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Curriculum

Riboflavin Riboswitch Regulation: Hands-On Learning about the Role of RNA Structures in the Control of Gene Expression in Bacteria †

Catherine E. Vrentas[1,*], Jacob J. Adler[2], Adam J. Kleinschmit[3], and Julia Massimelli[4]
[1]*The Engaged Scientist, Ames, IA, 50014;* [2]*Division of Mathematics and Natural Sciences, Brescia University, Owensboro, KY, 42301;* [3]*Department of Biology and Earth Sciences, Adams State University, Alamosa, CO, 81101;* [4]*Department of Molecular Biology and Biochemistry, University of California – Irvine, Irvine, CA, 92697*

American Society for Microbiology (ASM) Curriculum Guidelines highlight the importance of instruction about informational flow in organisms, including regulation of gene expression. However, foundational central dogma concepts and more advanced gene regulatory mechanisms are challenging for undergraduate biology students. To increase student comprehension of these principles, we designed an activity for upper-level biology students centered on construction and analysis of physical models of bacterial riboswitches. Students manipulate an inexpensive bag of supplies (beads, pipe cleaners) to model two conformations of a riboswitch in a bacterial transcript. After initial pilot testing, we implemented the activity in three upper-level classes at one research-intensive and two primarily undergraduate institutions. To assess student perceptions of learning gains, we utilized a pre/post-activity 5-point Likert-type survey instrument to characterize student perceptions of confidence in both their understanding of riboswitches and their ability to apply the central dogma to riboswitches. Median post-test ranks were significantly higher than median pre-test ranks ($p < 0.0001$) when compared by the Wilcoxon signed-rank test ($n = 31$). Next, we assessed post-activity knowledge via use of a rubric to score student responses on exam questions. More than 80% of students could correctly describe and diagram examples of riboswitches; data from initial iterations were used to enhance curriculum materials for subsequent implementations. We conclude that this riboswitch activity leads to both student-reported increases in confidence in the ASM curriculum dimension of gene regulation, including central dogma concepts, and demonstrated student ability to diagram riboswitches, predict outcomes of riboswitches, and connect riboswitches to evolutionary roles.

INTRODUCTION

Understanding the modes by which cells can regulate gene expression is critical in linking how organisms adapt to changes in environmental conditions. Indeed, the American Society for Microbiology's (ASM's) *Recommended Curriculum Guidelines for Undergraduate Microbiology Education* include the key concept: "The regulation of gene expression is influenced by external and internal molecular cues and/or signals" (1). Historically, most textbooks cover the topic of gene regulation focusing primarily on DNA-binding regulatory proteins that affect the interaction of RNA polymerase with the promoter region of the regulated genes. Additionally, many schematics in textbooks oversimplify RNA as a linear, unstructured molecule. However, the direct regulation of riboswitches at the RNA level is also an important example of the regulatory mechanisms controlling information flow in bacteria. In addition, class discussion of riboswitch regulation has the added benefit of reinforcing other supporting concepts in biochemistry, including hydrogen bonding, mRNA secondary structure, and complementary base pairing. Riboswitches constitute an excellent example to introduce the concept of regulation at the RNA level, with numerous associated elements: cis versus trans regulation; the ability of RNA to directly monitor a physiological signal, in the absence of any other cellular factor (such as a regulatory protein or a translating ribosome); and the role of RNA structural shifts in the regulation of gene expression (2).

Riboswitches are sections of the transcribed messenger RNA molecule (usually in the 5′ untranslated region of the mRNA) that can fold into alternative secondary structures in the presence or absence of a ligand that binds to the structure formed by the RNA. A variety of ligands can be sensed by riboswitches, including ions,

*Corresponding author. Mailing address: The Engaged Scientist, 332 Sunflower Drive, Ames, IA, 50014. Phone: 814-883-8581. E-mail: cevrentas@gmail.com.
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nucleic acid precursors, enzyme cofactors, and amino acids (reviewed by 3–7). Riboswitches are composed of two domains, the aptamer and the expression platform (5, 8). The aptamer is the domain that binds to the ligand, while the expression platform contains sequences that directly regulate gene expression. In between these two domains, a switching sequence is usually present; its placement (bound to the aptamer vs. bound to the expression platform) is what dictates the expression outcome of the mRNA. For example, an RNA can fold into conformation "A" (Fig. 1) in the absence of a metabolite binding to the aptamer in a bacterial cell. This "A" conformation allows ribosomes to access the Shine-Dalgarno binding sequence and start codon in the expression platform of the RNA, so that a protein product used to transform a cellular metabolite can be translated. Binding of the converted metabolite to the RNA induces aptamer conformation "B" (Fig. 1), which hides the Shine-Dalgarno sequence and start codon in the expression platform, blocking further translation. Riboswitch regulation is used by many bacteria as well as some archaea and eukaryotes, and regulation can occur at additional levels besides the translation mechanism focused on here, including regulation of transcription attenuation (reviewed by 5–7).

