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***In vitro* Selection of *glmS* Ribozymes**

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Summary

Riboswitches modulate gene expression in eubacteria and eukaryotes in response to changing concentrations of small molecule metabolites. In most examples studied to date, riboswitches achieve both metabolite sensing and gene control functions without the obligate involvement of protein factors. These findings validate the hypothesis that RNA molecules could be engineered to function as designer gene control elements that sense and respond to different ligands. We believe that reverse engineering natural riboswitches could provide an intellectual foundation for those who wish to build synthetic riboswitches. Also, natural riboswitches might serve as starting points for efforts to change ligand specificity or gene control function through mutation and selection *in vitro*. In this chapter, we describe how *in vitro* selection can be used to create variant *glmS* ribozymes. Additionally, we discuss how these techniques can be extended to other riboswitch classes.

Keywords

allosteric ribozyme; aptamer; hammerhead ribozyme; *in vitro* selection; riboswitch

1. Introduction

Riboswitches are metabolite-binding RNA elements that are commonly located within the noncoding regions of mRNAs where they regulate the expression of downstream genes via directly binding to small molecule metabolites (1–3). Riboswitch classes are distinguished by their natural aptamer domains (4) which are responsible for selectively sensing a variety of natural ligands, including key metabolites including coenzymes, amino acids, and nucleobases and their derivatives. Each riboswitch aptamer typically is associated with an expression platform (1,5) that transduces a ligand binding event into a change in gene expression. In many instances, natural riboswitches function as allosteric RNAs because ligand binding at one site alters folding of the mRNA at distal sites to control gene expression.

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One riboswitch class that has several remarkable characteristics compared to most riboswitches is the *glmS* ribozyme motif (6,7) (Fig. 1A). To date, all *glmS* ribozymes have been discovered in bacterial mRNAs that encode L-glutamine:fructose 6-phosphate amidotransferase. This biosynthetic enzyme produces glucosamine-6-phosphate (GlcN6P), which is a precursor compound used in the production of bacterial cell walls. GlcN6P is selectively recognized by *glmS* ribozymes from *Bacillus subtilis* (6) and *Bacillus cereus* (8,9), and GlcN6P binding results in efficient self-cleavage of *glmS* ribozymes. Since ligand binding induces *glmS* ribozyme self-cleavage, *glmS* ribozymes are ideally suited for use with *in vitro* selection methods that rely on the separation of functional from inactive RNAs via size purification.

We have used the methods described below to assess whether *glmS* ribozyme variants could be found that either changed or broadened their ligand specificity, which may yield ribozymes that trigger gene control using analogs of GlcN6P (10). Numerous variant ribozymes were isolated that retained function with the original ligand. However none were identified that had altered ligand specificity. Most likely, this was due to the fact that GlcN6P is not a simple allosteric effector that induces cleavage by changing the folding pattern of the RNA. Rather, GlcN6P serves as a cofactor used at the active site of the ribozyme most likely to protonate the 5'-oxyanion leaving group during RNA cleavage (11–13). Regardless, methods similar to those described here may be useful to select for true allosteric self-cleaving ribozymes that can be used for gene regulation applications.

2. General Selection Strategy

In vitro selection was initiated (10) by using a population of variant ribozymes that carry 122 mutagenized nucleotides of a total of 142 nucleotides that comprise the *glmS* ribozyme from *B. cereus* (Fig. 1B and Fig. 2). The initial population for generation zero (G0) was generated by reverse transcription extension of two synthetic DNAs synthesized with a degeneracy of 0.09 per mutagenized position (14). Transcription of approximately 200 pmol (1.2×10^{14} molecules) of the resulting DNAs yielded a population of RNAs that provide complete coverage of all 6-error mutants. Full-length precursor RNAs were purified by denaturing (8 M urea) 6% PAGE, extracted from the gel, concentrated by precipitation with ethanol, and resuspended in selection buffer.

Self-cleavage reactions were initiated by the addition of the effector compounds, and the cleavage reaction incubations were terminated by adding an equal volume of 2× denaturing gel-loading buffer. The mutagenized 3' cleavage products were purified by denaturing 6% PAGE and full-length RNAs were generated by reverse transcription and polymerase chain reaction (RT-PCR). The PCR products from this G0 round of *in vitro* selection were used to initiate the next round (G1) of selective-amplification by repeating the process as described.

