNAs targeting Tuschl motifs:

Column 1: target position (starting - ending)

Column 2: sense siRNA (5' → 3')

Column 3: antisense siRNA (5' → 3')

Column 4: GC content of target sequence

Column 5: antisense siRNA binding energy (kcal/mol)

Column 6: Pattern code for Tuschl target motifs

Filter criteria:

A) 40% ≤ GC % ≤ 60%;

B) Antisense siRNA binding energy ≤ −15 kcal/mol;

C) Exclusion of target sequence with at least one of AAAA, CCCC, GGGG, or UUUU.

43- 63	ACAGACAGAAUGGUGCAUCTT	GAUGCACCAUUCUGUCUGUTT	42.9%	-16.9	BCD
44- 64	CAGACAGAAUGGUGCAUCUTT	AGAUGCACCAUUCUGUCUGTT	42.9%	-16.0	B D
53- 73	UGGUGCAUCUGUCCAGUGATT	UCACUGGACAGAUGCACCATT	47.6%	-16.2	B
195- 215	CCUGUCCUCUGCAAAUGCUTT	AGCAUUUGCAGAGGACAGGTT	50.0%	-16.7	B D
243- 263	UGGCAAGAAGGUGCUGGCUTT	AGCCAGCACCUUCUUGCCATT	55.0%	-16.8	B D

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Statistical modeling of experimental data

Computational modeling of the RNAi pathway is not possible, because the molecular mechanism for gene silencing is as yet only partially understood. However, experimental data from RNAi experiments have been emerging. The melting temperature T_m of the siRNA duplex can be calculated with RNA thermodynamic parameters (Xia *et al.*, 1998). T_m, the antisense siRNA binding energy calculated by *S*fold, secondary structural features at target site, GC content, and other

primary sequence composition features can be included as variables for building statistical models, such as logistic regression for predicting the potency of siRNAs. In one study, T_m was not associated with the potency of siRNAs (Hohjoh, 2002). This suggests that thermodynamics of the siRNA duplex is not an important factor for potency. The lack of correlation is perhaps because duplex unwinding is a ATP-dependent process performed by an unknown helicase (Elbashir *et al.*, 2001a; Nykänen *et al.*, 2001). On the other hand, it has been speculated that low GC content at the 5' end of the duplex facilitates unwinding (G. Hutvagner, personal communication, 2003). Should experimental evidence emerges to support this speculation, inclusion of a rule in modeling and design could improve target prediction. Clearly, there is considerable room for improvement in siRNA design. We expect that statistical modeling of experimental findings will provide an efficient means to assess the contribution to silencing potency by each of these factors.

Specificity of siRNAs

Specificity of siRNAs is important for interpretation of experimental results and for the reliability of newly discovered phenotypes from large-scale RNAi screening. For *Drosophila melanogaster* embryo lysate, a single base mismatch in the center of the siRNA duplex prevented target RNA cleavage (Elbashir *et al.*, 2001b). The exquisite specificity is also supported by two studies using gene expression profiling (Chi *et al.*, 2003; Semizarov *et al.*, 2003). However, there is evidence that challenges the requirement of perfect identity between siRNA and its target mRNA. Holen and coworkers (2002) demonstrated that one or two central mutations in the siRNA targeting position 167 in human tissue factor (TF) did not abolish the siRNA' ability to reduce mRNA levels. Also for human TF, it was found that siRNAs generally tolerated mutations at the 5' end of the target, whereas they exhibited low tolerance for mutations at the 3' end (Amarzgouioui *et al.* 2003). More

strikingly, an expression profiling study revealed off-target effects on nontargeted genes that possessed as few as 11 contiguous nucleotides of identity to the siRNA (Jackson *et al.* 2003). A separate concern about specificity is that siRNAs can also function as microRNAs (miRNAs; Doench *et al.*, 2003; Zeng and Cullen, 2003). These authors report reduction in protein levels even through the use of siRNAs having only partial complementarity to the 3'UTRs of target mRNAs. miRNAs can trigger a translational repression mechanism, which is distinct from the catalytic mechanism for RNAi and is not yet well understood at the molecular level. However, this does not explain the off-target effects reported by Jackson and coworkers (2003), because translational repression does not trigger target mRNA cleavage, and it is thus unlikely to alter array profiling. For plants, a majority of predicted targets for miRNAs are transcription factors (Rhoades *et al.*, 2002). Regulation of transcription factors can alter the levels of mRNAs. Thus, the non-specific effects observed by Jackson and coworkers (2003) may result from secondary effects of siRNAs acting as miRNAs that induce regulation of transcription factors. Recently, Zeng and coworkers (2003) reported that miRNAs can act as (antisense) siRNAs. Furthermore, in a plant embryo extract, a miRNA that lacks perfect complementarity to its target acts as a siRNA, by guiding an endonuclease for efficient target cleavage (Tang *et al.*, 2003).

