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Applications of high-throughput sequencing to analyze and engineer ribozymes

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ABSTRACT

A large number of catalytic RNAs, or ribozymes, have been identified in the genomes of various organisms and viruses. Ribozymes are involved in biological processes such as regulation of gene expression and viral replication, but biological roles of many ribozymes still remain unknown. Ribozymes have also inspired researchers to engineer synthetic ribozymes that function as sensors or gene switches. To gain deeper understanding of the sequence-function relationship of ribozymes and to efficiently engineer synthetic ribozymes, a large number of ribozyme variants need to be examined which was limited to hundreds of sequences by Sanger sequencing. The advent of high-throughput sequencing technologies, however, has allowed us to sequence millions of ribozyme sequences at low cost. This review focuses on the recent applications of high-throughput sequencing to both characterize and engineer ribozymes, to highlight how the large-scale sequence data can advance ribozyme research and engineering.

1. Introduction

Since the first discoveries of ribozymes by Cech [1] and Altman [2], several classes of ribozymes have been found in various organisms and viruses. While the sequences and structures of these ribozymes are highly diverse, the majority of the naturally occurring ribozymes are involved in either splicing or self-cleavage of RNA strands. However, *in vitro* selection experiments from a large pool of random RNA sequences have resulted in ribozymes capable of catalyzing various chemical reactions beyond nucleic acid chemistry such as Diels-Alder [3] and aldol [4] reactions, underscoring the latent potential of ribozymes to catalyze diverse reactions. Regardless of the origins of the ribozymes, elucidation of the sequence-function relationship is of fundamental importance for understanding and engineering ribozymes. Conventional mutational analysis of a ribozyme involves preparation of individual ribozyme mutants followed by biochemical assay of their activities, for example, by gel electrophoresis. Single-base substitutions (single mutants) that compromise activity indicate that those nucleotides are critical for ribozyme function (and possibly structure). Base pairs that are functionally important can be confirmed by single mutants that disrupt the suspected base pair (inactive ribozyme) and double mutants that replace the original base pair with an alternative pair (active ribozyme). While such classical biochemical assays provide quantitative ribozyme activity information for the mutants examined, the number of ribozyme variants that can be characterized is limited. Therefore, mutants that are subjected to conventional mutational analysis are highly biased by the researcher.

2. Mutate, select, and sequence

Because RNA can be readily amplified by reverse transcription and polymerase chain reaction (PCR), ribozymes can be selected for activity *in vitro*. In fact, most artificial ribozymes have been selected from random RNA sequences through iterative selection cycles [5]. The prime advantage of ribozyme selection is its ability to enrich higher activity sequences from a large number (up to $\sim 10^{15}$) of sequences. Therefore, by starting from a pool of mutants of a ribozyme of interest instead of a random pool of sequences, *in vitro* selection can isolate only those mutants that retain a certain level of activity and eliminate those with weaker or no activity. Sequence analysis of the mutants that survive the selection should provide rich information on sequence-function relationship of the parental ribozyme. Ribozyme mutants can be prepared either by “doped” oligonucleotide synthesis or error-prone PCR. In doped oligonucleotide synthesis, the template DNA for *in vitro* transcription of a ribozyme is synthesized with a mixture of the wild-type nucleotide monomer doped with small amounts (e.g. 3% each) of three other monomers during the coupling steps. This results in a pool of statistically mutated DNA (and thus RNA). By varying the doping ratio, the researcher can control the composition of the mutants in the library relatively well. Error-prone PCR employs manganese (II) ion or

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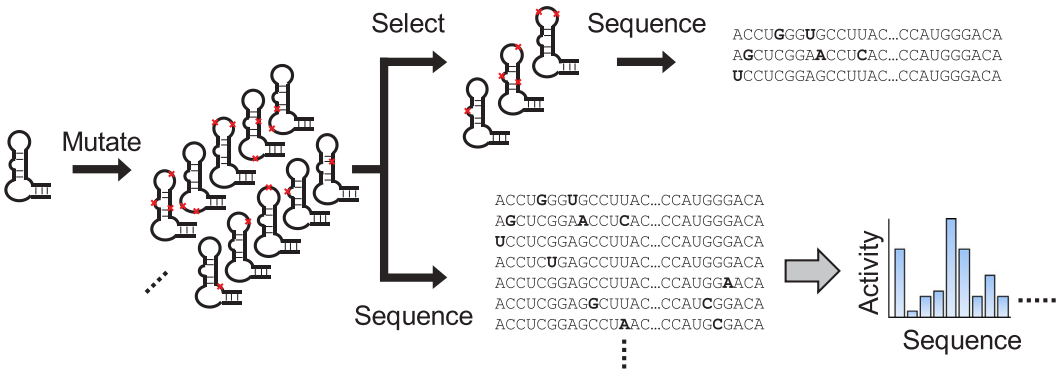

