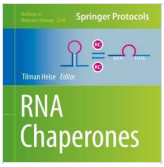

Sep 02, 2021

Assessment of the RNA Chaperone Activity with a Molecular Beacon

Book Chapter

In 1 collection

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ABSTRACT

This is part 3.2 of the "Fluorescent Molecular Beacons Mimicking RNA Secondary Structures to Study RNA Chaperone Activity" collection of protocols.

Collection Abstract: Molecular beacons (MBs) are oligonucleotide probes with a hairpin-like structure that are typically labelled at the 5' and 3' ends with a fluorophore and a quencher dye, respectively. The conformation of the MB acts as a switch for fluorescence emission. When the fluorophore is in close proximity to the quencher, fluorescence emission cannot be detected, meaning that the switch is in an OFF state. However, if the MB structure is modified, separating the fluorophore from the quencher, the switch turns ON allowing fluorescence emission. This property has been extensively used for a wide variety of applications including real-time PCR reactions, study of protein-DNA interactions, and identification of conformational changes in RNA structures. Here, we describe a protocol based on the MB technology to measure the RNA unfolding capacities of the CspA RNA chaperone from *Staphylococcus aureus*. This method, with slight variations, may also be applied for testing the activity of other RNA chaperones, RNA helicases, or ribonucleases.

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COLLECTIONS

Fluorescent Molecular Beacons Mimicking RNA Secondary Structures to Study RNA Chaperone Activity

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Fluorescent Molecular Beacons Mimicking RNA Secondary Structures to Study RNA Chaperone Activity

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MATERIALS TEXT

Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to reach a sensitivity of at least 18 MΩ at **25 °C**) and analytical grade reagents for use in molecular biology. Store solutions at **Room temperature** unless stated otherwise. Follow safety and waste disposal regulations when handling harmful products accordingly.

1. Molecular beacon mimicking the hairpin structure under study (*see* **Note 3**).
2. Spectrophotometer equipped with a UV lamp (e.g., NanoDrop).
3. Recombinant RNA chaperone CspA (or protein of interest).
4. Bovine Serum Albumin (BSA) dissolved in CspA storage buffer at the same concentration as the protein of interest (*see* **Note 4**).
5. TE buffer: **10 Milimolar (mM) Tris–HCl** pH **7.5** and **1 Milimolar (mM) EDTA** .
6. 96-well PCR plates suitable for the Real-Time PCR System available. Plate is sealed with optically clear adhesive film (*see* **Note 5**).
7. AriaMx Real-Time PCR System (Agilent Technologies) or any other equivalent thermal cycler including an optical system able to excite the FAM fluorophore and register the fluorescence emission at different temperature incubation times.
8. CspA storage buffer (*see* **item 17** in part 3.1, Materials for section "Purification of Recombinant CspA from Total Protein Crude Extracts").
9. **10 X reaction buffer** : **100 Milimolar (mM) MTris–HCl** pH **7.5** , **300 Milimolar (mM) KCl** , **200 Milimolar (mM)** NH_4Cl, **15 Milimolar (mM) DTT** , **50 Milimolar (mM)** $MgCl_2$ (*see* **Note 6**).
10. **4 U/µl Ribolock** (*see* **Note 7**).
11. Proteinase K (Sigma) stock solution. Dissolved Proteinase K powder in water to a final concentration of **20 mg/mL** (*see* **Note 8**). Store at **-20 °C** .

SAFETY WARNINGS

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

BEFORE STARTING

Prepare Buffers and Solutions as described in section 'Materials'.

Molecular Beacon Design

1

The success of this assay lies in an adequate MB design, which is based on two main principles:
(1) the presence of an RNA structure targeted by the RNA chaperone under study.
(2) fluorescence quenching exerted by a quencher dye (e.g., BHQ_1) on a fluorophore (e.g., FAM), which occurs when both molecules are in close proximity to one another.
Additionally, the selected RNA structure must keep the quencher close enough to the fluorophore at the working temperature (switch OFF). MB mimicking hairpin-like structures have been shown to comply these criteria before [7–9]. Likewise, alternative MB conformations can be tested according to the characteristics of the protein of interest. Having decided the MB configuration, the synthesis of the labelled probe may be ordered to any oligonucleotide supplier (*see* **Note 18**). Based on the binding capacity of the CspA protein and the stability and cost of the probe, we decided to use single-stranded DNA instead of RNA oligonucleotides (*see* **Note 19**).

