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Quality Control Certification of RNA Aptamer-Based Detection

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Aptamers are single-stranded DNA or RNA molecules with a defined tertiary structure for molecular recognition. Numerous RNA aptamers with excellent binding affinity and specificity have been reported; they constitute an attractive reservoir of molecular recognition elements for biosensor development. However, RNA is relatively unstable owing to spontaneous hydrolysis and nuclease degradation. Thus, RNA aptamer-based biosensors are prone to producing false-positive signals. Here, we present an RNA aptamer biosensor design strategy that utilises an internal control to distinguish target binding from false-positive signals. The sequence of a chosen RNA aptamer is expanded so that it can form three consecutive short RNA–DNA duplexes with 1) a quencher-labelled DNA strand (Q_1DNA), 2) a dual-fluorophore-labelled DNA strand (F_1DNAF_2) and 3) another quencher-labelled DNA strand (Q_2DNA). The addition of a target releases Q_2DNA from the duplex assembly, and produces the expected positive signal from F_2. However, the authenticity of target recognition is validated only if no signal is generated from F_1. We have successfully engineered two fluorescent reporters by using an RNA aptamer that binds thrombin and one that binds theophylline. Both reporters show the expected binding affinity and specificity, and are capable of reporting system malfunction when treated with nucleases and chemical denaturants. This strategy provides a simple and reliable way to ensure high-quality detection when RNA aptamers are employed as molecular-recognition elements.

Introduction

RNA is a functionally versatile biopolymer from which numerous molecular recognition elements, known as aptamers, have been derived in different ways. Nature, for instance, has employed a host of RNA aptamers to construct riboswitches to regulate gene expression.[1] With the development of the in vitro selection technique, a large battery of artificial RNA aptamers have been isolated from random-sequence RNA libraries to recognise wide-ranging targets.[2] Many naturally occurring and artificially developed RNA aptamers exhibit excellent binding affinity and specificity, thus making them attractive for biosensor development.[3] However, reports on exploring RNA aptamers for biosensing applications are relatively limited, largely owing to the poor chemical stability of RNA and its susceptibility to RNase degradation. To harness RNA aptamers as sensing elements, we previously developed a general approach to create structure-switching reporters from RNA aptamers.[4] More recently, we have shown that entrapment in sol-gel-derived materials conferred protection from nuclease degradation on these RNA-based reporters.[5] Nucleotide chemical modifications, spiegelmers and locked nucleic acids (LNAs) are other established methods of RNA protection.[6]

Working with RNA aptamer-based biosensors requires appropriate experimental controls to ensure the validity of the detection results. Although external experiments can be implemented to assess RNA integrity,[7] a biosensing strategy that incorporates a simple internal control for continuous monitoring of RNA quality would be highly beneficial. Many biosensing designs, such as structure-switching reporters, require the formation of DNA–DNA or RNA–DNA duplexes as critical structural elements.[4] Unintentional structural denaturation, however, can occur from a variety of factors, such as altered pH and suboptimal ion strength. RNA is also prone to spontaneous transesterification,[8] and degradation by ubiquitous RNases.[6] Therefore, having a simple internal control that can detect system malfunction would ensure the reliability and reproducibility of RNA aptamer-based sensing.

Herein, we present an aptamer biosensor design strategy that exploits an internal control as a quality control (QC) element to distinguish target binding from false-positive signals in real time. The sensing system consists of a multifunctional RNA (mfRNA) with three sequence domains (Figure 1 A): the first forms a duplex with a quencher-labelled DNA strand (Q_1DNA), the second forms a duplex with a dual-fluorophore-labelled DNA strand (F_1DNAF_2), and the third is an RNA aptamer sequence capable of forming a duplex with another quencher-labelled DNA strand (Q_2DNA). The addition of target releases Q_2DNA from the duplex assembly, and produces an expected signal from F_2 (Figure 1 B). Incorporation of the Q_1F_1