Fig. 1. Mutate-select-and-sequence (top) and mutate-and-sequence (bottom) strategies for high-throughput mutational analysis of ribozymes. In the mutate-select-and-sequence approach, sequences of a very small fraction of the mutants that survive the selection (thus are active) are obtained, and abundance of each sequence provides limited and qualitative information about the activity of such mutants. In the mutate-and-sequence approach, quantitative activity (e.g. fraction cleaved) is calculated for a large number (10^3–10^6) of mutants regardless of their activity.

engineered DNA polymerase to elevate the error rate during PCR [6,7]. While error-prone PCR can introduce errors in long DNA sequences, it is difficult to control the error rate and the mutational spectrum is known to be highly biased.

In the mutate-select-and-sequence strategy (Fig. 1), the goal of the selection is to recover the mutants that retain some level of activity (e.g. near wild-type) rather than to isolate the few sequences with the highest activities. By analyzing which base-substitutions survive the selection or not, functionally critical nucleotides for every position in the ribozyme can be inferred. Furthermore, covarying mutations can be identified from the sequencing data to discover base-base interactions that are functionally critical. Earlier studies have used Sanger sequencing of up to ~100 mutants to infer such functionally important nucleotides and base-base interactions. For example, Nehdi and Perreault randomized 25 positions in the antigenomic HDV ribozyme and sequenced active variants that survived *in vitro* selection to identify functionally critical bases and base pairs [8]. Curtis and Bartel studied mutational coupling effects of a large number of mutations in a kinase ribozyme to characterize the fitness landscape by Sanger sequencing [9]. Fitness landscape is a conceptual map of genotype (ribozyme sequence) to fitness (ribozyme activity) and its feature or "shape" is important for understanding the evolutionary processes.

While these efforts have yielded useful insights, the amount of data generated by Sanger sequencing severely limits the coverage of mutants that exist in the selected ribozyme populations. Naturally, high-throughput sequencing of the selected ribozymes guarantees more comprehensive coverage of mutants with fewer base substitutions (e.g. single and double mutants), therefore, can extract much richer sequence-function relationship information from selection experiments.

In a recent example, Hayden et al. selected active *Azoarcus* group I ribozyme mutants under high and low Mg^{2+} concentrations and used high-throughput sequencing to analyze the ribozyme populations that survived the selections. As low Mg^{2+} is unfavorable for ribozyme folding and activity, they identified and analyzed mutants that retain activity at high Mg^{2+} concentration but lose activity at low Mg^{2+} concentration, and discovered that such mutations are localized to the so called scaffold domain [10].

Similarly, high-throughput sequencing can be a powerful tool for analyzing the global fitness landscape and the evolutionary dynamics of ribozymes evolved in the laboratory. This was first demonstrated by Pitt and Ferré-D'Amaré who selected for active mutants of the class II ligase ribozyme [11] and sequenced the selected genotypes [12]. The researchers used genotype abundance in the selected populations as a proxy for biochemical activity to infer the fitness landscape of the ribozyme. The fitness landscape surrounding the ribozyme sequence was found to be rugged, meaning that minor mutations can drastically change the ribozyme activity. In another example, Hayden et al. demonstrated that cryptic mutations in the *Azoarcus* group I ribozyme that accumulate during purifying selection can be advantageous for the population to adapt to a new substrate by deep sequencing of the ribozyme populations evolved *in vitro* [13].

In a complementary approach, Jäschke and coworkers studied how ribozymes that catalyze Diels-Alder reaction emerged from random sequences via *in vitro* selection [14]. They sampled and sequenced RNA populations from different stages of the multi-round *in vitro* selection process, and found that the populations retain significant diversity even after ten rounds of selection. Similarly, Pressman et al. used high-throughput sequencing to analyze RNA populations from an *in vitro* evolution experiment of triphosphorylating ribozymes from a random pool [15]. They used the sequencing data to estimate the distribution of catalytic activity in the global RNA sequence space.

