

Computational Generation of RNA Nanorings

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Abstract

A variety of designed RNA ring structures (ranging from triangles to hexagonal rings) have been reported in the scientific literature. Designing self-assembling RNA ring structures from structural motifs is, however, a nontrivial problem as there are many combinations of motifs and linking helices. Moreover, most combinations of motifs and linker helices will not lead to ring closure. A solution to this problem was recently published using a “design-by-catalog” approach where motif combinations that lead to rings are precomputed and tabulated. Here we present a web-browser based workflow for creating RNA rings using Galaxy, a web-based platform that can be used for workflow management. An example of how these RNA rings are generated and processed to create a 3D model of the ring is discussed.

Key words RNA, Ring, Closure, Enumeration, Scaffold

1 Introduction

1.1 RNA Nanotechnology

RNA can be designed to self-assemble into a large variety of geometries (such as RNA tiles, hexameric rings, cubes, bundles, and arrays) [1–8] due to its ability to form a variety of base-pairs with at least two hydrogen bonds, in addition to the standard Watson–Crick base pairs [9]. RNA is also able to self-assemble in cellular conditions [10] through interstrand and intrastrand interactions [11]. RNA is not only able to form many shapes, but is also able to perform enzyme-like functions [12–15]. Also, a polyvalent RNA nanostructure can have different functional attachments, such as siRNAs, aptamers, peptides, or fluorescent tags [8, 10, 16]. Due to existing computational prediction capabilities, RNA is relatively conducive to rational design. In terms of therapeutics and medicine, RNA is biocompatible and biodegradable. Due to these properties, RNA is an attractive material to be used in nanotechnology and biomedical applications.

1.2 *Traditional Computational Design Approaches*

Through the use of computational tools [17–19] the process of designing nanostructures can be simplified and accelerated. We highlight two strategies for the computational design of RNA nanostructures: building a scaffold de novo or starting with existing RNA motifs [4, 16, 20, 21]. Here we discuss both of these approaches as well as a new catalog-based approach.

1.2.1 *De Novo Design of RNA Scaffolds*

There are some geometries and properties for which there are no naturally occurring RNA structural motifs. An example of this situation is the in silico designed RNA nanocube [8, 22]. For the case of the RNA cube, we could not identify any three-way junctions in the RNAJunction database that had substantial similarity to a cube corner [23]. Several approaches are possible for the creation of 3D models of such RNA nanostructures. One approach is to design the RNA nanocube with a de novo three-way junction [8]. Another approach is to focus on optimizing the placement of helices and then placing bridging RNA strands de novo [22]. Both methods resulted in 3D models of nanocubes [22].

1.2.2 *Building Block Approach*

Analysis of available RNA tertiary structures has led to the identification of RNA building blocks. These motifs have been compiled into various databases such as the k-turn database [24], SCOR [25], FRABASE [26], Motif Atlas [27], RNA CoSSMos [28], and RNA STRAND [29]. RNAJunction is a database of kissing loops, bulges, and n -way junctions [23]. These databases can be used to identify motifs with geometries of interest and serve as a good starting place for the designer. Note that these compilations of RNA motifs often utilize different conventions of how to define and group structural motifs. These motifs can be linked together with helices or single-stranded regions allowing for a building-block based approach. Given the building blocks, the designer is able to create a variety of shapes.

1.2.3 *Catalog-Based Design*

A new approach is the catalog-based design in the form of the Ring Catalog [30]. Rings are defined as repeated concatenations of one or two motifs, such that the motifs together with their connecting linker helices form a circular RNA complex. As described in reference [23], motifs are RNA structural elements such that each end of all involved RNA strands forms a base pair with another RNA strand end. In this fashion, the structural element possesses terminal base pairs that can be extended with helices. Two or more motifs can be connected with linker helices that extend the terminal base pairs. Most nonbranching concatenations of motifs and linker helices will not correspond to closed ring structures but rather to rods or spirals. The combination of which motifs and linker helices to use in order to obtain ring-closure is most likely not obvious to even the expert designer. A solution to this problem is the complete enumeration of motif combinations and linker helix lengths while