The value of active learning in the science classroom is well established in the literature (9). Research focused on the constructivist teaching approach recommends the use of models to teach advanced science concepts (e.g., 10). Examples of modeling in gene regulation curricula include hands-on models of the *lac* operon for the classroom, such as commercially available kits (#4738400, Ward's Science), as well as activities that make use of electronic simulation software (11). The *lac* operon has also been demonstrated to be effective as a basis for teaching students via model-based reasoning (12, 13). Riboswitch regulation requires visual understanding of folding and binding; therefore, an activity with manipulatives and model-based questioning has the potential to be very effective to teach this concept. Additionally, the use of 3D models provides the opportunity to challenge students to consider the diversity of biochemical structures involved in structure-function relationships in the cell.

Here, we describe a classroom activity that uses 3D materials to introduce and model the concept of riboswitches. The activity uses the flavin mononucleotide (FMN) riboswitch as an example, which regulates FMN biosynthesis and transport proteins (14, 15). The activity incorporates materials that include pipe cleaners, small lettered pony beads, and large colored beads to model riboswitches, which are commonly used to model the structure of RNA (16).

Intended audience and prerequisite student knowledge

The activity is appropriate for mid- to upper-level undergraduate courses in microbiology, molecular biology, or genetics, and could be adapted for graduate-level coursework. Prior to the activity, students should have exposure to the concept of hydrogen bonding and the molecular mechanisms of transcription and translation, with a particular focus on genetic elements associated with translation of coding mRNA.

In microbiology courses, riboswitches constitute an exceptional example of rapid gene regulation and the cross-talk between transcription and translation. Riboswitches also serve as a prime example to explore the secondary structure of RNA, the regulation of gene expression, and novel antibiotic targets. In a foundational biology course, instructors can use riboswitches as a model to explain transcription, translation, and mRNA structure from a