3. Mutate and sequence (without selection)

Mutate-select-and-sequence strategy exploiting high-throughput sequencing allows comprehensive mutational analysis throughout the ribozyme sequence which is impossible with conventional biochemical analysis of individual mutants. However, there are few drawbacks that should be considered. First, there are no quantitative information on the relative activities of individual mutants. It should be noted that abundance of individual mutants in the sequenced population does not necessarily reflect their relative ribozyme activity [16]. As the starting ribozyme population prepared by doped oligonucleotide synthesis or error-prone PCR is already biased before selection, it can easily be imagined that the selected population carries such biases as well. Furthermore, the composition of the selected ribozyme population can also depend on the stringency of the selection and any systematic biases introduced during the selection process (reverse transcription and PCR). Consequently, observation of mutations that survive a selection experiment should be interpreted as a *qualitative* indication that such mutations do not perturb activity below an arbitrarily set threshold based on the applied selection pressure.

We recognized the limitations of the conventional biochemical mutational analysis and the mutate-select-and-sequence strategy. Therefore, we decided to develop a new high-throughput and quantitative mutational analysis strategy for ribozymes. Focusing on self-cleaving ribozymes, the overall strategy is summarized in Fig. 2. First, a dsDNA library that contains all mutants of interest is synthesized by an appropriate method. Second, the ribozyme library is generated by *in vitro* transcription and allowed to undergo self-cleavage. The cleaved and uncleaved fragments are converted into dsDNA via reverse transcription and PCR while adding adapter sequences for high-throughput sequencing. Finally, the dsDNA library is sequenced, and the number of cleaved (N_{clv}) and uncleaved (N_{unc}) fragments for every mutant of

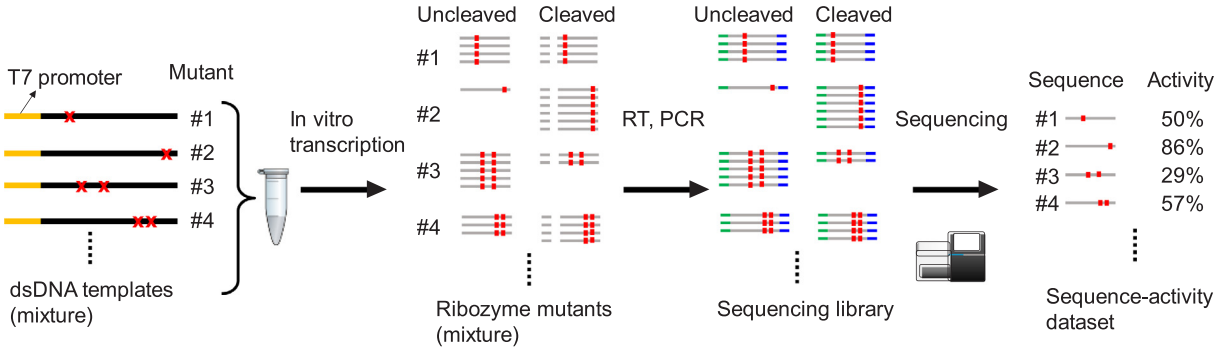

Fig. 2. High-throughput ribozyme self-cleavage assay by sequencing. Ribozyme mutants are transcribed *in vitro* from DNA templates as a mixture. The ribozyme mutants are then converted to dsDNA templates for high-throughput sequencing via reverse transcription and PCR during which adapter and barcode sequences are attached. Sequencing allows the researcher to count the numbers of cleaved and uncleaved reads for each mutant in the library.

interest are counted. Activity of each mutant can be calculated as fraction cleaved (FC) as follows:

$$FC = N_{clv}/(N_{clv} + N_{unc})$$

The number of mutants that can be analyzed is limited by the number of sequencing reads. Assuming a conservative average of 500 reads per mutant ($N_{clv} + N_{unc}$), a typical MiSeq output of 2×10^7 reads are sufficient for 4×10^4 mutants. Using a current state-of-the-art sequencer (e.g. NovaSeq 6000) that can generate up to 10^{10} reads per experiment, as many as 2×10^7 mutants should be amenable to quantitative analysis.