3. Materials

3.1. Construction of the Starting Pool

1. Appropriate chemically synthesized oligonucleotides that encode the mutagenized population (see Note 1).

2. 5× reverse transcription buffer: 250 mM Tris (pH 8.3 at 23°C), 375 mM KCl, 15 mM MgCl₂
3. 100 mM dithiothreitol (DTT).
4. 10× deoxynucleoside 5'-triphosphate (dNTP) mix: 2 mM each of deoxyguanosine 5'-triphosphate (dGTP), deoxyadenosine 5'-triphosphate (dATP), deoxythymidine 5'-triphosphate (dTTP) and deoxycytidine 5'-triphosphate (dCTP) (*see* Note 2).
5. Reverse transcriptase (e.g., SuperScript II, Invitrogen).
6. 10× transcription buffer: 500 mM HEPES (pH 7.5 at 23°C), 150 mM MgCl₂, 20 mM spermidine and 50 mM DTT (*see* Note 3).
7. 3 M sodium acetate (pH 5.2).
8. 100% ethanol.
9. 5× nucleoside 5'-triphosphate (NTP) mix: 12.5 mM each of guanosine 5'-triphosphate (GTP), adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP) and cytidine 5'-triphosphate (CTP).
10. T7 RNA polymerase (T7 RNAP).
11. [α -³²P] UTP and appropriate equipment for handling ³²P.
12. Crush/soak buffer: 200 mM NaCl, 10 mM HEPES (pH 7.5 at 23°C) and 1 mM ethylenediaminetetraacetate (EDTA) (*see* Note 3).
13. 2× denaturing gel-loading buffer: 32% (w/v) sucrose, 0.16% (w/v) sodium dodecyl sulfate, 0.08% (w/v) bromphenol blue, 0.08% (w/v) xylene cyanol, 14.5 M urea, 144 mM Tris-HCl, 144 mM borate and 200 mM EDTA.
14. Reagents and apparatus for denaturing PAGE.

3.2. Positive Selection

1. Appropriate chemically synthesized oligonucleotides for the amplification of recovered sequences (*see* Note 1).
2. 2× *glmS* assay buffer: 100 mM HEPES (pH 7.5), 20 mM MgCl₂ and 400 mM KCl (*see* Note 3).
3. 10× effector solution.

¹For each selection a total of 4 oligonucleotides should be synthesized. Two of the oligomers should encode the population of mutagenized RNAs. The 5' oligomer (primer 1) should encode a T7 promoter and overlap the 3' oligomer (primer 2) by 15 to 20 nucleotides. Two oligomers should be synthesized for use in RT-PCR. The 5' oligomer (primer 3) should include approximately 15 nucleotides downstream of the cleave site. The 3' oligomer (primer 4) should include nucleotides complementary to the last 15 to 20 nucleotides of the RNA construct. The DNAs should be purified by denaturing PAGE or high-performance liquid chromatography (HPLC) to ensure length homogeneity.

²The optimum concentration of dNTPs in a reaction containing SSII RT is 0.5 mM. However, we have observed that SSII RT is capable of producing full-length products in the presence of 0.2 mM dNTPs. This allows the same dNTP mix to be used in reactions containing SSII RT and *Taq* DNA Polymerase.

³All buffers that *glmS* ribozymes are exposed to before or during the *in vitro* selection reaction should not contain tris(hydroxymethyl)aminomethane (Tris) because this buffer is known to activate the ribozyme (8,9).

4. 100% ethanol.
5. 3 M sodium acetate (pH 5.2).
6. Crush/soak buffer: *see subheading 3.1., item 12.*
7. 2× denaturing gel-loading buffer: *see subheading 3.1., item 13.*
8. Reagents and apparatus for denaturing PAGE.
9. X-ray film and reagents for developing the X-ray film.
10. PhosphorImager cassettes and PhosphorImager (e.g. Storm PhosphorImager, GE HealthCare).
11. Appropriate equipment for handling ³²P.

3.3. Reverse Transcription and PCR Amplification

1. Appropriate chemically synthesized oligonucleotides for the amplification of recovered sequences (*see Note 1*).
2. 5× reverse transcription buffer: *see subheading 3.1., item 2.*
3. 100 mM DTT.
4. 10× deoxynucleoside 5′ triphosphate (dNTP) mix: *see subheading 3.1., item 4.*
5. Reverse transcriptase (e.g., SuperScript II, Invitrogen).
6. 10× PCR Buffer: 100 mM Tris-HCl (pH 8.3 at 23°C), 15 mM MgCl₂, 400 mM KCl and 0.1% gelatin.
7. *Taq* DNA polymerase.
8. 100% ethanol.
9. 3 M sodium acetate (pH 5.2).
10. Reagents and apparatus for denaturing PAGE.
11. Reagents and apparatus for agarose gel electrophoresis.
12. Appropriate equipment for handling ³²P.