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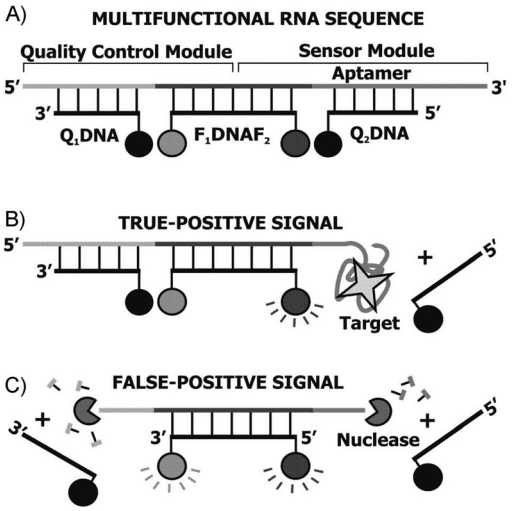

Figure 1. Structure-switching RNA aptamers with a quality-control element. A) Duplex assembly between a multifunctional RNA with Q_1DNA, F_1DNAF_2 and Q_2DNA. B) True-positive signal. Addition of the target (star) results in target–aptamer complex formation and the departure of Q_2DNA, which is accompanied by signal generation from F_2. C) False-positive signal. System destruction, for example, by a nuclease, would lead to signal production from both F_1 and F_2.

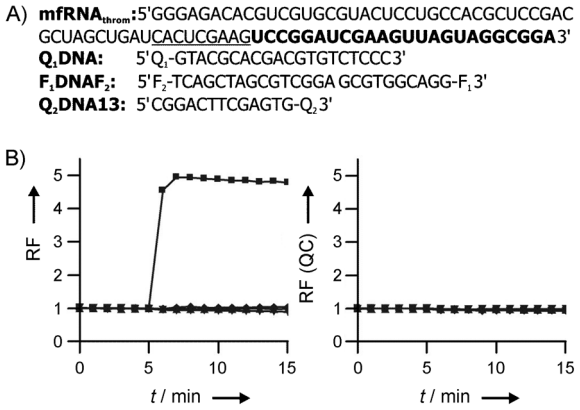

Figure 2. A thrombin reporter. A) The sequences of mfRNA, Q_1DNA, F_1DNAF_2 and Q_2DNA used. B) Specificity test. The signalling duplex was incubated for 5 min, then thrombin (■) or prothrombin (▲, 3 μM) was added. Mutants 1 (▼) and 2 (◆), modified mfRNAs with mutations in the aptamer domain, were both tested with thrombin. RF: relative fluorescence from F_2 (sensor module, left) and F_1 (QC module, right) , calculated as F_t/F_0, fluorescence intensity at time t and time 0. The data are the average of three independent experiments.

pair allows true-positive signals to be distinguished from false-positives that can arise from unexpected system malfunctions, such as the destruction of the DNA/RNA assembly by nucleases (Figure 1C). According to this strategy, we have successfully engineered two fluorescent reporters by using an RNA aptamer that binds thrombin and one that binds theophylline.

Results and Discussion

Construction of an aptamer reporter for thrombin

As the first demonstration, we chose an RNA aptamer that recognises human α-thrombin,[9] a serine protease that plays a critical role in the regulation of thrombosis, and haemostasis.[10] The DNA sequence encoding both the QC and aptamer elements was chemically synthesised, and amplified through polymerase chain reaction (PCR) with necessary primers to produce a double-stranded DNA template, which was then used for in vitro transcription to generate the required $mfRNA_{throm}$ (its sequence is given in Figure 2A, along with those of the relevant chromophore-containing DNA molecules). $mfRNA_{throm}$ was designed to form a 20 bp duplex with Q_1DNA, and a 25 bp duplex with F_1DNAF_2.

To determine the optimum length of Q_2DNA for the third duplex, which is critical to the sensing performance of the system, we examined thermal-denaturation profiles of the duplex assemblies with Q_2DNAs containing 13–16 nt (Figure S1A). As the 13-nt Q_2DNA (Q_2DNA13) produced the best signal enhancement with a relatively low melting point (which should facilitate the displacement of Q_2DNA by thrombin), this molecule was chosen for further study. Note that we incorporated several non-aptameric nucleotides (underlined letters) immediately upstream of the aptamer domain (boldfaced letters). This feature, which was established in our previous studies,[4] allows the formation of a stable Q_2DNA–mfRNA duplex without sequestering many nucleotides in the aptamer domain that are involved in target recognition (Figure 2A).

