



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

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### Abstract

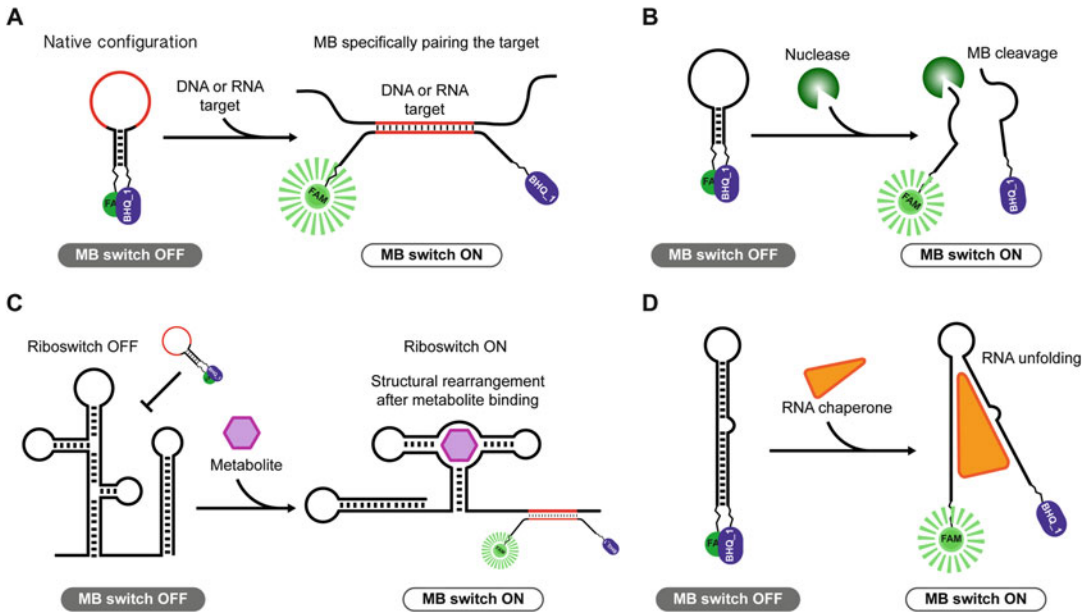
Molecular beacons (MBs) are oligonucleotide probes with a hairpin-like structure that are typically labelled at the 5' and 3' ends with a fluorophore and a quencher dye, respectively. The conformation of the MB acts as a switch for fluorescence emission. When the fluorophore is in close proximity to 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.

**Key words** RNA, Chaperone, RNA-binding protein, Hairpin, Stem loop, Molecular beacon, Fluorescein, Quencher, FAM

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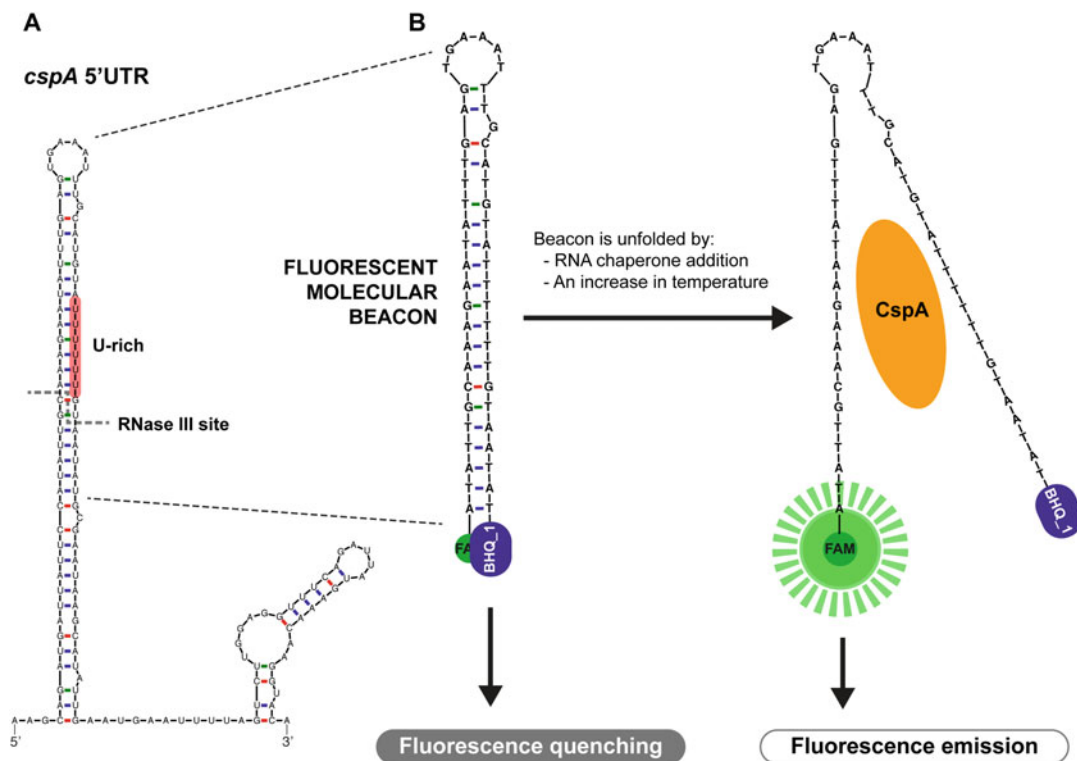
### 1 Introduction