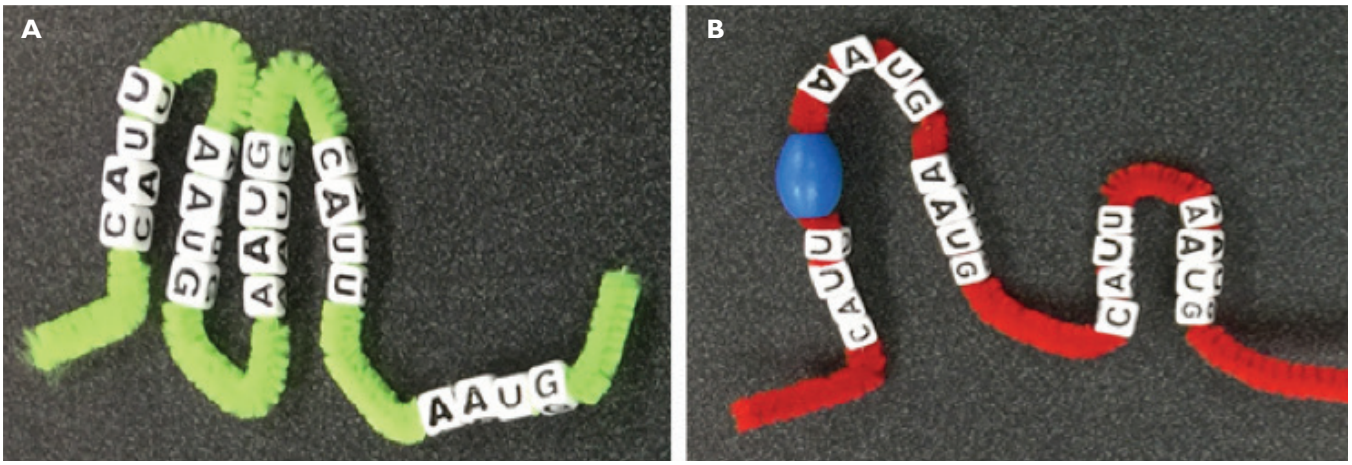

FIGURE 1. Riboswitch structures. Photographed models with the riboswitch in the ON (A) and OFF (B) conformations, respectively. Student groups were asked to assemble two models of the riboswitch using lettered pony beads (A, G, C, and U) to show base pairing of mRNA. The sequence and base pairing instructions were provided in a PowerPoint presentation (Appendix 4) or as a handout (Appendix 6). (A) A base pairing conformation that does not involve the start codon (AUG, right end of green pipe cleaner). The blue bead depicted in (B) represents the ligand binding. The ligand promotes a conformation change in the base pairing in the RNA. In the OFF conformation, the AUG codon is inaccessible due to interactions with other bases as part of a hairpin structure (second hairpin on the red pipe cleaner).

more applied perspective, describing riboswitches as attractive targets for the development of novel antibiotics aimed at halting the metabolism and growth of bacterial pathogens. Riboswitches predominantly control genes essential for bacterial survival, or genes that control the ability of bacteria to succeed at infection (17). Therefore, a drug designed to affect a riboswitch could be a powerful tool for targeting pathogenic bacteria. Using the FMN riboswitch as an example further exploits this point, as there are two well-characterized FMN riboswitch inhibitors (18–21).

The activity presented here can be easily integrated into a larger discussion of the use of regulation of gene expression to allow a cell to adapt to changes in its environment. Most riboswitch RNAs are examples of negative feedback regulation of biosynthetic operons, with the aptamer binding to metabolites that are the end products of biosynthetic pathways to regulate genes that are part of these pathways. Instructors may use riboswitches to discuss anabolic pathways and the conditions under which a particular metabolite would need to be synthesized by the cell.

Learning time

The hands-on activity described below can be presented in a single 50-minute class period or expanded up to a 90-minute class session within a classroom or laboratory-based course.

Learning objectives

At the conclusion of this activity, students should be able to:

1. Describe the structure and functionality of riboswitches
2. Identify at least one type of organism in which riboswitches are utilized
3. Diagram an example of a riboswitch and identify the step in the central dogma at which the diagrammed riboswitch regulates gene expression
4. Identify at least one potential evolutionary advantage of the use of riboswitches for the control of gene expression in bacteria
5. Describe and/or diagram the process by which a riboswitch can control a downstream metabolic activity (e.g., riboswitches that bind to a metabolite to control translation of an enzyme used to synthesize that same metabolite).

Implementation of the more advanced version of the activity, as outlined in Appendix 6, involves the following additional learning objectives:

6. Compare and contrast structure and functionality of riboswitches and other RNA regulatory elements, like attenuators
7. Analyze the structure and mode of action of riboswitches using data from published literature
8. Infer the importance of riboswitches for drug design.

All of these learning objectives can be assessed with the discussion questions provided on handouts and the exam questions. To assess the exercise, we have collected assessment data to validate learning objectives 1 to 5.

PROCEDURE

Materials

Required materials (outlined in Appendix 1) distributed in a plastic bag were sufficient for each group to construct one version of each of the "off" and "on" conformations of the riboswitch (Fig. 1). During our evaluative trials, we implemented the activity with two to four students per group. For a small class, it would be feasible for students to work individually, if desired. For a large course, the main challenge is instructor assembly of the bags of materials. However, an undergraduate teaching assistant or work-study student could perform this task. Alternatively, the activity could be implemented during the laboratory section(s) of such a course, and students could compile their own materials.

Student instructions and sample models

The description below is limited to the hands-on component of the activity; the pre- and post-activities associated with the hands-on component are presented under the teacher instructions below. As described above, students are provided with a bag of materials and a handout with assembly directions (Appendix 2). Students are first tasked with stringing the beads to match the two sequences, representing the riboswitch with and without the bound metabolite. Next, students are provided with the riboswitch conformation handout (Appendix 3) and instructed to recreate the two depicted conformations of the riboswitch along with the instructor. A projection screen is useful to show the final structures of these small riboswitch manipulatives to the class (Fig. 1). Students are instructed to make two separate conformations, using the green pipe cleaner for the ON conformation (Fig. 1A; lacking the FMN bead) and the red pipe cleaner for the OFF conformation (containing the FMN bead; Fig. 1B). Through the hands-on folding process, students experience RNAs as highly folded molecules, with hydrogen bonded stem-loops introducing significant 2D structures with regulatory potential. In this highly simplified model, the AUG start codon is accessible to allow for translation in conformation "A," whereas the AUG start codon is inaccessible in conformation "B" due to binding of the metabolite (Fig. 1). Therefore, binding of the metabolite would turn expression of the transcript off; this represents an example of negative feedback inhibition of a

transcript that is used in the synthesis of a macromolecule in the cell by a downstream product.