3.4. Analysis of the *in vitro* Selection Products

1. Reagents for cloning and sequencing of PCR products (e.g. TA cloning kit™, Invitrogen).

4. Methods

4.1. Construction of the Starting Pool

1. Use standard solid phase methods to chemically synthesize the appropriate DNA oligomers using a defined mixture of phosphoramidites for the randomized region (*see Note 1*).

2. To prepare the double-stranded DNA template for transcription, incubate 200 pmol each of the two oligomers that encode the G0 population in 25 mM Tris-HCl (pH 8.3 at 23°C), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 200 μM of the four dNTPs and 8 U/μL reverse transcriptase. The mixture may be heated to 95°C for 1 min and cooled to room temperature to allow the oligonucleotides to anneal before adding the reverse transcriptase (*see* Note 4).
3. Incubate at 37 to 42°C for 2 hours.
4. Run a small aliquot out on a 2% agarose gel to confirm that double-stranded DNA was synthesized and is the correct size.
5. Precipitate the double-stranded DNA by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold ethanol. Incubate the mixture at –20°C for 20 min and pellet by centrifugation at a maximum of 10,000g for 20 min.
6. Resuspend the DNA to yield a 200 μL transcription reaction using 50 mM HEPES (pH 7.5 at 23°C), 15 mM MgCl₂, 2 mM spermidine, 5 mM DTT, 2.5 mM of the four NTPs, 20 μCi of [α-³²P] UTP and 10 to 40 U/μL T7 RNA polymerase.
7. Incubate at 37°C for 1 to 2 hours (*see* Note 5).
8. To concentrate the RNA, precipitate with ethanol and centrifuge as in **step 5**.
9. Resuspend the RNA pellet in sterile deionized water and add an equal volume of 2× denaturing gel-loading buffer.
10. Separate full-length precursor RNAs from 3′ cleavage products, premature transcription products and unincorporated [α-³²P] UTP by denaturing 6% PAGE. (*see* Note 6).
11. Visualize the RNA product bands by UV shadowing, excise the gel band containing the full-length RNAs, and cut the excised band into ~1 mm cubes.
12. Elute the RNA from the diced gel slices overnight at 4°C using crush-soak buffer.
13. Transfer the solution containing the eluted RNAs into a fresh tube.
14. Concentrate the sample by precipitation with ethanol and centrifuge as in **step 5**.
15. Resuspend the RNA in sterile deionized water.

⁴The decision to begin the selection with 200 pmol of variant RNAs balances the need for a highly diverse sequence population with the ability to efficiently perform laboratory experiments. Increasing the diversity of the starting population is always advantageous because more sequence space is being searched. However, we do not recommend scaling up the initial population to greater than 2 nmol because populations larger than this are not easily manipulated. Conversely, we do not recommend reducing the initial population to amounts lower than 20 pmol because manipulation does not become easier, but the decreased sequence diversity of the G0 population could impede the selection.

⁵A white precipitate may form during the course of *in vitro* transcription, resulting from the formation of an insoluble complex between Mg²⁺ and inorganic pyrophosphate. Typically, the formation of the white precipitate indicates that a large amount of RNA has been produced. Inorganic pyrophosphatase can be added to the *in vitro* transcription reaction at a final concentration of 0.1 U/μL to prevent precipitation.

⁶*In vitro* transcription steps carried out in the absence of ligands and antisense oligonucleotides can serve as a negative selection step, wherein RNAs that cleave in the absence of ligands are removed from the population during gel purification of the full-length RNAs that remain.