Sensing performance of the thrombin reporter

The aptamer reporter produced an approximately fivefold increase in intensity at 665 nm upon the addition of thrombin (Figure 2B, left); the fluorescence of TYE665 as F_2 was indicative of Q_2DNA departure from the duplex assembly. The high-level signal enhancement from F_2 confirms that the reporter is capable of thrombin detection. Two experiments were conducted to verify specific molecular recognition between the aptamer and thrombin. First, prothrombin, a precursor of thrombin that has no affinity for the original RNA aptamer,[9] was tested. No signal enhancement was observed at F_2 (Figure 2B, left). Second, two mutated versions of mfRNA sequences, in which selective nucleotides critical for thrombin recognition were altered (Figure S2A), were examined. Neither mutant was able to register a signal increase at F_2 (Figure 2B, left). To assess the sensitivity of the reporter, the relative fluorescence from F_2 was determined at various concentrations of thrombin (Figure S3A, left). The detection limit was found to be 0.01 μM, and the dynamic detection window was between 0.01–3 μM.

Functionality test of the QC element

Two scenarios are expected of the QC element with regard to signal generation: when the reporter is fully functional, no signal change from F_1 should be observed; however, the observation of notable fluorescence changes from both F_1 and F_2 indicates system malfunction.

No intensity increase was observed at 520 nm (Figure 2B, right and Figure S3A; fluorescence from fluorescein as F_1 in the QC element) in the presence of the intended target, thrombin. However, when the system was treated with RNase A, DNase I and NaOH, notable signal enhancement for both F_2 (Figure 3A), and F_1 (Figure 3B) was observed. These results validate the complete function of the thrombin reporter.

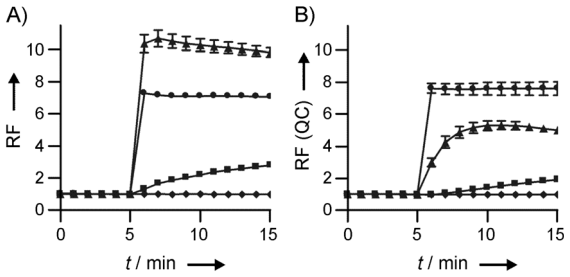

Figure 3. False-positive signal reporting. The signalling duplex was incubated for 5 min, then RNase A (▲), DNase I (■), or NaOH (●) was added. For the negative control (◆) no denaturant was added. Relative fluorescence (RF) was measured for A) F_2 and B) F_1. Data are the average of two independent experiments.

Construction of an aptamer reporter for theophylline

To demonstrate the generality of the approach, we designed a second reporter by using the well-characterised anti-theophylline RNA aptamer.[3d,4b] We directly incorporated many design features shown to be effective for the thrombin reporter into the construction of the theophylline reporter. As shown in Figure 4A, the theophylline reporter used the same Q_1DNA and F_1DNAF_2. The mfRNA sequence ($mfRNA_{theo}$) possessed the same non-aptameric nucleotides upstream of the aptamer domain (underlined letters), and only differed in the actual aptamer sequence (boldfaced letters). To determine the optimal Q_2DNA length, we examined the thermal denaturation profiles of four different Q_2DNA sequences (13–16 nt) in duplex with $mfRNA_{theo}$, F_1DNAF_2 and Q_1DNA (Figure S1B). As for the thrombin reporter, the optimum length of Q_2DNA for the theophylline system was determined to be 13 nt (Q_2DNA13; best signal enhancement with relatively low melting point), and this sequence was selected for further experiments.