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). 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



**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)

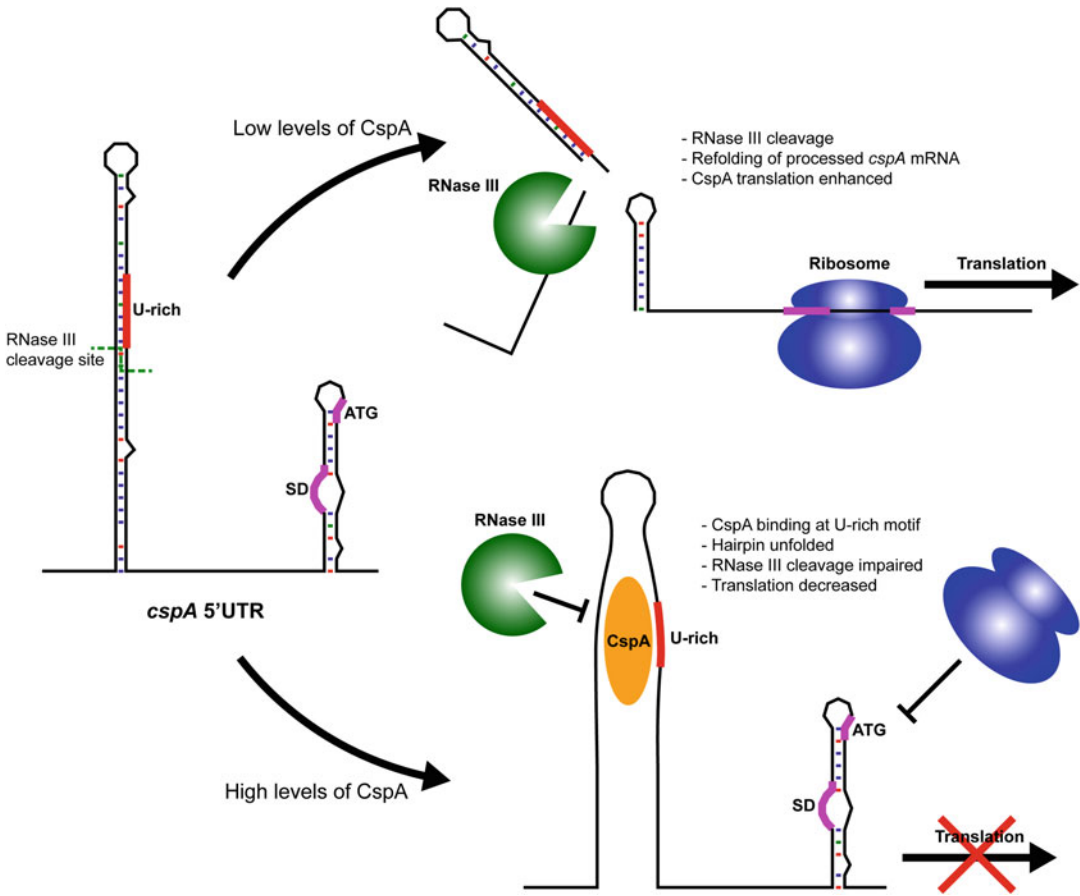
each other, allowing fluorescence emission (switch ON) (Fig. 1a). Since MBs tolerate very versatile designs, they have been used for various applications [1, 2]. Molecular biologists have taken advantage of their potential for studying different mechanisms such as protein-DNA interactions [3], single-stranded 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