Faculty instructions

As a part of our development of this activity, we have prepared a series of PowerPoint slides that can be used to introduce the activity, to provide guidance during the hands-on component, and to introduce questions for post-activity group work (Appendix 4). After completion of the hands-on riboswitch modeling activity, students are asked to answer a series of questions in small groups, followed by large-group sharing of student responses (Appendix 5). To promote equal participation, working groups can split duties, with some students assigned to construct the riboswitch and some students going over the discussion questions and keeping track of the group's answer for each question.

Suggestions for determining student learning

For formative assessment purposes, instructors may collect responses to questions completed by student groups in class (Appendix 5). Alternatively, to wrap up the activity, instructors may opt to have students complete a minute paper, where students individually reflect on and summarize their understanding of riboswitches as well as any points of confusion. At the beginning of the following class session, the instructor may address student questions or address any misconceptions identified as part of the formative assessment analysis.

Safety issues: None

DISCUSSION

Field testing

This activity was initially conceived and implemented as a part of a teaching demonstration for undergraduate students in a genetics course at a public, liberal arts, primarily undergraduate institution (PUI) in the southeastern United States in fall 2014. In the initial version of the activity, the riboswitch models were presented as a part of a larger discussion about the role of gene regulation in modulating the differences in the phenotypes of different tissues. However, we decided that a stronger student connection to practical implications could be generated by focusing the class presentation on the role of the FMN riboswitch in the context of microbial systems, which led to the development of a PowerPoint presentation (Appendix 4).

Use of the FMN riboswitch facilitates the understanding of gene regulation in a practical context by demonstrating the connection between riboflavin (vitamin B2) and riboswitch regulation. Riboflavin is needed as a precursor for flavin coenzymes, such as FMN, which are required for the activity of multiple different enzymatic pathways. Riboflavin is converted into FMN by biosynthetic enzymes in bacteria, and FMN is then further converted to flavin adenine dinucleotide. When FMN levels increase in the cell of a bacterium like *Bacillus subtilis*, production of these biosynthetic enzymes is turned "off" by the binding of FMN to a riboswitch in the RNA of the riboflavin biosynthesis operon. The FMN riboswitch can control both transcription (attenuation via regulation of early transcription termination) and translation (via occlusion of the ribosome binding site). Primary literature articles describing these findings (22, 23) could be assigned as reading for a graduate or advanced upper-level course. Additionally, Vitreschak *et al*. (14) describe the riboflavin biosynthesis operons and RNA regulatory elements across multiple bacterial genomes, with several potentially useful images for instruction.

In this activity, we have used a simplified model to demonstrate one example of a riboswitch regulatory mechanism that targets the control of translation initiation. To further validate the tool, we used the American Society for Microbiology's Biology Scholars network to assemble a group of faculty from three universities to test the activity in upper-level biology-focused classroom settings (Table 1).

The first iteration of the activity was implemented in a required 300-level, single-semester genetics course at a small, private PUI located in the southeastern United States. Students enrolled in this course were junior or senior level and had no previous encounters with riboswitches. Students had recently completed an examination of structures of RNA and pathways for protein production in the course prior to implementation. This course is considered a general education elective and consists of biology majors and nonmajors. The small class size and diverse student abilities in biology made this an excellent location for the initial test of the activity. After an informal evaluation of the activity by the instructor following our first iteration, we made modifications to the activity that were then implemented in the second and third iterations. Specifically, we modified the slide set to add additional background information and details, with a particular focus on highlighting the impact of the two conformations of our model riboswitch on translation.