cataloging the subset of structures that are generated in this fashion that are forming closed ring structures. One issue is that iterating over many linker helix lengths and motif combinations may lead to a “combinatorial explosion,” making searching for rings computationally demanding. A subset of motifs was chosen by creating clusters and using representatives of those clusters in the ring search. A cluster was defined as a set of motifs with the same sequence and same NC-IUBMB nomenclature notation [31]. This ensures there are no redundant motifs. Also, the types of motifs chosen were three-way junctions, internal loops/bulges, and kissing loops. By avoiding the “combinatorial explosion,” a trade-off was made in terms of comprehensiveness. NanoTiler, a Java-based multifunctional software that is able to create different RNA-based nanostructures [17], was used to perform the search using its “growscan” command. This used all pairs of motifs and iteratively changed the length of the linking helices. Rings with a ring-closure score below a threshold were stored and then classified based on topology. Ring structure coordinate data is available online at <https://rnajunction.ncifcrf.gov/ringdb>. This catalog-based approach allows the user to sift through ring structures based on topologies of interest, thus decreasing the effort needed to design ring structures from scratch.

Here, we demonstrate how to generate ring structures, with the square ring shown in the Ring Catalog publication as the example [30], and generate sequences that can be purchased to assemble the ring experimentally.

1.3 Galaxy

Galaxy is a web-based platform for workflow management, data integration, and data analysis [32]. While originally developed for genomic data, Galaxy has become a general workflow management system for bioinformatics and other areas that require numerical processing of data sets. Galaxy functions as a web server and the user interacts with Galaxy via a web browser. The system allows the user to upload data sets, to manipulate them with computational tools and to store the results in a history. This is achieved by encapsulating each of the programs or algorithms into web-based tools where the user typically only specifies input parameters via clickable menu items. This simplifies the access to these computational tools compared to a command line interface. Particularly useful for computational analysis is the history feature of Galaxy. Whenever a user launches a computational tool, the corresponding results are stored in a history. This helps with repeating workflows and reproducing results at a later time because the user is able to see exactly what was run as well as what the inputs and outputs were. Galaxy also has an extensive tool library called Tool Shed where users can install tools created by other users onto their local Galaxy instance [33]. Galaxy also handles any dependencies those tools

may require, which simplifies the installation process. Here we use Galaxy as a web-based front-end for 3D structure manipulation in general and for the design of RNA rings in particular.

2 Materials

Readers interested in how rings are generated in the Ring Database should follow all of this section and download the junction used to generate the square. Otherwise, Subheadings 2.5 and 3.1 may be skipped, as the downloaded structure from Subheading 2.4 will be the same as the structure generated from Subheading 3.1.

2.1 Virtual Operating System for Hosting a Unix-Like Operating System

The work described in this chapter is based on the VirtualBox system. The VirtualBox virtual machine environment allows one to run a variety of Linux distributions on non-Linux host machines such as Apple OS X or Microsoft Windows.

1. Download a version of VirtualBox that is appropriate for the operating system of your host computer from <https://www.virtualbox.org/wiki/Downloads>
2. Follow the installation instructions available in the User Manual available on the VirtualBox website.

2.2 Download and Start Custom Linux Distribution

The Galaxy web server requires a Unix-like operating system that can host the other needed software components. In this chapter, we describe a VirtualBox virtual machine-based Linux distribution that is based on Ubuntu-14 and has been customized by us in order to facilitate RNA 3D structural bioinformatics and computational biology. Note that a Linux distribution that runs within VirtualBox is called an appliance.

1. Download the virtual machine image from <https://binkley2.ncifcrf.gov/users/bshapiro//software.html>.
2. Start the VirtualBox software installed in the previous step.
3. Within VirtualBox, click **File**, then **Import appliance**.
4. Click the folder icon and navigate to the virtual machine image downloaded in the first step.
5. Click **Next**, **Next**, then **Import**.
6. A pop-up will appear indicating the virtual machine is being imported.
7. Once the importing is complete, double click on the new virtual machine.
8. Once the login screen appears log-in with username and password provided with the download instructions.

2.3 Starting and Accessing Galaxy

1. After starting the hosted Linux virtual machine, and a screen prompting for username and password appears, log in with the username rnalab (the password is provided as part of the download instructions). The custom Linux distribution has been prepared by us such that the local Galaxy web server starts automatically upon logging into the prepared user account named rnalab. No additional steps are needed to start Galaxy. If, however, a different user account is utilized or if the Galaxy web server has terminated, Galaxy may need to be started manually. For more details *see* **Note 1**.
2. In order to access the running Galaxy web server, open a web browser (for example by double-clicking the **Firefox** icon on the desktop or by choosing the web browser in the Applications pull-down menu).
3. In the address bar of the web server type `http://localhost:8080` or equivalently `http://127.0.0.1:8080`. This is the web address of the local Galaxy instance.