16. Determine the concentration of RNA by measuring the extent of incorporation of [α - 32 P] UTP (*see* Note 7).

4.2. Positive selection

1. Incubate half of the RNAs (minimum of 200 pmol) in the presence of 50 mM HEPES (pH 7.5 at 23°C), 10 mM MgCl₂ and 200 mM KCl. This reaction contains no added effectors and serves as the negative control reaction used to monitor the extent of cleavage without effector. Incubate the remaining RNAs (minimum 200 pmol) in the presence of 50 mM HEPES (pH 7.5 at 23°C), 10 mM MgCl₂, 200 mM KCl, and effectors. This reaction is the positive selection reaction. (*see* Note 8 and Note 9). Both reactions are incubated for 30 min at 37°C (*see* Note 10).
2. To concentrate the RNAs from the reactions, precipitate with ethanol and centrifuge as in **Subheading 4.1., step 5**.
3. Resuspend the RNAs in sterile deionized water and combine with an equal volume of 2× denaturing gel-loading buffer.
4. Separate cleaved RNAs from full-length RNAs by denaturing 6% PAGE. A marker lane should be included (e.g. wild-type RNAs allowed to self-cleave to 50% completion) as a marker for the full-length and cleaved RNAs from the population. Leave at least one lane between the control lane (no effectors), cleavage marker and positive selection lanes to avoid cross contamination (*see* Note 11).
5. Image the resulting gel using a phosphorimager and calculate the fraction of the RNAs that are cleaved (*see* Note 12).

⁷Typically, *in vitro* transcription reactions produce approximately 10 to 20 copies of RNA for each DNA. However, we have determined that *in vitro* transcription reactions of several *glmS* ribozyme constructs produce only 1 to 2 copies of each sequence. Therefore, to insure that there is no loss of sequence diversity during *in vitro* transcription, the researcher should confirm that the amount of RNA produced is greater than the amount of template DNA. Sometimes the number of copies of RNAs can be increased by slightly changing the length of the DNA template or by adding 0.1 U/ μ L inorganic pyrophosphatase to the transcription reaction.

⁸The order of addition of the components of the positive selection is critical. First, the deionized water and assay buffer are combined. Then the population of RNAs are added to the reaction, and the effectors are added last. This order of addition allows the RNAs to pre-fold in the presence of Mg²⁺ before the effectors are added to the reaction.

⁹The concentration of possible effectors in the positive selection reaction were chosen based on the known properties of the parental RNA. It was known that the apparent K_D of GlcN6P and glucosamine-6-sulfate (two known *glmS* ribozyme effectors) are approximately 200 μ M and 20 mM, respectively, assuming that the reduced activity of glucosamine-6-sulfate is entirely due to its poorer affinity (6). Furthermore, nonspecific interactions between RNA and some effectors can be observed when the concentration of the effector is above 1 mM. Therefore, we chose 200 μ M as a starting point for all the effector candidates.

¹⁰The incubation time for the positive selection reaction must be chosen to favor effector-dependent self-cleaving RNAs relative to RNAs that display increased rate constants for self-cleavage in the absence of effector. Typically, during the early rounds of selection the incubation time is long (30 min) to favor the recovery of the maximal number of effector-dependent self-cleaving RNAs. During the later rounds of selection, the duration of the incubation time is drastically reduced (~1 min) over several rounds of selection. This change favors the isolation of the most efficient effector-dependent ribozymes and should further reduce the number of RNAs selected that cleave without requiring effector binding.

¹¹It is possible for the sizes of the RNAs undergoing selective-amplification to change as the rounds of *in vitro* selection progress. Therefore, it is often useful to include a marker on the gel to insure that the size of the full length and 3' cleavage products are the correct lengths.

¹²The fraction RNAs cleaved in the positive selection reaction should be approximately the same as the negative control during the initial rounds of selection, because inactive variants and variants with wild-type-like activity dominate the population. If the fraction of RNAs cleaved in the positive selection reactions is reduced compared to the negative control reaction, it could be an indication that one of the effectors within the cocktail is inhibiting self-cleavage. Conversely, if there is significantly more cleavage observed in the positive selection reaction compared to the negative control, before effector-dependent RNAs have been enriched, one or more of the

6. Use an autoradiogram of the gel to precisely locate the 3' cleavage products from the positive selection lane, excise the gel band containing the cleaved RNAs and cut the excised band into ~1 mm cubes (*see* Note 13).
7. Elute the RNAs as in subheading 4.1., step 12.
8. Transfer the solution containing the eluted material into a fresh tube.
9. To concentrate the sample, precipitate with ethanol and centrifuge as in subheading 4.1., step 5 (*see* Note 14).
10. Resuspend the cleaved RNAs in sterile deionized water.