Testing the Effectiveness of the Designed MB and Setup of the Working Conditions 20m

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2

Before assessing if the RNA chaperone is able to unwind the MB structure, the effectiveness of the designed MB must be tested following two main criteria. On the one hand, no fluorescence emission should be detected when the MB is in an OFF state. On the other hand, maximum fluorescence levels should be registered when the MB is completely unfolded (ON state). In other words, a MB design will be appropriate when a large ON/OFF fluorescence ratio is detected. Incubating different MB concentrations at increasing temperatures (that denature the oligonucleotide and open the structure leading to FAM fluorescence emission) helps determining both the background fluorescence (*see* **Note 20**) and the lowest quantity of oligonucleotide needed to obtain good fluorescence levels when the MB is in an ON state (*see* **Note 21**). To test if the MB design was successful, proceed as follows:

3 Dissolve the MB in TE buffer to obtain a concentration of **100 Micromolar (µM)**, following the manufacturer recommendations (*see* **Note 22**). Concentration of the MB should be corroborated with a spectrophotometer (e.g., NanoDrop).

4 Program the AriaMx thermal cycler to incubate the MB samples as follows: **37 °C**, **00:05:00**; **45 °C**, **00:05:00**; **55 °C**, **00:05:00**; and **65 °C** during **00:05:00** (*see* **Note 23**). 20m

Register the emission of FAM fluorescence **every minute**.

5

Make serial dilutions of the MB in an optical 96-well plate as indicated in Table 1, which shows mixtures of the components to analyze different concentrations of the MB. Triplicates are highly recommended.

	MB concentration (pmol)							
	0	**0.5**	**1**	**2**	**5**	**10**	**15**	**20**
MB 10 µM	-	-	-	-	0.5	1	1.5	2
MB 1 µM	-	0.5	1	2	-	-	-	-
CspA storage buffer [b]	12.5	12	11.5	10.5	12	11.5	11	10.5
10X reaction buffer	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Ultrapure water	10	10	10	10	10	10	10	10
Final volume	25 µL							

Table 1
Preparation of dilution mixes for testing MB effectiveness[a]

[a]Volumes of each reactive are indicated in µL
[b]Since the CspA protein is diluted in CspA storage buffer, the MB effectiveness test is performed including this buffer

6

Seal the plate with an optically clear adhesive film (*see* **Note 5**) and load it into the thermal cycler. Start the incubation program.

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7

Once the incubation time is finished, plot the obtained fluorescence signals in function of the MB concentration at the different temperatures. If replicates are used, plot the means of the fluorescence signals. The instrument background signal should be previously subtracted. Figure 4 shows an example of the results obtained with the MB designed for the analysis of *S. aureus* CspA activity [9] (Fig. 4). In this example, when the MB was incubated at **55 °C** and **65 °C**, fluorescence emission was registered, indicating that the MB was in an ON state. These fluorescence levels were directly proportional to the MB concentration. In contrast, when the MB was incubated at **37 °C** and **45 °C**, the fluorescence values were close to those of the background confirming that the MB was in an OFF configuration. This experiment validated the functionality of the designed MB (*see* **Note 24**).

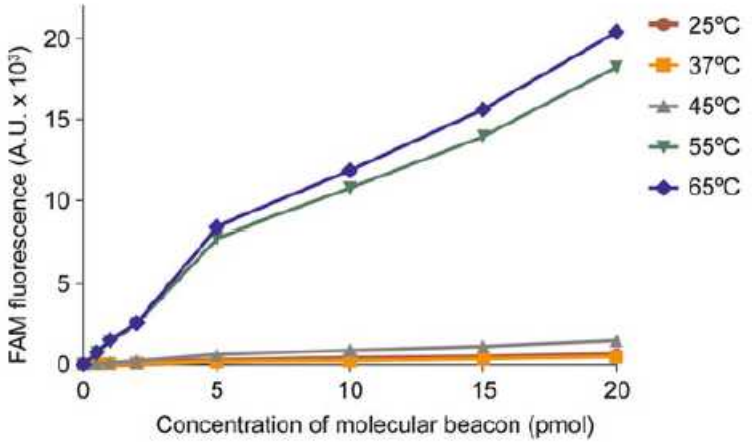

Fig. 4 Test of the molecular beacon functionality. Different concentrations of the MB mimicking the hairpin structure located at the 5'UTR of the cspA mRNA were incubated at different temperatures and fluorescence emission was registered. The experiment was carried out using the AriaMx thermal cycler

Determination of the RNA Chaperone Activity Using the Designed MB 1h 55m

8 Based on the data obtained from the MB effectiveness test, select the lowest MB concentration that gives a good ratio between the fluorescence and background signals (*see* **Note 21**).