2.4 Download Junction Structure

1. Download the three-way junction used by opening a web browser and navigating to <https://rnajunction.ncifcrf.gov/index.php> and typing **12316** in for **Entry ID**.
2. Click **Download** next to **Extracted Structure** to download the PDB-formatted coordinate file of the ring. This webpage also has the sequence of the motif which may be useful later during sequence optimization.

2.5 Download Ring

1. Download draft ring by opening web browser and navigating to <https://rnajunction.ncifcrf.gov/ringdb>.
2. Click item **Three-way Junctions**, then scroll down and click on the ring image that is square-shaped. (At the time of writing this, it is the fourth image).
3. The ring of interest has the following characteristics:
 - Motif 1: __20GM.rnaview.pdb_j3_0-A2073_0-C2179_0-A2204.pdb__
 - Motif 2: NA
 - Descriptor: 1_1_1_3_5
4. Once that ring has been found, click **PDB** under **PDB** to download the pdb file of the ring.

3 Methods

If interested in generating the ring (Subheading 3.2), follow Subheading 3.1 and upload the junction from Subheading 2.4. If not, follow Subheading 3.1 and upload the ring from Subheading 2.5, then skip to Subheading 3.3.

3.1 Upload File

1. Start a web browser. Preinstalled with the virtual-machine distribution is the FireFox web browser. This browser can be started by clicking on the FireFox logo or by selecting the pull-down menu Applications → Network → FireFox web browser at the top left of the window of the virtual machine. View within the web browser the status of the Galaxy server by choosing the website <http://127.0.0.1:8080>. This link is also provided in the bookmarks toolbar of the preinstalled FireFox browser. This web address is referred to as the local Galaxy homepage.
2. It is optional but recommended that you log into the Galaxy web server. Without logging in, some functionality of the Galaxy web server (such as the 3D visualization) will not be available. You can use a prepared guest account: At the top right of the web browser window, choose User → Login. At the prompt, enter as e-mail address `guest@rna.lab` and the password provided with the download instructions. Once logged in, you can change the password under User → Preferences → Change your password. Alternatively, you can register a new local Galaxy account under User → Register. At the end of this protocol step, you should be logged into the local Galaxy web server instance.
3. On the left side of the local Galaxy homepage, under **Tools**, upload the downloaded pdb file by clicking **Get Data**, then **Upload File**.
4. In the new window that appears, click **Choose local file** and navigate to the location of the downloaded pdb.
5. Click the drop-down menu under **Type** and choose data type **pdb**.
6. Click **Start** and a history element corresponding to a new compute job will appear on the right side of the local Galaxy homepage. Once the background color of this history element is green, the compute job is completed (*see* **Note 2**).

3.2 Generate Draft Ring Structure

3.2.1 Scan for Rings

1. Under **Tools** on the left side, click **NanoTiler**, then **Grow Scan** (*see* Fig. 1-h).
2. For the **Junction** menu of the **Grow Scan** tool, make sure the junction uploaded (as described in Subheading 3.1) is selected. All other settings can be left at default values. Click **Execute** to begin the compute job (*see* **Note 3**).
3. Once the compute job is completed, refresh the history by clicking the icon with two arrows in a circle on the top right of the browser page next to the gear.
4. A few more computational results should appear—they correspond to additional ring structures that the program identified. The data set named **Grow Scan on data<some number>**

