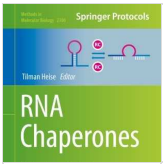

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Fluorescent Molecular Beacons Mimicking RNA Secondary Structures to Study RNA Chaperone Activity

Book Chapter

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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.

ATTACHMENTS

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

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GUIDELINES

1 Introduction

Molecular beacons (MBs) are oligonucleotide probes commonly used to target DNA for real-time monitoring of polymerase chain reactions (RT-PCRs). The central nucleotides of the MB are complementary to a specific DNA (or RNA) target and do not base pair with one another, while the five to seven nucleotides at each terminus are complementary to each other, creating a hairpin-like conformation (Fig. 1a).

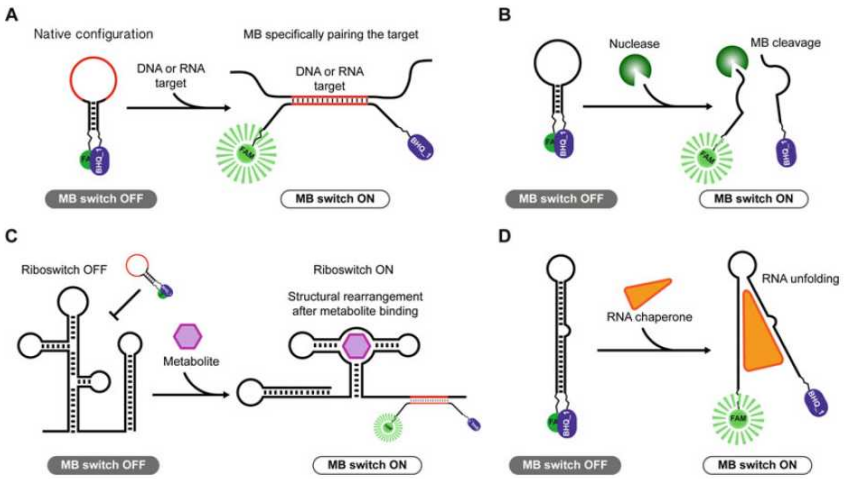

Fig. 1 Examples of different MB designs dedicated to (**a**) quantifying specific DNA or RNA molecules, (**b**) analyzing the single-stranded DNA cleavage by specific nucleases [4], (**c**) studying the structural changes on ribozymes and riboswitches [5, 6], or (**d**) determining the RNA chaperone activity on hairpin-like structures by cold shock proteins (CSPs) [7–9]. In all cases, the MB switch turns on when the fluorophore (FAM) folds away of the quencher (BHQ_1) due to the base-pairing of the MB with its specific target (**a, c**) or to the MB cleavage or unfolding by the activity of an RNA-binding protein (**b, d**)

Since the 5' and 3' ends are labelled with a fluorophore and a quencher, respectively, the MB acts as a switch. In their native conformation, the extremes are close enough for the quencher to prevent fluorescent emission from the fluorophore (switch OFF). When the MB hybridizes with its specific target, its native structure is disrupted, and both dye molecules fall apart from each other, allowing fluorescence emission (switch ON) (Fig. 1a). Since MBs

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tolerate very versatile designs, they have been used for various applications [1, 2]. Molecular biologists have taken advantage of their potential for studying different mechanisms such as protein-DNA interactions [3], single-stranded DNA cleavage by specific nucleases (Fig. 1b) [4], and structural changes on ribozymes and riboswitches (Fig. 1c) [5, 6]. In this last case, RNA conformational changes have been determined by the use of MBs that target specific RNA regions that become free for hybridization. This usually occurs after binding of the metabolite, which induces the subsequent RNA structural change on the ribozyme or riboswitch (Fig. 1c) [5, 6]. Thus, only when the MB is bound to its RNA target, the probe structure unfolds and becomes fluorescent. On the other hand, in order to study RNA chaperone activity, a more direct approach by using a MB that mimics the regulatory RNA hairpin targeted by a specific RNA-binding protein (RBP) has been adopted [7, 8]. This strategy assumes that binding of the RNA chaperone to the MB may cause a similar RNA conformational rearrangement to the one occurring on the native RNA. Therefore, the MB may act as a direct reporter of its own structural rearrangement (Fig. 1d). We choose this approach to demonstrate that the RNA chaperone CspA of *Staphylococcus aureus* unfolds the RNA hairpin present in the 5'UTR of its own mRNA [9] (Fig. 2a). This hairpin (ΔG = −24.60 kcal/mol) is cleaved by endoribonuclease III (RNase III) mainly at position G-53, generating a shorter *cspA* mRNA version that is more efficiently translated than the unprocessed mRNA [10]. CspA would repress its own expression by unfolding the hairpin and thus antagonize the function of RNase III [9].