A) **$mfRNA_{theo}$:** 5'GGGAGACACGUCGUGCGUACUCCUGCCACGCUCCGACG CUAGCUGAUCACUCGAAGGGCGAUACCAGCCGAAAGGCCCUUGG CAGCGUCCAACACAUCG3'
Q_1DNA: 5'Q_1-GTACGCACGACGTGTCTCCC3'
F_1DNAF_2: 5'F_2-TCAGCTAGCGTCGGAGCGTGGCAGG-$F_1$3'
Q_2DNA13: 5'CGCCCTTCGAGTG-$Q_2$3'

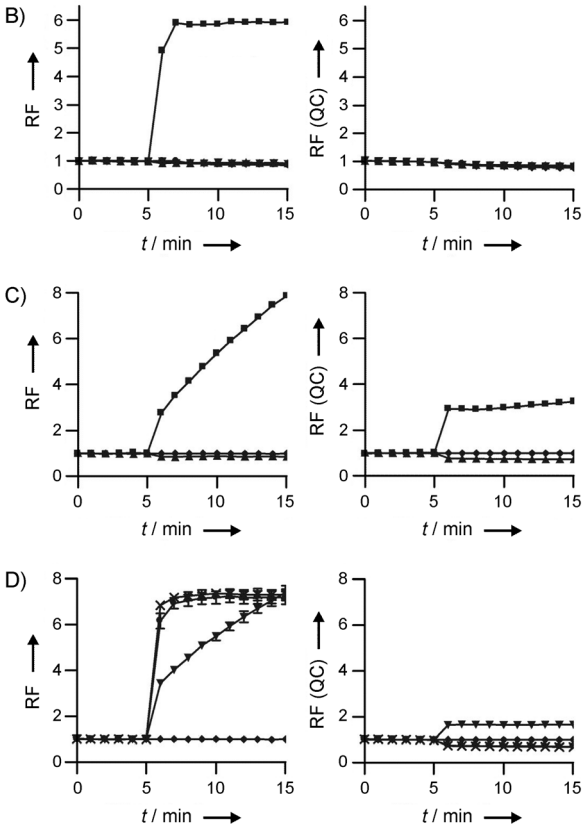

Figure 4. A theophylline reporter. A) The sequences of mfRNA, Q_1DNA, F_1DNAF_2 and Q_2DNA used. B) Specificity test. The signalling duplex was incubated for 5 min then theophylline (■), caffeine (▲) or theobromine (▼; 1 mM) was added. Mutants 1 (◆) and 2 (●), modified mfRNAs with mutations in the aptamer domain, were both tested with theophylline. RF from F_2 (sensor module, left) and F_1 (QC module, right) are presented (same layout for subsequent panels). C) and D) Tests in human serum comparing the effects produced by the addition of unfiltered and filtered human serum doped with theophylline. C) ■: serum, ▲: filtered serum, ◆: negative control; D) ▼: serum + target, ×: filtered serum + target, ●: filtered target only, ◆: negative control.

Sensing capability of the theophylline reporter

Consisting of $mfRNA_{theo}$-Q_1DNA-F_1DNAF_2-Q_2DNA, the theophylline reporter generated an approximately sixfold signal enhancement at 665 nm upon addition of theophylline (Figure 4B, left). This F_2 signal was verified to be target-specific, as addition of the closely related structural derivatives caffeine or theobromine (Figure 4B, left) generated no signal at F_2. Likewise, the F_2 signal increase from theophylline addition was also demonstrated to be aptamer sequence-specific, as mutations of critical recognition nucleotides in the mfRNA sequence (Figure S2B) abolished the binding activity, as shown by the absence of F_2 signal for mutants 1 and 2 (Figure 4B, left). After evaluation of the sensitivity of the reporter (Figure S3B, left), the detection limit was determined to be 1 μM, and the dynamic detection window was between 1–1000 μM.