The second iteration of the physical model riboswitch activity was implemented in a required 400-level, second-semester molecular biology course at a public liberal arts PUI located in the mountain west United States. All participating students were biology majors who had completed a 300-level genetics course, a 400-level microbiology course, and the first of a two-semester 400-level molecular biology course sequence. While participants had previously been exposed to riboswitches, here participants explored details of riboswitches as part of a unit focused on classes of regulatory and non-coding RNA and their biological applications. The riboswitch activity was well received by the molecular biology student participants. Activity presentation slides designed to hook student interest and highlight the importance of dietary riboflavin for biological organisms were newly implemented

TABLE 1.
Classroom setting associated with riboswitch class module implementation.

Institution Type	Course	Course Enrollment	Implementation Iteration
Public Liberal Arts University	300-level Genetics	10	Pilot Test
Private Liberal Arts University	300-level Genetics	9	1[a]
Public Liberal Arts University	400-level Molecular Biology	8	2[a]
Public Research University	400-level Microbial Genetics	19	3[a]

[a]Student perceptions survey and summative assessment data collected

in this iteration. These slides also focused on the riboflavin operon and biosynthesis pathway to provide student participants with a solid understanding of the biochemical components associated with the FMN riboswitch.

The third iteration was implemented in an elective 400-level microbial genetics course at a large public research university in the western coastal United States. All participants were biology or genetics major seniors. Most of these students were exposed to the basic idea of riboswitches in previous microbiology and genetics courses. The activity was taught as part of the class module dealing with bacterial gene regulation, and it was combined with the concept of attenuation. This upper-level course is designed to involve little direct instructor lecturing. Therefore, the instructor used a handout introducing the concepts of attenuation and riboswitch control that included the instructions to assemble RNAs with pipe cleaners and beads, modeling both an attenuator and the FMN riboswitch in all of their configurations. The handout includes instructions to create these models, as well as questions students were instructed to answer in groups after the modeling activity (Appendix 6). The class was two hours long, providing plenty of time for modeling and discussion.

For instructors spending more time on RNA regulation in advanced courses, comparing and contrasting attenuators versus riboswitches using the 3D models can be an excellent exercise to understand the difference between regulation at the early transcriptional termination and translational levels. The exercise also allows for understanding of applications in drug design and can include data analysis from journal articles as demonstrated in the attached handout (Appendix 6).

Potential challenges for the implementation of this activity include material preparation (as addressed above) and diversity in the level of student prior knowledge of central dogma and RNA pairing concepts. To address the latter concern, one option is to conduct a review activity related to these concepts before undertaking the activity, either in class or as a homework assignment.

Evidence of student learning

To assess perceptions of student learning gains in each course setting, we presented students with a set of assessment questions (Appendix 7) immediately before and after the activity. We used a pre/post-activity 5-point Likert-type survey instrument to assess student perceptions of self-confidence in understanding of riboswitches and in application of the central dogma to riboswitches. Pre- and post-survey responses ($n = 31$) were compared using the Wilcoxon signed-rank test. For all questions, median post-test ranks were statistically significantly higher than median pre-test ranks ($p < 0.0001$) (Table 2). We conclude that this activity leads to self-reported student increases in confidence in the ASM curriculum dimension of gene regulation, including understanding of central dogma concepts.

Summative assessment of student learning gains was embedded within course examinations as short-answer questions two to five weeks after the classroom activity. Short-answer examination questions based on the activity's student learning objectives were assessed via a common three-point rubric (Appendix 7). From post-activity student learning gains assessment questions, we determined that more than 80% of students could correctly describe and diagram examples of riboswitches. In the first round of summative evaluation, student scores were comparatively lower, likely attributable to the lower-level (300-level) background of these students compared with upper-division (400-level) iterations. In a second-year iteration of this activity at the same institution, students performed better (class average = 57%) compared with the first year (class average = 24%) but still below scores from other 400-level biology courses. This may indicate that this activity is better suited to upper-level courses.

We did use the data from our initial implementation in an attempt to improve student performance on central dogma-related questions in two subsequent implementations (Table 3). Specifically, we refined and expanded on information about the translation step affected in the riboswitch example provided in this activity, developing slides and images in Appendix 3 and Appendix 4 to address learning objectives relevant to question 1 (Table 4). It is important to note that not all iterations involved students with equivalent pre-activity background knowledge, and that the summative assessments were delivered at different times post-activity. Therefore, average performance from each iteration illustrates the potential range of student achievement that instructors may observe when using our exam questions. Overall, these summative assessment data

TABLE 2.
Results of Wilcoxon signed-rank test for the pre- and post-activity riboswitch survey items listed below[a].