**Fig. 2** Molecular beacon (MB) design to study the CspA RNA chaperone activity on the RNA hairpin structure of the *cspA* mRNA [9]. (a) The proposed RNA structure for the 5'UTR of *cspA* 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]

RNA chaperone CspA of *Staphylococcus aureus* unfolds the RNA hairpin present in the 5'UTR of its own mRNA [9] (Fig. 2a). This hairpin ( $\Delta G = -24.60$  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].

Specifically, we designed a MB that comprised a 49-mer ssDNA oligonucleotide, which included the central functional sequence of the *cspA* 5'UTR hairpin ( $\Delta G = -13.70$  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 *cspA* 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



**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 *cspA* 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 *cspA* mRNA is not processed by RNase III and CspA translation is decreased

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 *cspA* 5'UTR and, thus, interfered with *cspA* mRNA processing by RNase III. When CspA levels were low, the *cspA* 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 *cspA* 5'UTR RNA hairpin to avoid RNase III cleavage [9] (Fig. 3).

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.

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## 2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to reach a sensitivity of at least 18 M $\Omega$  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.

### 2.1 Purification of the Recombinant RNA Chaperone CspA

#### 2.1.1 Growth of Bacteria Expressing GST-CspA Fusion Protein

1. *E. coli* BL21 (DE3) harboring pGEX-6P-2::*cspA* (see Note 1).
2. Sterile material for bacterial growth: 10-, 100- and 1000- $\mu$ L pipette tips, test tubes, 2-L Erlenmeyer flasks, graduated cylinders, 250-mL centrifuge tubes, petri dishes, 1.5-mL Eppendorf tubes.
3. 100 mg/mL ampicillin stock solution sterilized by filtration. Store at  $-20$  °C.
4. 40% Glucose solution sterilized by filtration.
5. Luria Bertani (LB) agar plates supplemented with 100  $\mu$ g/mL ampicillin.
6. Microbiological incubator at 37 °C.
7. Luria Bertani (LB) medium sterilized by autoclave and supplemented with glucose and ampicillin to a final concentration of 1% and 100  $\mu$ g/mL, respectively.
8. Shaking incubator at 37 °C and 200 r.p.m.
9. Spectrophotometer.
10. Centrifuge with rotor for Eppendorf tubes.
11. 200 mg/mL Isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) stock solution, sterilized by filtration. Store at  $-20$  °C.

12. Refrigerated centrifuge with rotor for 250-mL centrifuge flasks.
13. Phosphate buffered saline (PBS): pH 7.3, 140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> sterilized by autoclave.