4.3 Reverse Transcription and PCR Amplification

1. Add 50 pmol of the 3' primer (primer 4) to the resuspended RNAs (unless it has been added as a carrier as in subheading 4.2., step 10; *see* Note 14).
2. Reverse transcribe the recovered RNAs by mixing the RNAs and primer 4 with 25 mM Tris-HCl (pH 8.3 at 23°C), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 200 μM of the four dNTPs and 8 U/μL reverse transcriptase (e.g., Superscript II, Invitrogen). The mixture may be heated to 95°C for 1 min and cooled to room temperature to allow the oligonucleotides to anneal before adding the reverse transcriptase.
3. Incubate at 37 to 42°C for 2 hours.
4. PCR amplify full-length double stranded DNAs by adding 5 to 10 μL of the reverse transcription reaction and 20 to 50 pmol each of primers 3 and 4 to a 100 μL PCR reaction containing 200 μM each of the four dNTPs, 10 mM Tris-HCl (pH 8.3 at 23°C), 1.5 mM MgCl₂, 40 mM KCl, 0.01% gelatin and 1 U/μL of *Taq* DNA polymerase (*see* Note 15 and Note 16).
5. Amplify using standard PCR cycling parameters. Segment 1: 94°C for 3 min; Segment 2: 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec; Segment 3: 72°C for 5 min (*see* Note 17).

effectors could be nonspecifically enhancing enzyme function. In either case, such an effector will interfere with the isolation of the desired RNAs and should be removed from the effector cocktail and the selection restarted.

¹³The amount of ribozyme cleavage product present in early RNA populations may be sufficient to allow detection on an autoradiogram after a brief exposure. However, if an overnight exposure is required, the film cassette should be stored at 4°C to reduce the rate of diffusion of the RNAs within the gel. If an overnight exposure does not permit detection of the 3' cleavage product, the size marker can be used to infer the location of these products.

¹⁴Since the presence of efficient effector-dependent ribozymes in the RNA populations of the early selection rounds, it is important to maximize the recovery of the 3' cleavage product from the positive selection reaction. Sometimes the amount of 3' cleavage product formed during the positive selection is too low for detection, and there is a high risk of losing some (or all) of the enriched molecules during the ethanol precipitation step. To improve the yield of RNAs recovered during the early rounds of selection it is recommended that additional material be added as a "carrier" to aid precipitation by ethanol. The preferred carrier is the 3' PCR primer (primer 4; 50 pmol) because it is subsequently required in the reverse transcription step. However, other molecules such as glycogen (20 μg) or linear acrylamide (5 μg) can be used as well.

¹⁵During the early rounds of selection, it is recommended that most (or all) of the cDNA be amplified. This will increase the probability that even the rarest of the active molecules will be amplified.

¹⁶To avoid the amplification of undesired non-specific PCR products it is suggested that the concentration of KCl in the PCR be optimized. Typically, this can be accomplished by using PCR to amplify a small amount of template DNAs in the presence of a range of KCl (10 to 50 mM). We have found that a final KCl concentration of 40 mM reduces or eliminates the amplification of undesired PCR products.

6. Separate a small aliquot of the PCR reaction via a 2% agarose gel to confirm that the PCR reaction has produced sufficient product to completion and that the DNAs synthesized are of the correct size (*see* Note 18).
7. Use the amplified DNAs as templates to transcribe the RNA pool for the next round of selection, as in **subheading 4.1., step 6**. Save a portion of the PCR product for archiving purposes.

5. Evaluating the Progression of the Selection

Rounds of selection should be performed by iterations of the protocol described above with reduced amounts of RNA (~10 pmol) in the positive selection and negative control reactions. Once the amount of 3' cleavage product within the positive selection reaction (effector-dependent RNAs) are at elevated levels relative to the negative control reaction (*see* Note 19), the selective pressure on the population of RNAs can be increased. This can be achieved by reducing the duration of the positive selection reaction (and subsequently the negative control reaction) or by reducing the concentration of effectors (*see* Note 20). In response to this pressure, the rate of self-cleavage of the positive selection reaction should start to increase because the population is being enriched for efficient effector-dependent self-cleaving RNAs. Although effector-independent self-cleaving RNAs could also survive the positive selection reaction, these RNAs should be selected against because they should also cleave during transcription. When satisfactory effector-dependent activity is exhibited by the population, DNAs encoding representative members of the population can be cloned and sequenced (*see* Note 21). The individual RNAs from these DNA clones can be prepared and assayed to determine the performance characteristics of these molecules. Important functional parameters include fold activation in the presence of the appropriate effector, apparent K_D of the RNA for its effector, rate constant for RNA cleavage, and the extent of RNA cleavage.