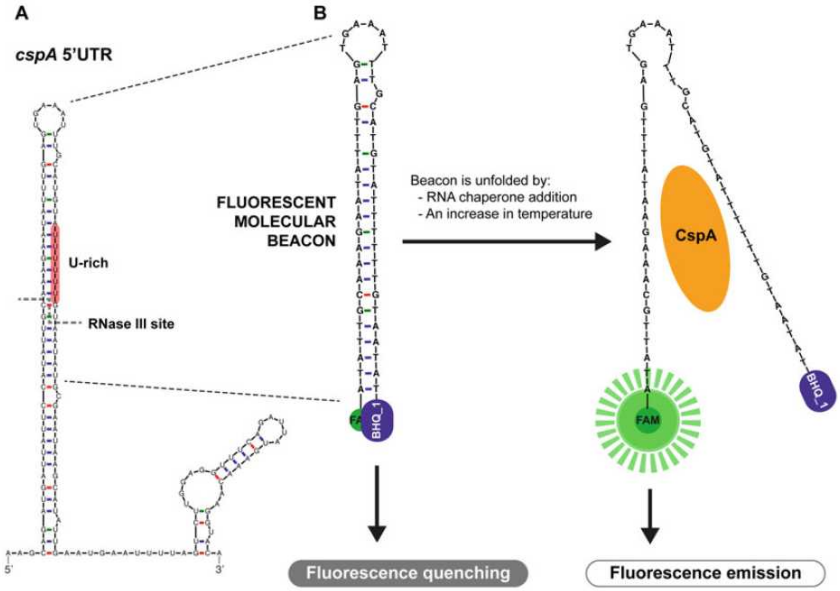

Fig. 2 Molecular beacon (MB) design to study the CspA RNA chaperone activity on the RNA hairpin structure of the *csp*A mRNA [9]. (**a**) The proposed RNA structure for the 5'UTR of *csp*A is shown [10]. The U-rich motif required for CspA interaction is highlighted in red. (**b**) The MB consisted of a 49-mer ssDNA oligonucleotide labelled with the FAM fluorophore and the BHQ_1 quencher at its 5' and 3' ends, respectively [9]

Specifically, we designed a MB that comprised a 49-mer ssDNA oligonucleotide, which included the central functional sequence of the *csp*A 5'UTR hairpin (ΔG = −13.70 kcal/mol). A molecule of fluorescein (FAM) and a Black Hole Quencher (BHQ_1) were attached to the 5' and 3' ends, respectively (Fig. 2b). In the native MB conformation, BHQ_1 efficiently quenched the fluorescence from FAM, indicating that the designed MB accurately mimicked the *csp*A 5'UTR hairpin. In contrast, when the MB structure was disrupted (separating FAM from BHQ_1) either by the presence of the RNA chaperone CspA or by an increase in the temperature of incubation, fluorescence emission was registered. The folded conformation of the MB could be efficiently restored (indicated by the ceasing of fluorescence emission) either by adding Proteinase K, which eliminated the chaperone activity by degrading CspA, or by decreasing the temperature of incubation. The specificity of CspA on the designed MB system was verified by the incubation of the MB with an unrelated protein. This strategy allowed us to demonstrate that CspA unfolded the regulatory hairpin located at the *csp*A 5'UTR and, thus, interfered with *csp*A mRNA processing by RNase III. When CspA levels were low, the *csp*A 5'UTR RNA hairpin was targeted and cleaved by RNase III. The resulting processed mRNA suffered a conformational change that favored CspA translation [10]. When CspA levels rose, CspA decreased its own expression by unfolding the *csp*A 5'UTR RNA hairpin to avoid RNase III cleavage [9] (Fig. 3).