*2.1.2 Bacterial Cell Lysis and Recovery of Total Protein Crude Extract*

1. Sterile 15-mL and 50-mL conical tubes.
2. Sterile PBS pH 7.3 (*see item 13* in Subheading [2.1.1](#)).
3. 50 mg/mL Lysozyme stock solution sterilized by filtration. Store at  $-20^{\circ}\text{C}$ .
4. 10 mg/mL RNase A stock solution. Store at  $-20^{\circ}\text{C}$ .
5. 100 mM Phenylmethanesulfonyl fluoride (PMSF) stock solution prepared in isopropanol. Store at  $-20^{\circ}\text{C}$  (*see Note 2*).
6. Shaking incubator at  $30^{\circ}\text{C}$  and 200 r.p.m.
7. Branson sonifier 250 with microtip.
8. Centrifuge with a rotor for 50-mL tubes, which allows centrifugation at  $16,000 \times g$ .
9. High speed centrifuge tubes.
10. 5 mg/mL DNase I stock solution prepared by dissolving DNase I powder in 0.15 M NaCl.
11. 0.45  $\mu\text{m}$  filters and 1.5-mL Eppendorf tubes.
12. 12% SDS-polyacrylamide gels.
13. 6 $\times$  Sample buffer: 375 mM Tris-HCl pH 6.8, 9% SDS, 50% glycerol, 9%  $\beta$ -mercaptoethanol and 0.03% bromophenol blue. Store at  $-20^{\circ}\text{C}$ .
14. Tris-glycine running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS.
15. Protein molecular weight marker. Store at  $-20^{\circ}\text{C}$ .
16. Heating block.
17. Electrophoresis chamber for polyacrylamide gels.
18. Power supply.
19. Coomassie brilliant blue R250 solution.
20. Orbital shaker.
21. Destaining solution: 40% ethanol and 10% acetic acid in water.
22. Fixation solution: 10% ethanol and 3% glycerol in water.

*2.1.3 Purification of Recombinant CspA from Total Protein Crude Extracts*

1. AKTAprime plus chromatography system.
2. GSTrap FF 5-mL column.
3. GSTrap FF 1-mL column.
4. HiPrep 16/60 Sephacryl S-100 HR column.

5. Ultrapure water, sterilized by autoclave and degassed.
6. 20% ethanol solution sterilized by autoclave and degassed.
7. Binding Buffer: degassed sterile PBS pH 7.3 (*see item 13* in Subheading 2.1.1).
8. PreScission Protease buffer: 50 mM Tris-HCl pH 7, 150 mM NaCl, 1 mM EDTA, 1 mM DTT sterilized by autoclave and degassed.
9. PreScission Protease.
10. PreScission Protease mix: mix 100  $\mu$ L (200 units) of PreScission Protease with 4.9 mL of PreScission Protease buffer at 4 °C.
11. 5-mL syringe with Luer tip.
12. 1.5-mL Eppendorf tubes and 15-mL conical tubes.
13. Elution buffer: 50 mM Tris-HCl pH 8, 10 mM reduced glutathione sterilized by autoclave and degassed.
14. Gel Filtration buffer: 20 mM Tris-HCl pH 7.4, 500 mM NaCl.
15. Slide-A-Lyzer Dialysis Cassettes.
16. 0.22  $\mu$ m filters.
17. CspA Storage buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA, 50 mM potassium chloride and 10% glycerol.
18. Bio-Rad protein assay.
19. 96-well standard plates.
20. MultiSkan EX (Labsystems) or any other equivalent microplate photometer.

**2.2 Assessment  
of the RNA Chaperone  
Activity  
with a Molecular  
Beacon**

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 mM Tris-HCl pH 7.5 and 1 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 Subheading **2.1.3**).
9. 10× reaction buffer: 100 mM Tris–HCl pH 7.5, 300 mM KCl, 200 mM NH<sub>4</sub>Cl, 15 mM DTT, 50 mM MgCl<sub>2</sub> (*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.