9 Program the AriaMx thermal cycler to incubate the MB samples as follows: **37 °C**, **00:05:00**; PAUSE, **37 °C** (1h), **00:15:00**; PAUSE, **37 °C**, **00:30:00**; **65 °C**, **00:10:00**; STOP (Table 2).

Register the fluorescence emission every minute (*see* **Note 25**).

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	Samples [b]							
	1	2	3	4	5	6	7	8
MB tube labelling	-	+	+	+	+	+	+	+
CspA tube labelling	-	-	+	+	+	-	-	-
BSA tube labelling	-	-	-	-	-	+	+	+
Water (Vf: 100 µL)	39	38	38	38	38	38	38	38
CspA storage buffer	50	50	30	15	-	30	15	-
10X reaction buffer	10	10	10	10	10	10	10	10
MB 1 µM	-	1	1	1	1	1	1	1
Ribolock 4 U/µL	1	1	1	1	1	1	1	1
	Seal the plate with adhesive film							
	Incubate 37 °C—5 min							
	Register fluorescence emission every minute							
	PAUSE incubation program[c]							
CspA stock (~200 µM)	-	-	20	35	50	-	-	-
BSA stock (~200 µM)	-	-	-	-	-	20	25	50
	Re-seal the plate with adhesive film							
	Incubate 37 °C—15 min							
	Register fluorescence emission every minute							
	PAUSE incubation program							
Proteinase K 20 mg/mL	10	10	10	10	10	10	10	10
	Re-seal the plate with adhesive film							
	Incubate 37 °C—30 min							
	Incubate 65 °C—10 min							
	Register fluorescence emission every minute							
	Collect the fluorescence data from AriaMx thermal cycler							
	Plot the data accordingly							

Table 2
Determination of RNA chaperone activity: preparation of reaction mixes[a]

[a]Volumes of each reactive are indicated in µL
[b]Replicates of samples should be included
[c]If the thermal cycler software allows it, the entire incubation protocol can be pre-programed including the corresponding PAUSE times

10 Prepare an optical 96-well plate including the reaction mixes as indicated in Table 2 (*see* **Note 26**).

Note that the CspA and BSA proteins should be added later.

11

Seal the plate with adhesive film (*see* **Note 5**) and load it into the thermal cycler. Start the incubation program.

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At the first pause of the incubation program, pull out the 96-well plate from the thermal cycler, remove the adhesive film and add the appropriate quantity of CspA and BSA. Re-seal the plate with a new adhesive film. This step must be performed swiftly.

13 15m

Reintroduce the plate into the thermal cycler and continue the incubation at **37 °C** during **00:15:00** .

Register the fluorescence emission every minute.

14

During the second incubation pause, pull out the plate, remove the adhesive film and add **10 µl proteinase K (20 mg/mL)** . Re-seal the plate with a new adhesive film. This step must be performed swiftly.

15 40m

Reintroduce the plate into the thermal cycler and continue the incubation for **00:30:00** at **37 °C** and then increase the temperature up to **65 °C** during **00:10:00** .

Register fluorescence emission **every minute**.

16 Once the incubation program is finished, collect the result data sheet.

17

Plot the obtained data subtracting the background fluorescence levels. If the experiments work as expected, fluorescence emission should be registered after addition of the RNA chaperone. This fluorescence should disappear after treatment with Proteinase K, showing the specificity of the reaction. Finally, increasing the temperature at **65 °C** should lead to maximum levels of fluorescence, indicating that the MB remains functional through the course of the experiment. Logically, in the negative controls, no fluorescence emission should be detected until the last step, when temperature is raised (e.g., *see* ref. 9).

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