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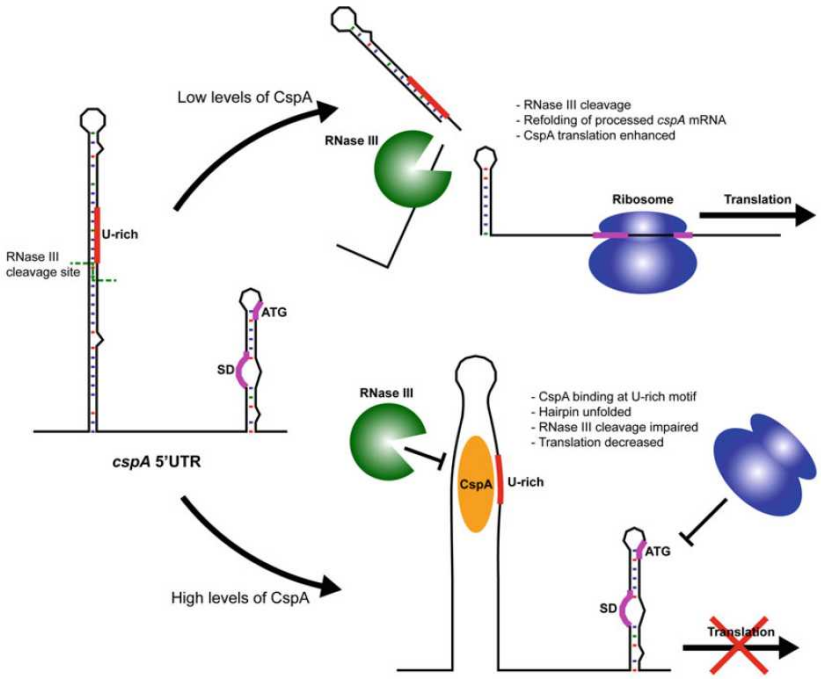

Fig. 3 Schematic representation of the putative auto-regulatory mechanism modulating CspA expression as previously described by Caballero and colleagues [9]. The 5'UTR of the *csp*A mRNA forms a hairpin structure that is cleaved by RNase III to enhance CspA translation when CspA levels are low [10]. When the concentration of CspA inside the cell is high, the protein is able to interact with the hairpin structure through a U-rich motif and unfold it. As a consequence, the *csp*A mRNA is not processed by RNase III and CspA translation is decreased

Here, we describe in detail the different steps that should be followed to determine the RNA folding rearrangements caused by the binding of any RBP by using a MB that mimics a natural target (whose synthesis could be ordered from a regular oligonucleotide supplier company). The protocol requires commonly available equipment at a molecular biology research center. It is noteworthy that, with slight modifications, this protocol may be adapted to test (1) any DNA or RNA folding structure that allows close proximity of BHQ_1 to FAM and that provides enough separation between them when disrupted; (2) the activity of RBPs such as RNA chaperones, RNA helicases and ribonucleases that target and/or process hairpin-like structures; and (3) the function of small regulatory RNAs that produce conformational changes on hairpin-like structures of their mRNA targets.

4 Notes

1. Although we purified the RNA chaperone CspA from *E. coli* using the glutathione S-transferase (GST) gene fusion system, any other recombinant purification alternative can be used for your protein of interest. We fused the *csp*A coding sequence to the GST gene in the pGEX-6P-2 plasmid. This allowed the convenient site-specific cleavage by the PreScission Protease between the GST domain and CspA at low temperature, minimizing the degradation of the protein of interest. Additionally, it provided the recovery of the recombinant CspA protein with only a few extra amino acids in its sequence (for details about the construction of this strain *see* ref. 9). The fact that the PreScission Protease was engineered with a GST tag, permitted an on-column cleavage so that the GST moiety of the tagged protein and the PreScission Protease itself remained bound to the Glutathione Sepharose column. Thus, at the end of the procedure CspA was not contaminated with the protease.
2. PMSF is unstable in the presence of water. A stock solution should be prepared in anhydrous isopropanol or anhydrous absolute ethanol.
3. Our designed MB consisted of a 49-mer single-stranded DNA oligonucleotide, which was synthetized and labelled at its extremes with the 6-FAM molecule and Black Hole Quencher (BHQ_1) by the Integrated DNA technologies company. This length was enough to include the functional part of the RNA hairpin (Fig. 2).
4. We used bovine serum albumin (BSA), a protein without capacity to bind nucleic acids, as a negative control. Any alternative protein lacking DNA/RNA binding domains can also be used.
5. We preferred to seal the 96-well plates with adhesive film because removing it and re-sealing the plates is faster than using flat caps. A quick sealing helps registering fluorescence emission sooner, after the RNA chaperone is added to the MB solution.