Test of QC element function within the theophylline reporter

No F_1 fluorescent increase was observed at 520 nm in assessment of the specificity and sensitivity of the theophylline reporter (Figure 4B, right and Figure S3B, right); this verifies the ability of the QC element to detect true-positive signals. To test for false-positive signalling, we evaluated the performance of the theophylline reporter in human serum, a medium routinely used in medical testing, and a known source of nucleases. The addition of serum produced signal enhancement for F_2,

as well as F_1 (Figure 4C), thus signifying separation of the fluorophores from their respective quenchers. This observation was confirmed to be the result of nuclease degradation, as filtration of the serum on a 10 kDa molecular weight cut-off column before addition to the reporter removed nucleases on the basis of molecular size and eliminated both F_2 and F_1 signals (Figure 4C). Together, these results validate the complete function of the theophylline reporter. Notably, a small signal decrease in F_1 and F_2 was produced upon the addition of filtered serum. Given the complex components found in serum, smaller molecules (peptides, hormones, small molecules, electrolytes, etc.) can still pass through the filter, and cause slight fluctuations in fluorescence due to molecular crowding of the fluorophores.[11]

Elimination of false-positive signals guided by QC element

We have already established that the QC element was able to detect the degradation effects of serum in the theophylline reporter (Figure 4C). False-positive signalling was also exhibited when testing the effects of serum and theophylline together (as would be the case in clinical blood testing for drugs and other substances). Here, we found that any theophylline-binding effects were "masked" by the F_2 and F_1 signals produced by serum degradation (Figure 4D). To correct this detection error, we removed contaminating nucleases by membrane column filtration, while retaining theophylline. By filtering serum doped with theophylline before addition, we were able to accurately detect theophylline (F_2 signal enhancement from aptamer-sensing module; Figure 4D, left), without any system malfunction (absence of F_1 signal from QC element; Figure 4D, right). Furthermore, we found that, after filtration, serum did not alter the signalling ability of the reporter, as both the filtered serum plus target sample (Figure 4D, left), and the filtered target-only sample (Figure 4D, left) produced the same magnitude of F_2 signal.

The thrombin reporter was also able to detect the degradation effects of serum, as high F_2 and F_1 signals were generated (Figure S4). However, given that thrombin possesses a molecular weight in the same range as those of most nucleases, simple column-filtration of the protein–serum mixture would not be a feasible remedy method for the detection of protein targets. In this case, adding RiboLock RNase inhibitor to the target–serum mixture successfully inactivated most nucleases, and the F_1 signal was substantially reduced compared to that of the serum sample without treatment with nuclease inhibitor (Figure S4, right). The likely reason why not all the F_1 signal was abolished is the capability of RiboLock to inhibit common nucleases, but not all. Further studies could benefit from the use of a cocktail of common nuclease inhibitors to inactivate a wider range of nucleases in complex samples.

Conclusions

In summary, we have demonstrated a new general approach to creating RNA aptamer-based biosensors that exploit an internal quality control element to distinguish target binding from false-positive signals. The sensing strategy is based on a multifunctional RNA sequence that forms a duplex with one dual-fluorophore-modified oligonucleotide and two quencher-modified DNA oligonucleotides, which function in coordination to form the QC element and sensor module. To the best of our knowledge, this is the first example of an aptamer-based sensor that is capable of monitoring the quality of detection.

The QC element was able to report true-positive signals for both reporters (production of an F_2 signal and lack of an F_1 signal) and also reliably reported system malfunction when treated with known chemical denaturants and nucleases (production of both F_1 and F_2 signals). These results demonstrate the utility of the QC element and showcase the generality of the design strategy. In testing the human serum samples doped with theophylline, we discovered that simple filtration of the drug–serum mixture prior to addition to the theophylline reporter removed all contaminating nucleases, but retained the drug target for detection. This approach is likely to be applicable for the detection of other small-molecule targets in serum. However, for the detection of protein targets, which have molecular weights in the same range as most nucleases, simple filtration would not be a feasible method. One possible solution that we have demonstrated is to treat protein–serum mixtures with common nuclease inhibitors. Hence, incorporation of a QC element will be useful to help identify remedy methods to eliminate false-positive signals.