3.1. Mutational analysis of ribozymes

An important distinction of this sequencing-based analysis from the mutate-select-and-sequence strategy is the lack of selection step which can be time consuming and is intrinsically a biased process. Furthermore, it provides quantitative activity (FC) for every mutant of interest in the library ($> 10^4$) comparable to the results that would be generated by biochemical assays of individual mutants (e.g. by gel electrophoresis). The number of mutants that can be analyzed ($> 10^4$) is also sufficient to cover all single and double mutants of a small ribozyme. This was demonstrated by analyzing all single and double mutants (10,296 mutants) within a 48-nt stretch of a small self-cleaving ribozyme (Osa-1–4 twister ribozyme) [17]. The exhaustive and quantitative mutational analysis provided a number of interesting observations. For example, while there are 10 bases that are phylogenetically conserved in 97% or more of the twister ribozymes, only two of them were found to be invariable for retaining residual activity. Additionally, global mutational analysis of single and double mutants revealed that some structural elements were more robust against mutations than others. The exhaustive mutational analysis data allowed another group to evaluate the extent of epistatic interactions of mutations and it was concluded that negative epistasis dominate in this ribozyme and other small noncoding RNAs [18].

The results of the mutational analysis, however, represent "endpoint" ribozyme assays, and do not provide detailed kinetic constants for each mutant. In theory, the ribozyme library can be reacted and sampled at multiple time points to acquire kinetic data. While it has not yet been applied to ribozymes, we recently reported parallel kinetic analysis of a self-cleaving deoxyribozyme based on the method. In this work, rate constants of 533 active mutants were obtained by fitting the time course data to a first-order rate law. The rate constants were then used to calculate activation energies using the Arrhenius equation [19]. This further allowed us to quantitatively assess the epistatic nature of 105 double mutants based on the activation energies of the corresponding single mutants.

3.2. Engineering ribozymes

Mutate-and-sequence approach was also used to engineer ribozymes for synthetic biology applications. An aptazyme is a ribozyme fused with an RNA aptamer that can specifically bind to a target ligand (e.g. small molecule or protein). It has been shown that insertion of an aptazyme in the 3′ UTR of an mRNA enables chemical regulation of gene expression by the aptamer ligand in eukaryotic cells [20]. Self-cleavage of an aptazyme in the 3′ UTR detaches the poly(A) sequence resulting in mRNA destabilization and translation repression. Aptazymes are typically constructed by inserting an aptamer in a structurally essential stem loop of a ribozyme via a "communication module" that couples ligand binding and ribozyme activity either positively or negatively. Some communication modules have been designed rationally or through trial-and-error, as well as through various screening or selection platforms. Rational or iterative design and test cycles have yielded functional aptazymes, but it is a time-consuming and laborious process, success is not guaranteed, and the sequence space that can be experimentally tested is very limited.

The majority of the aptazyme engineering efforts have relied on some forms of screening or selection from a large pool of aptazyme candidates with randomized communication module. A variety of selection and screening methods exist for discovery of genetic switches in bacteria and yeast, some of which has been used to screen functional aptazymes in these organisms [21]. While these methods allow sampling of a larger sequence space, they are laborious and time-consuming to implement, often requiring several rounds of screening or selection. Moreover, sequences are typically obtained only for very few "hits", and vast majority of the mutants generated for screening or selection are simply discarded.

We applied the mutate-and-sequence approach described above to assay 512 aptazyme mutants in which the nucleotides connecting the guanine aptamer and an HDV-like ribozyme were randomized [22]. The mutants were *in vitro* transcribed from a mixture of DNA templates with or without guanine, and then converted to a sequencing library. "Barcode" sequences that distinguish the reaction conditions (with or without guanine) were included in the reverse transcription primers. This allowed us to calculate FC values of all mutants in the presence and absence of guanine, regardless of their performance as switches. Not only could best switch candidates be identified from the results, performance of many of their mutants could also be accessed without additional experiments. For example, we could quickly confirm that stabilizing the stem region connecting the aptamer and the ribozyme generally increases the ribozyme activity (regardless of presence or absence of guanine), and destabilizing the stem region generally renders the ribozyme less active (even in the presence of guanine). Such analysis facilitates researchers to generate mechanistic hypotheses of aptazymes. The guanine aptazymes identified were then shown to function as riboswitches in mammalian cells.