¹⁷The number of cycles required for the complete incorporation of all primers in a PCR reaction is dependent on the amount of template DNA. Therefore, it is critical to stop the PCR amplification of the cDNA before undesired higher molecular weight products are formed. The optimal way to determine the number of cycles for complete incorporation of the PCR primers is to analyze aliquots of the PCR reaction withdrawn from a scout PCR as the number of cycles increase.

¹⁸Typically, smaller undesired PCR products are transcribed more efficiently than the larger specific products that encode the desired RNAs. If smaller PCR products are present, it is recommended that the desired PCR products be purified by cutting the appropriate agarose band and recovering the DNA by using crush/soak elution, electro-elution or by using a Qiagen gel extraction kit.

¹⁹If a cocktail of effectors has been used in the positive selection reaction, it is likely that this response is resulting from a single effector or a subset of the effectors and it is highly unlikely that it is resulting from all of the effectors. Assays should be performed on the RNAs with each individual effector to determine which effectors are responsible for promoting the observed increase in activity. In subsequent rounds of selection, the population should be split into two pools. To optimize ribozyme activity for the most active effector, one pool should be subjected to selection only with the effector responsible for the increased activity. The second pool can be subjected to further rounds of selection with a mixture of the remaining effectors. This process can be repeated each time a dominant effector-dependent class of RNAs is identified.

²⁰The duration of the positive selection reaction (and subsequently the negative control) should be gradually decreased over several rounds of selection. If the duration of the positive selection reaction (and subsequently the negative control) is reduced too much, there will be a loss of selective advantage for effector-dependent activity and the round of selection should be repeated using a longer incubation time. This loss of effector-dependent activity might not manifest itself until the subsequent round of selection. If this occurs, both rounds of selection should be repeated. Alternatively, several incubation times can be used for the positive selection reaction (and subsequently the negative control). This will allow for several populations of RNAs to be isolated after each round of selection, decreasing the rounds of selection that need to be repeated. Furthermore, if three or more incubation times are used, the observed rate constant for the positive selection reaction (and subsequently the negative control) can be determined.

²¹It is very convenient to clone the DNAs encoding the RNAs using a TOPO TA cloning kit (Invitrogen). This method does not require restriction enzyme sites at the 5' and 3' ends of the PCR product.

6. Evolving Non Self-Cleaving Riboswitches for Altered Specificity

The methods described herein can be adapted for use with various ribozyme and aptamer constructs. For some applications it might be beneficial to employ *in vitro* selection to modify the function of an aptamer domain of a riboswitch that does not undergo self-cleavage. One way to achieve this is to employ allosteric selection techniques (15–18). This can be accomplished by fusing a population of mutagenized riboswitch aptamer domains to a hammerhead ribozyme (or another self-cleaving ribozyme type) using a communication module (Fig. 3) (*see* Note 22). These RNAs can then be enriched using the above techniques for effector-dependent activation of hammerhead self-cleavage. The resulting engineered riboswitch aptamer domains can be subsequently isolated by disintegration of the aptamer domain from the corresponding allosteric ribozymes (18).

The methods described above have proven to be useful for isolating *glmS* ribozyme variants. However, there are numerous strategies in which effector-independent RNAs can survive an allosteric selection. It worth noting that these effector-independent RNAs can even dominate the population of RNAs despite the constraints imposed by the selection. Therefore, it is critical to implement an appropriate selection strategy to remove these molecules from the population of RNAs.

One common way for effector-independent RNAs to survive a selection is to exhibit slow observed rate constants for self-cleavage. While the total number of effector-independent RNAs will be drastically reduced during the *in vitro* transcription step (*see* Note 4), some will inevitably survive intact. These effector-independent molecules then self-cleave during the positive selection step. Since these effector-independent molecules can be more prevalent in the initial population compared to effector-dependent RNAs, they can dominate a population of RNAs. One way to remove these RNAs from the population is to employ more aggressive negative and positive selection steps (*see* Note 23). The most common way to determine if effector-independent RNAs have overtaken a population is to observe an increase in the amount of RNAs cleaving during the transcription and/or the negative selection step over several rounds of selection.