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### 3 Methods

#### 3.1 Purification of the Recombinant RNA Chaperone CspA

##### 3.1.1 Growth of Bacteria Expressing the GST-CspA Fusion Protein

1. Streak the *E. coli* BL21 (DE3) pGEX-6P-2::*cspA* strain in an LB agar plate supplemented with 100 μg/mL ampicillin and incubate at 37 °C overnight.
2. Inoculate a colony of the previous culture into a sterile test tube containing LB medium supplemented with 100 μg/mL ampicillin and 1% glucose. Grow culture at 37 °C and 200 r.p.m. overnight.
3. Inoculate 500 μL of the bacterial preculture (1/1000 dilution factor) into two sterile pre-warmed 2-L Erlenmeyer flasks containing 500 mL of LB medium supplemented with 100 μg/mL ampicillin and 1% glucose. Mix and incubate the cultures at 37 °C and 200 r.p.m. until an optical density (OD<sub>600nm</sub>) of 0.5 is reached.
4. Induce the expression of CspA by addition of IPTG to a final concentration of 0.4 mM. Save 1 mL of culture of one of the flasks and centrifuge it for 3 min at 18,000 × *g*. Store the bacterial pellet at –20 °C. This aliquot sample corresponds to the pre-induction control (*see* **Note 9**). Resume bacterial growth for another 5 h at 37 °C and 200 r.p.m.
5. Save 1 mL of culture of one of the flasks and centrifuge it for 3 min at 18,000 × *g*. Store the bacterial pellet at –20 °C (post-induction control) (*see* **Note 9**). Harvest the rest of the cultures in 250-mL tubes and centrifuge for 10 min at 5000 × *g* (*see* **Note 10**). Discard the supernatant and resuspend the pellets in 1 volume of PBS pH 7.3. Repeat the centrifugation step, discard the supernatant and store the bacterial pellets at –80 °C (*see* **Note 11**).

##### 3.1.2 Bacterial Cell Lysis and Recovery of Total Protein Crude Extract

1. Thaw the bacterial pellets, resuspend them in 25 mL PBS pH 7.3 in 50-mL conical tubes (per pellet) and add lysozyme, RNase and PSMF to a final concentration of 1 mg/mL, 10 μg/mL, and 1 mM, respectively. Incubate the samples for 30 min at 30 °C and 200 r.p.m.



2. Sonicate the samples on ice as follows: 3 cycles of 30 s power 4, 2 cycles of 30 s power 5. Leave the samples on ice for 1 min in between cycles.
3. Centrifuge the samples at  $16,000 \times g$  for 30 min at 4 °C (*see Note 10*). Transfer the supernatant (soluble fraction) to new tubes and store the pellet at  $-20$  °C. Pellets (insoluble fraction) contain inclusion bodies (IB), and constitute the IB control (*see Note 9*).
4. Supplement the soluble fraction with DNase I and RNase A to a final concentration of 10  $\mu\text{g}/\text{mL}$  and 5  $\mu\text{g}/\text{mL}$ , respectively, and incubate on ice for 30 min. Store 50  $\mu\text{L}$  of the sample at  $-20$  °C (pre-filtered soluble fraction control) (*see Note 9*).
5. Filter the soluble fraction using a 0.45  $\mu\text{m}$  filter whilst on ice (*see Note 12*). Store 50  $\mu\text{L}$  of the sample at  $-20$  °C (post-filtered soluble fraction control) and the rest of the soluble fraction at  $-20$  °C (*see Note 9*).
6. Mix aliquots of the different control samples (pre-induction control, post-induction control, IB control, pre-filtered soluble fraction and post-filtered soluble fraction), collected in the previous steps (*see Note 9*), with  $6\times$  sample buffer to a final concentration of  $1\times$ . Denature mixtures at 95 °C for 5 min and load them in a polyacrylamide gel (a Molecular Weight Marker should be included) (*see Note 13*). Run the gel with  $1\times$  running buffer at 130 V until the front reaches the bottom of the gel (*see Note 14*).
7. Stain the gel with Coomassie blue for at least 4 h at room temperature on an orbital shaker. Destain the gel with several washes of destaining solution at room temperature and shaking. Once protein bands are visible and the background level is low, incubate the gel with fixing solution for 15 min at room temperature and shaking. Optimal results are reached when most of the GST-CspA fusion protein appears in the soluble fraction and not in the inclusion bodies fraction (*see Note 15*).

### 3.1.3 Purification of the Recombinant CspA Protein

1. Thaw the post-filtered soluble fraction and purify the GST-CspA fusion protein with the use of a GSTrap FF 5-mL column and an AKTAprime plus chromatography system, following the recommendations of the manufacturer.
2. Clean the system with 20% ethanol and ultrapure water.
3. Connect the column to the AKTAprime plus system “drop to drop” to avoid introducing air into the column. Equilibrate the column with 25 mL of binding buffer at a flow rate of 5 mL/min.
4. Apply the sample at a flow rate of 0.2 mL/min (*see Note 16*).