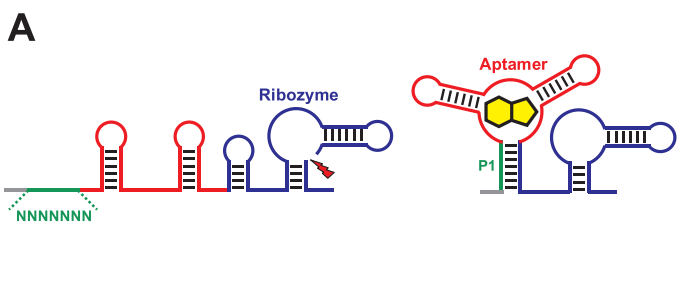

Fig. 3. Ribozyme libraries characterized by sequencing. (A) Tandem aptazyme architecture. Aptamer (red) and the pistol ribozyme (blue) structures are exclusive because formation of P1 is essential for the aptamer structure but it interferes with the ribozyme fold. Optimal P1 stability allows switching between these two structures in the presence and absence of the aptamer ligand [23]. (B) Stability of the P4 stem loop in an HDV-like ribozyme was varied by randomizing 6 bases [24]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In another example, we devised a tandem aptazyme architecture in which an aptamer sequence is fused directly upstream (5′ end) of a ribozyme so that the two structures are formed exclusively (Fig. 3A). It was envisioned that the strength of the P1 stem of the aptamer plays a key role in the performance of the aptazyme. If P1 is too strong, ribozyme would be inactive even without the aptamer ligand. On the other hand, if P1 is too unstable, aptazyme would be active even in the presence of the aptamer ligand. Therefore, it was anticipated that when the stability of P1 stability is optimally adjusted, the ribozyme would be active without the ligand but would be inactive in its presence. This system was implemented by fusing the guanine aptamer with a pistol ribozyme, and randomizing seven bases upstream of the aptamer that could potentially constitute the P1 stem, resulting in 16,384 aptazyme mutants [23]. Analysis of the results allowed us to extract specific sequence requirements for aptazyme function. In another study, three base pairs in the P4 stem adjacent to the catalytic site of an HDV-like ribozyme was exhaustively mutated and analyzed to understand how the stem stability affects ribozyme activity (Fig. 3B) [24]. A number of ribozyme mutants with varying activities were then inserted into the 3′ UTR of a reporter gene mRNA to fine tune gene expression level.

Finally, the mutate-and-sequence approach was implemented in mammalian cells. It has been pointed out that ribozyme and aptazyme activities are often context dependent, for example, where the ribozyme is inserted, or the cell type in which the ribozyme is used [25]. Therefore, it is desirable to perform high-throughput assay in the cells in which the ribozyme is intended to be used. We observed that the pistol ribozyme we used in a previous study did not self-cleave efficiently in mammalian cells even though it was highly active *in vitro* [23]. Therefore, we decided to assay 376 natural pistol ribozyme variants that have been computationally identified by the Breaker group [26] for activity in mammalian cells. Additionally, we designed 2625 artificial pistol ribozyme mutants by recombining structural elements (stems, linker, pseudoknot) of naturally occurring ribozymes *in silico*. Total of 3001 pistol ribozyme sequences were synthesized by on-chip parallel DNA synthesis and fused to U6 promoter by PCR. The linear ribozyme transcription template was tranfected into HEK 293 cells, and total RNA was extracted after 6 h. The total RNA was separated by gel electrophoresis, and the regions that correspond to cleaved and uncleaved ribozymes were isolated and sequenced. This allowed us to identify the first pistol ribozymes demonstrated to be active in mammalian cells [27].

An alternative strategy for assaying and engineering ribozyme activity in living cells was reported by the Smolke group. Townshend et al. constructed aptazyme libraries in *Saccharomyces cerevisiae* by inserting aptazyme variants in the 3′ UTR of EGFP mRNA [28]. The cells were then sorted in bins according to their EGFP expression level. By analyzing the frequencies of each variant in the sorted populations by high-throughput sequencing, they were able to identify intracellularly functional aptazymes which were subsequently validated individually. This sort-seq approach may allow rapid and direct identification of aptazymes that are functional in living cells.