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6. Storage of the 10X reaction buffer will require it to be prepared without DTT. DTT should be added just before use.
7. Any alternative RNase inhibitor can be used. If ssDNA is used as the backbone of the MB, RNase inhibitors are not required.
8. To demonstrate the specificity of chaperone activity, proteinase K (or any alternative protease) may be included to degrade the protein under study. This should eliminate the RNA chaperone activity and restore the MB folding. If fluorescence is not quenched again after Proteinase K treatment (in other words, the MB cannot be refolded), it might indicate a contamination of the RNA chaperone solution with nucleases that affect the MB integrity.
9. It is important to collect samples at various steps during the purification procedure to monitor the yield of the recombinant protein. Comparison of these control samples helps evaluating if (1) the induction of the recombinant protein expression is adequate (pre-induction vs post-induction control), (2) the recombinant protein is present in the soluble and/or the insoluble fraction (IB control vs soluble fractions), and (3) the recombinant protein is lost during the step of sample clearance by filtration (pre-filtered vs post-filtered soluble fractions).
10. Centrifuge should be pre-cooled before use.
11. Bacterial pellets can be stored at –80 °C for several days.
12. We recommend the use of filters with a pore size of 0.45 µm instead of 0.2 µm to avoid filter saturation.
13. Pre-cast or custom-made gels may be used with the appropriate percentage of acrylamide according to the protein of interest (we used 12% PAGE).
14. Adjust voltage of the electrophoresis system accordingly.
15. If the protein of interest is not in the soluble fraction, bacterial growing conditions should be modified to force its solubilization. Alternatively, protein purification methods from inclusion bodies may be applied.
16. Due to the slow binding kinetics between GST and glutathione, it is very important to keep the flow rate as low as possible during sample application for maximum binding capacity.
17. The column used in this protocol is specific for separating proteins with a small size. If the RNA chaperone of interest has a bigger size, the column should be changed accordingly.
18. Some oligonucleotide supplier companies limit the synthesis of labelled oligonucleotide probes to 50 nucleotides (nt). In our design, the functional RNA hairpin region could be included in an oligonucleotide probe smaller than 50 nt. For larger regulatory structures, the synthesis of a MB may prove more challenging. This problem could be solved by dividing the MB synthesis into two shorter oligomers that can afterwards be ligated as previously described [7].
19. The reason for using a labelled DNA oligonucleotide as a MB is that it has been proven that CSPs can bind ssDNA as efficiently as RNA molecules [11]. Nevertheless, testing other RNA-binding proteins may require synthesis of RNA-based MBs.
20. If the region of the RNA structure under study is not strong enough to maintain the MB beacon in an OFF state, the basal level of fluorescence might be too high to obtain reliable results once the RNA chaperone is added.
21. Sometimes the quantity or the concentration of the chaperone under study can be limited. We recommend using the lowest concentration of the MB that gives good fluorescent levels in an ON state. This will help saving RNA chaperone sample.
22. The fluorophores of the MB are sensitive to the light; therefore, keep the stock and any other dilutions wrapped in aluminum foil and protect them from exposure to light to maintain their integrity.
23. The selected temperature might vary depending on the melting temperature of the MB structure.
24. If the control of the MB functionality does not show clear differences on the fluorescence signals between ON and OFF states, and/or the fluorescence background is too high, the MB should be redesigned.
25. The entire incubation protocol to be carried out with the AriaMx thermal cycler (or any equivalent equipment) can be programed from the beginning, including the corresponding pause times required to add the different components of the reactions.
26. Volumes of each reactive should be adjusted according to the concentration of the RNA chaperone.

Acknowledgments

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

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ATTACHMENTS

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FILES

Purification of the Recombinant RNA Chaperone CspA
Version 1
by satyavati Kharde

Assessment of the RNA Chaperone Activity with a Molecular Beacon
Version 1
by satyavati Kharde

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