Item	Associated Learning Objective	Survey Item: Strongly agree (5) to Strongly disagree (1)	Pre[b]	Post[b]	*P* value
Describing a Riboswitch	1	I can describe what a riboswitch is.	3	4	< 0.0001
Organisms	2	I know what organisms riboswitches are found in.	3	4	< 0.0001
Central Dogma	3	I know at what point of the central dogma of molecular biology riboswitches function.	3	5	< 0.0001
Diagramming Ability	3	I am confident in my ability to diagram riboswitch regulation right now.	2	4	< 0.0001

[a]The pre- and post-activity median responses (n = 31) from a 5-point Likert-type scale survey instrument were compared using the Wilcoxon signed-rank test.
[b]Represents median answer.

TABLE 3.
Analysis of rubric-scored criteria on students' summative assessment responses pertaining to the riboswitch class module.

Criteria	Associated Learning Objective	Level of Performance[a]		
		Exemplary	Beginner	Unsatisfactory
Interpret the impact of an altered riboswitch structure on gene expression	1, 5	61.1%	13.9%	25%
Remember and recognize the process affected by the altered riboswitch structure	3	75%	0%	25%
Understand and explain what makes riboswitches good antibiotic targets	1, 2, 4	41.7%	25%	33.3%

[a]Scores represent the percentage (%) of total students (all iterations; n = 36) who scored in each rubric category (Exemplary, Beginner, and Unsatisfactory) for each criterion. Full rubric details are provided in Appendix 7.

TABLE 4.
Average score obtained for riboswitch quiz questions after implementation of the riboswitch class module[a].

Iteration	Average % (Mean ± SD)	Number of Students (*N*)
1[a]	24 ± 26	9
2	70 ± 24	8
3	80 ± 33	19

[a]Questions were included on a summative final exam that took place several weeks after the riboswitch class module.

demonstrate student ability to diagram riboswitches, predict outcomes of riboswitches, and connect riboswitches to evolutionary roles.

Possible modifications

To introduce the activity, instructors may choose to start with publisher videos or animations of relevant aspects of gene expression.

For use after implementation of the activity, to provide a basis for follow-up assignments, we have created a series of discussion questions (Appendix 5). These questions can be used for group discussion, post-activity homework assignments, online discussion board questions post-class, and/or for expansion into larger assignments, such as short essays or group or individual projects. For example, students could be asked to create a single slide about one riboswitch they researched and present the overall regulatory mechanism to the rest of the class as an oral presentation extension.

Additionally, for some upper-level courses, additional complexity may be required, depending on the learning goals of the course as well as the students' background. As described above, the upper-level microbial genetics course fell into this category. Therefore, we designed a supplementary activity on attenuation that also uses pipe cleaner models of nucleic acid structures. This activity was pilot tested as a part of the microbial genetics course (Appendix 6).

Finally, there is a wealth of potential extensions for this activity that could integrate other elements beyond gene expression into the learning experience. For example, students can explore the 3D structure of the riboswitch at

an atomic level. The Protein Data Bank's (PDB) "Molecule of the Month" feature on riboswitches within PDB's Educational Portal highlights a purine-binding riboswitch (http://pdb101.rcsb.org/motm/130) (24). Classes could go even a step further to manipulate the riboswitch PDB structure after loading the file on a viewing program like PyMOL, which could serve as the basis for short student oral presentations showing screenshots of structures or be accompanied by an in-class worksheet for a computer lab work session.

CONCLUSION

The riboswitch activity presented here provides a variety of pedagogical options for undergraduate classrooms. Our iterative process of testing and improvement of the materials provided the opportunity to refine the curriculum with the input of multiple experienced instructors. Student feedback indicates that this experience with hands-on manipulation was well-received by students, who collectively perceived gains in their understanding of the complex genetic regulatory concepts associated with this activity. Furthermore, summative assessment questions validated student perceived learning gains. Future implementation and analysis could be used to examine student learning gains related to learning objectives 6 to 8 for advanced courses.

SUPPLEMENTAL MATERIALS

Appendix 1: Required materials for activity implementation
Appendix 2: Student handout—assembly instructions
Appendix 3: Student handout—riboswitch conformations
Appendix 4: Word version of PowerPoint slides for classroom use
Appendix 5: Post-activity challenge questions
Appendix 6: Upper division class handout (includes attenuation materials)
Appendix 7: Assessment questions, rubric, and IRB information

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