5. Wash the column with 50 mL of binding buffer at a flow rate of 5 mL/min.
6. Equilibrate the column with 50 mL of PreScission Protease buffer at a flow rate of 5 mL/min and disconnect the column from the AKTAprime plus chromatography system.
7. Prepare the PreScission Protease mix at 4 °C and load it manually onto the column using a syringe at a flow rate of 1 mL/min. Seal the column with the top and bottom stop plugs and incubate overnight at 4 °C.
8. Connect a GSTrap FF 1-mL column to the AKTAprime plus system and equilibrate it with 5 mL of PreScission Protease buffer at a flow rate of 1 mL/min.
9. Place the GSTrap FF 5-mL column on top of the GSTrap FF 1-mL column. This tandem column scheme acts as a filter to capture any released cleaved GST proteins, uncleaved GST-tagged proteins and unbound PreScission Protease. Elute CspA with 15 mL of PreScission Protease buffer at a flow rate of 1 mL/min. Collect 1 mL fractions containing the CspA protein and place them on ice.
10. Elute the GST and GST-PreScission Protease from the columns with 30 mL of elution buffer at a flow rate of 1 mL/min. Clean the system and columns with ultrapure water and 20% ethanol and remove columns from the system.
11. Dialyze the CspA fractions against Gel Filtration buffer using a Slide-A-Lyzer Dialysis Cassette overnight at 4 °C. Collect CspA from the Dialysis Cassette and filter the solution using a 0.22 µm filter. Keep the CspA sample on ice until its purification by size exclusion chromatography.
12. Connect a HiPrep 16/60 Sephacryl S-100 HR Column (*see Note 17*) to the AKTAprime plus system “drop to drop” to avoid introducing air into the column. Equilibrate the column with 60 mL of ultrapure water at a flow rate of 0.5 mL/min and then with 240 mL of Gel Filtration buffer at a flow rate of 1 mL/min.
13. Inject the CspA sample into the column and run it with 120 mL of Gel Filtration buffer at a flow rate of 0.5 mL/min. Collect 3 mL fractions and place them on ice.
14. Clean the column with 480 mL of ultrapure water and 480 mL of 20% ethanol at a flow rate of 1 mL/min. Remove the column from the system and clean the system with ultrapure water and 20% ethanol.
15. To select fractions containing CspA, mix an aliquot of each peak fraction with sample buffer 6× and perform a 12% PAGE as described above.

16. Load the CspA selected fractions into a Slide-A-Lyzer Dialysis Cassette and dialyze against CspA Storage buffer overnight at 4 °C.
17. To assess protein purity, mix an aliquot of the recombinant CspA chaperone with sample buffer 6× and perform a 12% SDS-polyacrylamide gel electrophoresis (PAGE) as described above.
18. Determine the recombinant protein concentration by the Bio-Rad protein assay.

### **3.2 Assessment of the RNA Chaperone Activity with a Molecular Beacon**

#### *3.2.1 Molecular Beacon Design*

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 and (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*).

#### *3.2.2 Testing the Effectiveness of the Designed MB and Setup of the Working Conditions*

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:

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

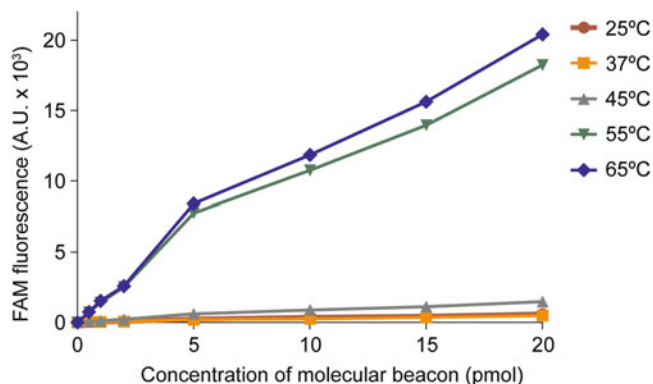
**Table 1**  
**Preparation of dilution mixes for testing MB effectiveness<sup>a</sup>**