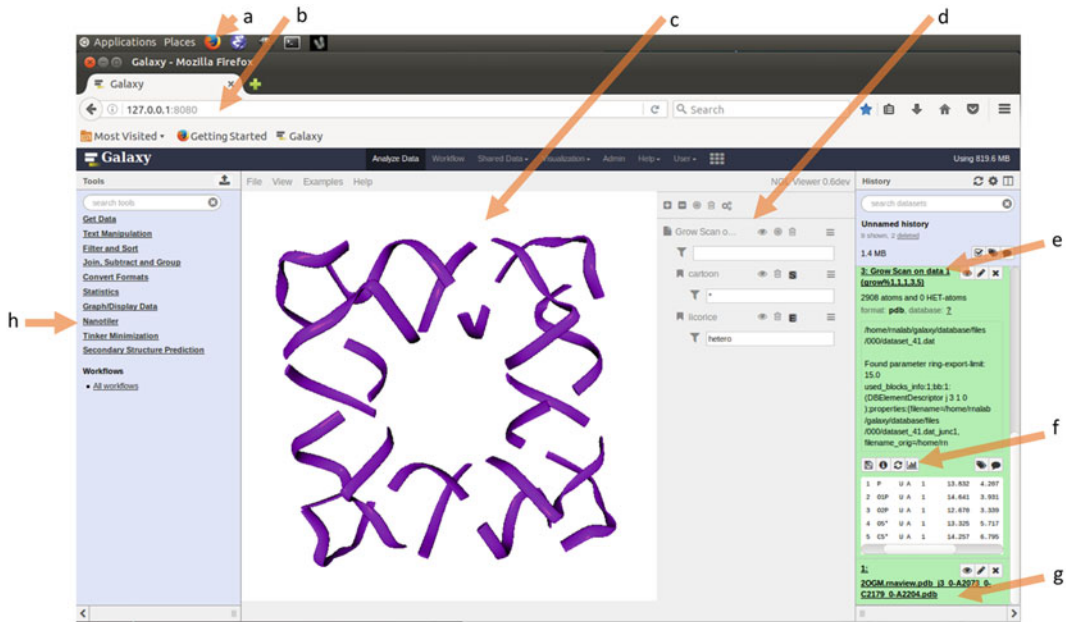


Fig. 1 Screenshot of local Galaxy instance after an initial ring structure has been found. (a) icon of the Firefox web browser; (b) text area for specifying the website of the browser; (c) main window of the Galaxy web server site. It can be used to configure tools to be launched, to inspect data sets or (as shown for this case) display the 3D structure of an RNA via the NGL viewer software; (d) parameter settings for the molecular viewer; (e) clickable title of the generated RNA ring data set. (f) icon that launches the NGL molecular viewer; (g) clickable title of the uploaded structure of the three-way junction; (h) link and header corresponding to a variety of NanoTiler tools

(**grow%1,1,1,3,5**) corresponds to the found ring structure (see Fig. 1-e and Note 4). Additional ring structures as well as versions of generated structures that do not contain linker helices are also available in the history pane.

5. Visualize the generated ring structure by selecting the visualization icon of the data set (the icon has the appearance of a histogram, see Fig. 1-f).

3.3 Postprocessing of Ring Structure

3.3.1 Optimize Ring Structure

3.3.2 Fuse Strands

1. On the left side of the local Galaxy homepage, click **NanoTiler**, then **Improve Ring Closure**, select the file named **Grow Scan on data<some number> (grow%1,1,1,3,5)** (see Fig. 1-h).

2. Press **Execute** to launch the program that optimizes ring closure.

In the presented example, there are 20 RNA strands. These can be fused resulting in a total of five strands using **Ring fuse**, **Fuse all strands**, and **Fuse strands** (see Note 5).

1. On the left side of the local Galaxy homepage, click **NanoTiler** (Fig. 1-h), then **Ring fuse**, selecting the file that was the

output of **Improve Ring Closure**. Click **Execute** to begin the compute job.

2. On the left side of the local Galaxy homepage, click **NanoTiler**, then **Fuse all strands**, selecting the file that was the output of **Ring fuse**. Click **Execute** to begin the compute job.
3. The number of chains in the obtained result can be verified on the level of the raw coordinate file by clicking on the “eye” icon within the history pane of Galaxy.
4. Click on the graph icon of the resulting data set in order to visually inspect the result structure using the **NGL Molecule Viewer** (see Fig. 1-f). In the molecular viewer, choose a representation that colors the 3D molecular image by strand. Note that structural gaps will be closed at a later stage—instead focus on strand color as indication of the strand fuse operation. If the resulting structure consists of more unfused strands than intended (if there are more than five strands in this example), one can attempt to rerun the **Fuse all strands** tool with a higher distance threshold. Alternatively one can use the molecular viewer in order to determine which strands should be fused explicitly.
5. To fuse specific strands perform the following steps:
 - Within the left tool pane of the local Galaxy homepage, click **NanoTiler**, then **Fuse strands**.
 - Choose the **Fuse all strands on data** as Junction, then enter the letters of the two strands in **Strand that is elongated** and **Strand to append** (see Note 6). Click **Execute** to begin the compute job.