Despite the mixed reports on specificity, it is advisable to BLAST siRNAs against NCBI's EST or UniGene database, to ensure that a selected siRNA does not have strong homology to nontargeted genes. It is understood that the RNA-induced silencing complex (RISC) can take either strand of the siRNA duplex after duplex unwinding, because RISC does not know the identity of its substrate before the contained siRNA strand identifies the target, presumably through complementary base pairing (G. Hutvagner, personal communication, 2003). Thus, both the sense

siRNA strand and the antisense strand may be available for binding to nontargeted genes, before they are digested by nucleases. It is therefore a good practice to perform alignment for both strands. The UniGene database contains transcript sequences that are either of known genes or are longest region of high-quality sequence data derived from ESTs. An Encyclopedia of DNA Elements (ENCODE) project was recently launched by the National Human Genome Research Institute (http://www.genome.gov/Pages/Research/ENCODE/). The goal of this project is to comprehensively identify functional elements in the human genome sequence. Such elements include alternative splicing sites, transcriptional start sites, translational start sites, polyadenylation sites, and protein-coding and non-protein-coding regions. The reliability of the BLAST search will improve with more accurate genome annotation. BLAST capability on the *S*fold server is currently under development and will be made available in the future.

Enhancing specificity through potency

Potency and specificity are related issues. In RNAi screening applications based on siRNA libraries, a design method with high likelihood for potent silencing can improve specificity, because off-target effects will be minimized when as few siRNAs as possible are needed to knock down their targets. Off-target effects are more likely to occur for higher concentration of siRNAs (Semizarov *et al.*, 2003). The strong binding by a potent siRNA to its target may discourage cross-hybridization with genes of partial complementarity, because cross-hybridization is less energetically favorable. On the other hand, an ineffective siRNA can bind only weakly to its target, and is thus more readily available for cross-hybridization with nontargeted genes.

In the construction of a siRNA library for high throughput RNAi screening, the issues of

potency and specificity must be addressed together. For each target gene, numerous accessible target sites are usually revealed by *S*fold that satisfy the empirical criteria. Thus, how to select one or two siRNAs for each of hundreds or even thousands of genes is a design challenge. Proper computational optimization is necessary to maximize both potency and specificity. This can be achieved through integration of target selection based on accessibility prediction and empirical rules and sequence homology analysis.

Conclusions

RNAi has become a powerful reverse genetics tool that promises to revolutionize molecular biology. Currently, there is no golden rule for the design of siRNAs. Improved design may be achieved through a combination of empirical rules, target accessibility evaluation, and statistical modeling. *S*fold aims to provide such tools to the scientific community through Web service. Specificity is an important issue for RNAi applications, and more experimental work is needed to reconcile conflicting results. Specificity can be enhanced through improvement in potency of silencing. Integration of computational approaches for maximizing both potency and specificity will facilitate high throughput applications to functional genomics, drug target validation, and development of RNAi-based human therapeutics (Fig. 2).

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*S*fold Web URLs: http://sfold.wadsworth.org/; http://www.bioinfo.rpi.edu/applications/sfold/.