	MB concentration (pmol)							
	0	0.5	1	2	5	10	15	20
MB 10 $\mu$ M	–	–	–	–	0.5	1	1.5	2
MB 1 $\mu$ M	–	0.5	1	2	–	–	–	–
CspA storage buffer <sup>b</sup>	12.5	12	11.5	10.5	12	11.5	11	10.5
10 $\times$ 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 $\mu$ L							

<sup>a</sup>Volumes of each reactive are indicated in  $\mu$ L

<sup>b</sup>Since the CspA protein is diluted in CspA storage buffer, the MB effectiveness test is performed including this buffer

2. Program the AriaMx thermal cycler to incubate the MB samples as follows: 37 °C, 5 min; 45 °C, 5 min; 55 °C, 5 min; and 65 °C during 5 min (*see Note 23*). Register the emission of FAM fluorescence every minute.
3. 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.
4. Seal the plate with an optically clear adhesive film (*see Note 5*) and load it into the thermal cycler. Start the incubation program.
5. 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

### 3.2.3 Determination of the RNA Chaperone Activity Using the Designed MB

1. 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*).
2. Program the AriaMx thermal cycler to incubate the MB samples as follows: 37 °C, 5 min; PAUSE, 37 °C, 15 min; PAUSE, 37 °C, 30 min; 65 °C, 10 min; STOP (Table 2). Register the fluorescence emission every minute (*see Note 25*).
3. 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.
4. Seal the plate with adhesive film (*see Note 5*) and load it into the thermal cycler. Start the incubation program.
5. 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.
6. Reintroduce the plate into the thermal cycler and continue the incubation at 37 °C during 15 min. Register the fluorescence emission every minute.
7. During the second incubation pause, pull out the plate, remove the adhesive film and add 10  $\mu$ L of proteinase K (20 mg/mL). Re-seal the plate with a new adhesive film. This step must be performed swiftly.
8. Reintroduce the plate into the thermal cycler and continue the incubation for 30 min at 37 °C and then increase the temperature up to 65 °C during 10 min. Register fluorescence emission

**Table 2**  
**Determination of RNA chaperone activity: preparation of reaction mixes<sup>a</sup>**

	Samples <sup>b</sup>							
	1	2	3	4	5	6	7	8
MB tube labelling	–	+	+	+	+	+	+	+
CspA tube labelling	–	–	+	+	+	–	–	–
BSA tube labelling	–	–	–	–	–	+	+	+
Water (Vf: 100 $\mu$ L)	39	38	38	38	38	38	38	38
CspA storage buffer	50	50	30	15	–	30	15	–
10 $\times$ reaction buffer	10	10	10	10	10	10	10	10
MB 1 $\mu$ M	–	1	1	1	1	1	1	1
Ribolock 4 U/ $\mu$ L	1	1	1	1	1	1	1	1
Seal the plate with adhesive film								
Incubate 37 °C—5 min								
Register fluorescence emission every minute								
<i>PAUSE incubation program<sup>c</sup></i>								
CspA stock (~200 $\mu$ M)	–	–	20	35	50	–	–	–
BSA stock (~200 $\mu$ M)	–	–	–	–	–	20	35	50
Re-seal the plate with adhesive film								
Incubate 37 °C—15 min								
Register fluorescence emission every minute								
<i>PAUSE incubation program</i>								
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								

<sup>a</sup>Volumes of each reactive are indicated in  $\mu$ L<sup>b</sup>Replicates of samples should be included<sup>c</sup>If the thermal cycler software allows it, the entire incubation protocol can be pre-programmed including the corresponding PAUSE times

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

9. 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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## 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 *cspA* 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 synthesized 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.
6. Storage of the 10× 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  $-80^{\circ}\text{C}$  for several days.
12. We recommend the use of filters with a pore size of  $0.45\ \mu\text{m}$  instead of  $0.2\ \mu\text{m}$  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 programmed 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.

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