3.3. Experimental considerations

The mutate-and-sequence strategy is still evolving and there are several important experimental or technical points to be considered for successful implementation. Ribozyme library can be constructed by several methods. If the desired mutations are localized such as in the case of aptazyme engineering, degenerate oligonucleotides can be synthesized with randomized bases. It is highly recommended to use “hand-mixed” bases for the randomized positions because standard “machine-mixed” bases often result in highly biased base compositions. If the desired mutations are spread over a long stretch of sequence as in the case of mutational analysis of a ribozyme, doped oligonucleotide synthesis can be used. This requires careful adjustment of the doping ratio of each nucleotide to maximize the representation of the desired set of mutants. Alternatively, on-chip parallel synthesis of up to $\sim 10^5$ sequences offers a more flexible option to synthesize arbitrary sequences as a mixture. Unlike doped oligonucleotides, on-chip synthesis allows each mutant to be represented approximately equally. However, on-chip synthesis is costly and suffers high synthetic errors. Another important limitation is that all mutations must reside in the same fragment of the cleaved products.

High-throughput sequencing requires the sequencing template to be flanked by adapter sequences for cluster amplification and for binding of sequencing primers. The first step in converting the ribozyme reaction products to sequencing templates is reverse transcription. The reverse transcription primer, therefore, must bind to a constant 3′ sequence of both cleaved and uncleaved ribozymes which also must be downstream of any mutations that must be sequenced. The 5′ end of the primer that does not hybridize with the ribozyme can contain an adapter sequence as well as a barcode sequence to encode reaction conditions, for example, the presence or absence of a ligand. Alternatively, if the cleaved and uncleaved ribozyme fragments are separated (e.g. by gel electrophoresis) prior to reverse transcription, the primer can contain a barcode sequence that distinguishes whether the fragment was cleaved or not. There are several methods to attach another adapter (and a barcode) sequence to the 3′ end of the newly generated cDNAs. In the earlier work, splint oligonucleotide mediated ligation was performed [22]. However, this requires careful optimization of stoichiometry and reaction conditions. It is also not applicable to systems where the ligation junction contains mutations. Another possibility is to exploit the template-switching activity of some reverse transcriptases which allows attachment of an adapter sequence during the reverse transcription reaction [17]. Again, reaction conditions must be carefully optimized and the products need to be purified after reaction. If there is sufficient length of constant sequence at the 3′ end of the cDNAs, one can simply use PCR to both amplify the library and attach the remaining adapter sequence. Furthermore, purification and PCR steps must be performed with care to minimize introduction of biases that result in altered quantities of cleaved vs uncleaved fragments. Overall, sequencing library construction strategy must be carefully planned according to the constraints (e.g. relative positions of cleavage site and mutations, positions of constant and variable sequences) of each experiment.

After sequencing, the researcher can analyze the data by filtering for quality, removing adapter sequences, sorting by barcode sequences, and enumerating cleaved and uncleaved reads of every mutant. Some of the data processing can be performed using existing scripts and software such as FASTX-Toolkit for quality filtering and barcode splitting. However, as ribozyme sequence and library design vary greatly from one experiment to another, sorting and counting of ribozyme reaction products are most conveniently executed using custom scripts such as those written in Python or Perl. The fraction cleaved values obtained should be validated by conventional biochemical assay. Several mutants with varying catalytic activities should be cloned and individually assayed by gel electrophoresis under the same condition as in the library preparation. Ribozyme activities of the mutants based on conventional assay should be correlated with the fraction cleaved values calculated from sequencing.

The reliability of activity data of individual ribozymes based on sequencing depends on the number of sequencing reads which vary statistically among mutants. Empirically, we have used a minimum of ~200 reads per mutant as a guide to observe reasonable correlation with gel analysis. Similarly, if detection limit and precision are critical, sequence-based activity should be empirically checked against gel analysis because errors or biases introduced during library preparation and sequencing can become significant. In the future, more rigorous statistical analysis may be desirable to assist interpretation of the mutate-and-sequence results.

4. Conclusion

High-throughput sequencing has had a profound impact on many fields in life sciences. The technology is also useful for ribozyme research because the genotype and the phenotype of ribozymes are encoded in the same molecule, the typical lengths of ribozymes are short, and ribozymes can be easily converted to sequencing templates. Mutate-select-and-sequence strategies allow more comprehensive coverage of the large sequence space represented by pre- and post-selection populations of ribozyme mutants, from which useful sequence-function relationship knowledge can be extracted. Mutate-and-sequence approach, on the other hand, offers more quantitative information on the mutational landscape of ribozymes. Future advances in high-throughput sequencing technologies such as direct single molecule RNA sequencing should bring additional opportunities to study and engineer ribozymes.

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