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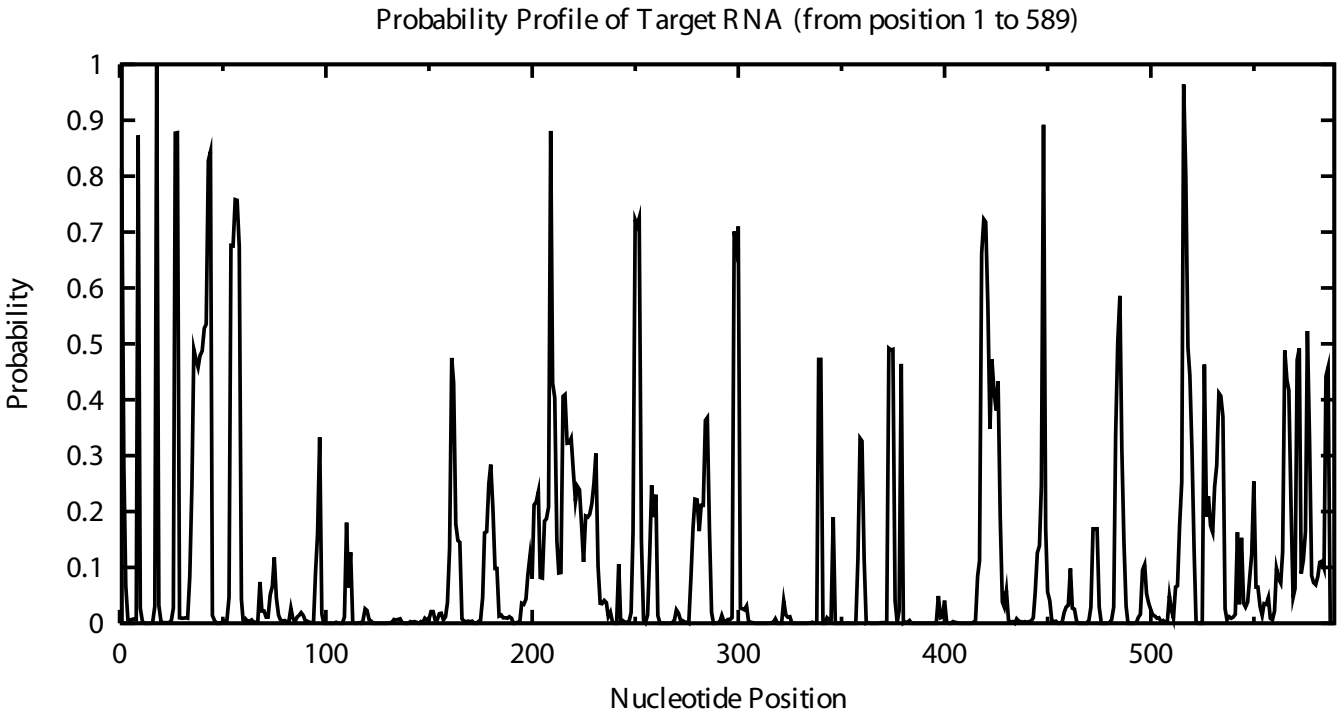
Figure Legends

Fig. 1. Probability profile for rabbit β-globin mRNA produced by *S*fold. Sites with high probabilities are predicted to be accessible for effective targeting by siRNAs.

Fig. 2. A computational framework for high throughput applications: design of potent siRNAs is integrated with sequence homology search in order to maximize specificity in applications to functional genomics, drug target validation, and development of RNAi therapeutics.


Probability Profile of Target RNA (from position 1 to 589)
Probability
Nucleotide Position
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0.1
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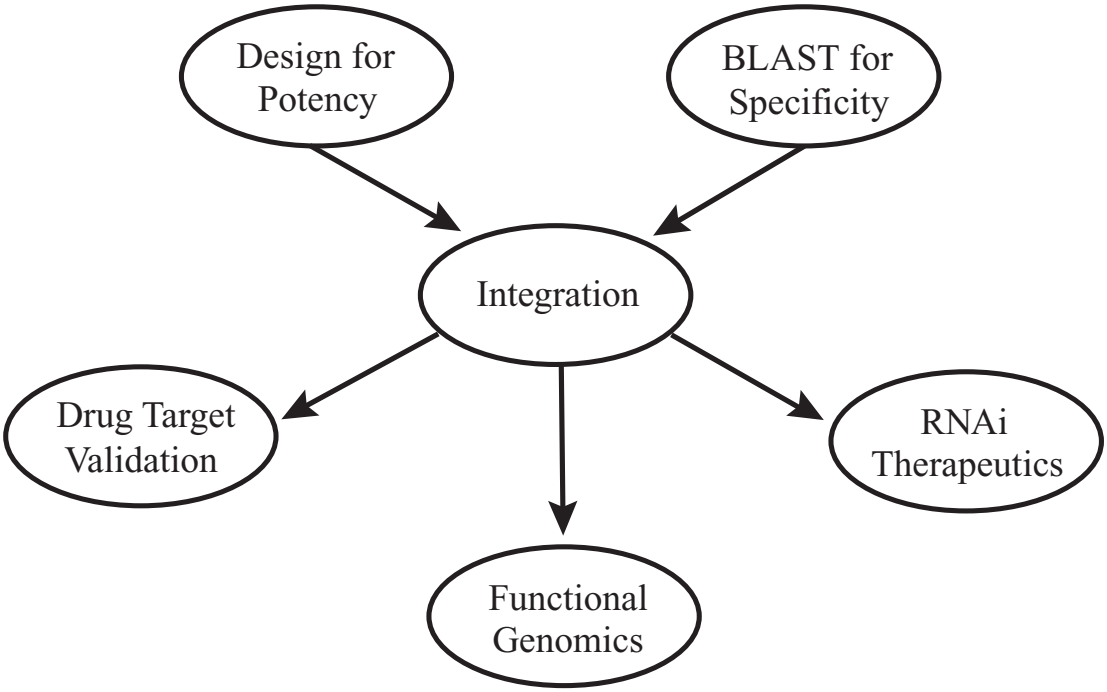
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
100
200
300
400
500


Design for Potency
BLAST for Specificity
Integration
Drug Target Validation
RNAi Therapeutics
Functional Genomics