3.3.3 Extend Helices

1. On the left side of the local Galaxy homepage, click **NanoTiler**, then **Extend Helix** (see Note 7).
2. Choose the result of the fusing from the last step for the **Structure**, **10** for **Number of basepairs**. For **Sequence and basepair of where to extend** enter the strand and base number separated by a colon (for example, A:1 will extend a helix off of the first base of strand A). Click **Execute** to begin the compute job.
3. Repeat for each applicable strand each time using the newly created file with the extended helices for structure.
4. To fuse the newly created helices to the square structure, use **Fuse all strands** and **Fuse strands**. Use **NGL Molecule Viewer** to see which strands need to be fused. Once each of the helices are fused, there will be five strands again (see Fig. 2a and Note 8).

3.3.4 Move Nick

1. Sometimes, the RNA strand breaks (i.e., nicks) that are utilized as a result of the previous steps are not at the desired positions.

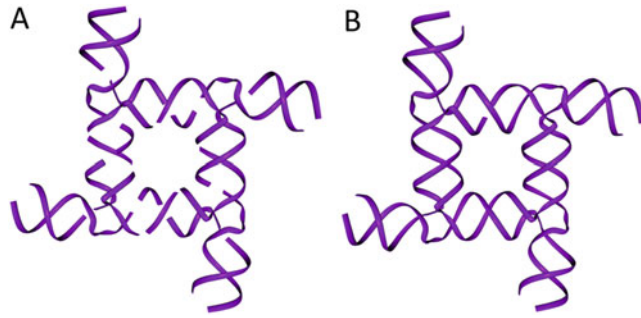


Fig. 2 Structures at different processing stages. (a) RNA square structure after helices has been added. (b) The RNA structure after energy minimization

In the case of the RNA square utilized in this example, use the **NGL Molecule Viewer** to find the 5' end of the inner strand (this strand corresponds to strand E in Fig. 5 in reference [30]). Estimate the number of nucleotides needed to be split and then rejoined for the 5' end of the strand to move toward the middle of a side of the RNA square. Note down the position of the base **after** the split (*see Note 9*).

2. On the left side of the local Galaxy homepage, click **NanoTiler**, then **Split strands**. Click **Execute** to launch the compute job.
3. Type the letter identifier of the strand representing the inner circular strand, for *Strand*.
4. Then use position noted down from **step 1**. The strand is cut before this position (*see Note 10*).
5. On the left side of the local Galaxy homepage, click **NanoTiler**, then **Fuse strands**. We will fuse the new strand to the old one. For **Strand that is elongated** type the name of **new strand**, and then the name of **old strand** for **Strand to append**. Click **Execute** to launch the compute job (*see Note 11*).
6. Use the NGL viewer and verify the nick is moved near the center.

3.3.5 Optimize Sequences

Initially, all generated linker helices correspond to consecutive all GC base pairings. Therefore, one needs to optimize individual strand sequences next.

1. Sequence optimization is necessary if the ring structure contains generated helical segments consisting of unoptimized placeholder sequences (in this example corresponding to GC base pairs).
2. On the left side of the local Galaxy homepage, click **NanoTiler**, then **Secondary Structure**.

3. Select the file of the structure with the adjusted nick moved in the drop-down menu. For format pick **Server**. Click **Execute** to begin the compute job.
4. Once it is completed, open the file by clicking the eye icon and copy the contents.
5. Point a web browser to the NanoFolder homepage at <http://matchfold.abcc.ncifcrf.gov/>. For a background of this computational tool for multistrand structure prediction and sequence optimization please consult the original publication [34].
6. Paste the contents from the results of the secondary structure tool (called “Secondary Structure”).
7. NanoFolder optimizes bases that are capitalized. Lowercase nucleotides are left unchanged by the optimization algorithm. The aim is to optimize bases that are part of the helices but not part of the motif. This can be achieved by visually inspecting the RNA draft structure and classifying strand regions as either motif regions or as linker helix regions. Helices not part of the motif will initially be only G or only C nucleotides. There are stretches of G’s in the motif so it may be helpful to also have the sequence of the motif available as well (see **Notes 12–14**).