Another common way an effector-independent RNA can survive an allosteric selection is to partition itself between active and inactive conformation during the negative selection step. The fraction of these molecules that fold into the active conformation can be removed during the negative selection step. However, the fraction of the molecules that do not fold correctly during the negative selection will survive if they correctly fold and cleave during the positive selection step. Altering the incubation times of the negative and positive selection steps will not remove these molecules because these RNAs will always portion into active and inactive

²²Caution has to be used whenever a preexisting communication module is employed to generate an allosteric ribozyme because communication modules are not always functional when placed in new contexts. Therefore, the observed rate constant of the parent allosteric ribozyme (containing the wild-type sequence of the natural aptamer domain) should be determined in the presence and absence of the small molecule metabolite. If the ribozyme does not sufficiently activate in the presence of the small molecule metabolite, a selection should be performed to isolate a communication module that provides sufficient switching characteristics (19).

²³One way to employ more aggressive negative selection is to conduct a conventional negative selection step prior to the positive selection step. During this negative selection step, the population of RNAs is incubated in selection buffer in the absence of effector. The full-length RNAs are then purified and subjected to the positive selection step using a shorter incubation time to favor the isolation of effector-dependent RNAs. See reference 17 for a detailed discussion of negative selection strategies.

conformation. The best way to remove these molecules from the population is to iteratively perform denaturing and refolding treatments during the negative selection reaction. For example, periodic heat spikes (65°C for 1 min) at regular intervals (10 min to 30 min) followed by incubation at room temperature (10 to 30 min) over a 2-hour period can help reduce the selective advantage of these misfolding variants. Even thermal denaturation will lose effectiveness as rounds of selection proceed because some RNAs will emerge that resist denaturation at high temperature. The most effective way to remove these molecules is to perform iterations of negative selection followed by chemical denaturation. Detailed protocols of allosteric selection techniques including these negative selection methods have been previously published (17).

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Fig. 1. Consensus and *Bacillus cereus glmS* ribozymes. (A) Consensus sequence and secondary structure model for *glmS* ribozymes. Nucleotide numbers conform to those for the *B. cereus* RNA (Fig. 1B). Boxed nucleotide is absent from the *B. cereus* sequence, and circles represent the two extra nucleotides present in *B. cereus* and *Bacillus anthracis* ribozymes (Fig. 1B). Nucleotides in black circles are conserved in at least 97% of natural *glmS* ribozyme representatives. N represents any nucleotide identity, where some stretches are variable in length. Nucleotides denoted Y are either C or U, and nucleotides denoted R are

either G or A. Arrowhead identifies the site of ribozyme cleavage. **(B)** The sequence and secondary structure model for the *glmS* ribozyme from *B. cereus* based on comparative sequence analysis (6,7) with revisions based on additional bioinformatics (unpublished data) and x-ray structural data (17,18). Nucleotides 61 and 62 (circled) are found in strains of *B. cereus* and *B. anthracis*, but are typically not present in *glmS* ribozymes from other bacteria (6,9). Nucleotides depicted in gray were mutagenized to a degeneracy level of 0.09 (11). Mutations were not introduced at nucleotide 52 due to restrictions of the method used to generate the DNA templates for transcription of the G0 RNA population. Arrowhead identifies the site of ribozyme cleavage and the boxed nucleotides identify the minimal functional core of the ribozyme (6). Images reproduced from reference 10 with permission.

Fig. 2.

Outline of the basic *glmS* selection scheme. (A) Two synthetic oligonucleotides are extended using SSII RT to generate double stranded DNAs encoding the mutagenized *glmS* ribozymes. (B) The double stranded DNAs encoding the mutagenized *glmS* ribozymes are transcribed using T7 RNA polymerase. (C) The full-length RNAs are purified and the RNAs are subjected to positive selection in the presence of the effectors. (D) The 3' cleavage products carrying the randomized ribozyme domains are gel purified and reverse transcribed. (E) The cDNA is then amplified in a PCR reaction that generates double stranded DNAs encoding the selected *glmS* ribozyme variants.

Fig. 3. Construct design for the *in vitro* selection of allosteric ribozymes. A mutagenized riboswitch aptamer can be grafted onto stem II of the hammerhead ribozyme via a communication module to create a population of RNAs. Allosteric ribozymes that are controlled by ligand binding to the aptamer undergo self-cleavage at the site indicated by an arrowhead.