3.3.6 Mutate 3D Model to Reflect Optimized Sequence

1. On the left side of the local Galaxy homepage, click **NanoTiler**, then **Mutate 3D structure**.
2. Select the file from the output of Subheading 3.3.5.
3. Then click the **Insert Mutations** button five times to bring up five input areas. Each one corresponds to the chain of the same number (except the chains are alphabetically named, so the max is 26), the first is for chain A, 2 is B, 3 is C, etc.
4. Copy the result of optimization into each corresponding area and then click **Execute** to begin the compute job.
5. Verify the mutations using the **Secondary Structure** tool.

3.3.7 Refine 3D Model

1. Molecular dynamics software suites such as Tinker can be used to refine the RNA 3D structure using molecular mechanics energy minimization [35]. Energy minimization will close gaps and reduce structural problems.
2. On the right side of the local Galaxy homepage, click **Tinker Minimization**, then **Prepare pdb**.
3. Set input to output file of **step 4** and click **Execute** to begin the compute job.
4. Once the compute job is completed, on the right side of the local Galaxy homepage, click **Tinker Minimization**, then **Tinker**.

5. Set the input to the output of the previous step and set the minimization termination parameter called “RMSG” to 1 for coarse refinement, and .1 for fine refinement (*see* **Note 15**).
6. The minimization can take several minutes or even an hour to complete, depending on the size of the RNA structure. Visualize or download the resulting structure (*see* Fig. 2b).

4 Notes

1. The installation directory of the Galaxy server is called “galaxy.” Modifying the behavior of the local Galaxy web server (such as adding computational tools to it) is entirely possible, but is beyond the scope of this chapter. Please consult the Galaxy documentation for questions related to the administration of the Galaxy server (<https://wiki.galaxyproject.org/Learn>). The prepared user account is set up in such a way that the Galaxy server starts automatically. In principle, it is possible to start the Galaxy server using two additional ways: (a): Select from the pull-down menu (top left) Applications → RNA Lab Applications → Galaxy. (b): Open a Unix shell terminal window by clicking on the Terminal icon on the desktop. In the command line of the obtained window type `cd galaxy; sh run.sh`. This starts up an instance of the Galaxy web server at port 8080. Note that the web server startup script only succeeds if no other web server is active at the same port number.
2. A gray background of the element of the Galaxy history pane indicates that the corresponding compute job is waiting to start, while a yellow background indicates that the compute job is currently running. A green background indicates that the compute job is completed.
3. This step may take some time to complete.
4. There will be other files with names like: **Grow Scan on data<some number> (grow%1,1,1,3,5_nohelices)**. These are the same structure with and without included linking helices.
5. The strand “fuse” family of commands merely change strand names, while atom coordinates are not changed.
6. The order does not matter unless the strands need to be appended in a specific order (can be fused on both ends such as in a ring).
7. Adding helix-forming complementary regions to a nucleic acid nanostructure may improve its thermal stability and assembly properties.

8. Because the chain letter labels change after each fusing operation, it is recommended to use a molecular viewer (such as the integrated **NGL Molecule Viewer**) to identify RNA strands that should be fused.
9. Checking the structure in the viewer will show that the inner strand starts and ends close to a junction. This means that during assembly, one end of the helix will only have a few base pairs and the other will have a lot more. Balancing it stabilizes the shorter helix. This can be achieved by splitting off nucleotides at the end of the strand corresponding to the shorter helix and adding them to the strand corresponding to the helix in need of stabilization.
10. To verify that the strand was split, click the “eye” icon in the history pane element corresponding to the data set and inspect the resulting pdb text file. At the end of the file, there will be a chain with the same number of bases as was split off.
11. The old strand is the strand that is deleted, because the old chain is appended to the new strand. If we were to reverse it, we would end back up where we were before this.
12. Any additional sequence regions that would need to be added (such as sequence regions at the 5' end related to a utilized RNA polymerase (8)), should be added before the sequence optimization step—either in form of an expanded 3D model or in form of an expanded secondary structure text input for the utilized sequence optimization program.
13. Ascertain the quality of the optimized sequence by inspecting the output of the optimization program and by utilizing RNA secondary structure prediction programs.
14. This step may take some time to complete.
15. The smaller the minimization tolerance parameter (“RMSG”) is set, the better the structure will end up, but it will take longer (e.g., a run with RMSG set to 0.1 can frequently take more 30 min for the presented example).

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