Generating aptamers that are intrinsically resistant to degradation would be an attractive option to avoid the need for remedy methods altogether. Chemical modification of nucleotides can prolong the lifetime of RNA aptamers for several days,[6] and sol–gel entrapment of RNA structure-switching reporters has demonstrated long-term chemical stability of up to one month.[5] However, generating modified RNA libraries for SELEX remains expensive, and some modified nucleotide building blocks are not commercially available. Furthermore, some modifications can only be incorporated into aptamers post-SELEX, and this can alter aptamer binding properties. This is especially the case for naturally occurring RNA aptamers, which offer a plethora of possibilities for biosensor development, yet still remain relatively underexplored. To utilise these aptamer species for biosensing applications, having a QC element can ensure the validity of the results obtained and provide greater user confidence.

Structure-switching reporters have been expanded to detect many small-molecule and protein targets,[12] and applied in many secondary applications, such as enzymatic reaction monitoring and inhibitor screening,[13] designing nanodevices and solid-phase assays,[14] and in vivo sensing.[15] Additionally, the fluorescence detection platform has been expanded to include other signal-transduction methods such as electrochemical, and colourimetric detection.[16] It is conceivable that aptamer reporters with a QC element could be useful for many of the aforementioned purposes, as well as for other future applications in which high-quality detection is needed.

Experimental Section

Materials: All DNA oligonucleotides were synthesised by using automated DNA synthesis (Integrated DNA Technologies, Coralville, IA). F_1 and F_2 of F_1DNAF_2 are 3′-fluorescein and 5′-TYE665, respectively; F_2 was introduced by using TYE665 phosphoramidite, whereas F_1 was introduced by using fluorescein-derivatised controlled-pore glass (CPG). Q_1DNA contains a 5′-Iowa Black FQ dark quencher introduced by using Iowa Black FQ phosphoramidite. Q_2DNA contains a 3′-Iowa Black RQ dark quencher introduced by using Iowa Black RQ-derivatised CPG. Unmodified oligonucleotides were purified by 10% denaturing PAGE, and modified DNAs were purified by HPLC before use. Human α-thrombin, and human prothrombin were obtained from Haematological Technologies (Essex Jct., VT). Unless otherwise noted, all other materials were purchased from Sigma–Aldrich and used without further purification.

Polymerase chain reaction: The synthetic DNA template for the thrombin reporter has the sequence 5′-GAATTC TAATAC GACTCA CTATAG GGAGAC ACGTCG TGCGTA CTCCTG CCACGC TCCGAC GCTAGC TGATCA CTCGAA GTCCGG ATCGAA GTTAGT AGGCGG A-3′. It was amplified by PCR from primers 5′-GAATTC TAATAC GACTCA CTATA-3′ (forward) and 5′-TCCGCC TACTAA CTTC-3′ (reverse). The theophylline reporter has the sequence 5′-GAATTC TAATAC GACTCA CTATAG GGAGAC ACGTCG TGCGTA CTCCTG CCACGC TCCGAC GCTAGC TGATCA CTCGAA GGGCGA TACCAG CCGAAA GGCCCT TGGCAG CGTCCA ACACAT CG-3′ and was amplified from primers 5′-GAATTC TAATAC GACTCA CTATA-3′ (forward) and 5′-CGATGT GTTGGA CGC-3′ (reverse). PCR was conducted in Tris·HCl (pH 9.0, 75 mM), $MgCl_2$ (2 mM), KCl (50 mM), $(NH_4)_2SO_4$ (20 mM), primers (0.5 μM of each), DNA template (3 nM), dNTPs (0.5 mM of each) and Taq DNA polymerase (five units, Biotools, Madrid, Spain). Thermal cycling steps were 94 °C for 1 min, 20 cycles of 94–42–72 °C (30 s for each temperature), and finally 72 °C for 8 min.

RNA transcription: The transcription reaction was conducted at 37 °C for 3 h in Tris·HCl (150 μL, 40 mM, pH 7.9), $MgCl_2$ (6 mM), dithiothreitol (DTT, 10 mM), NaCl (10 mM), spermidine (2 mM), PCR-amplified DNA (25 pmol), NTPs (2.5 mM of each), RiboLock ribonuclease inhibitor (1.07 units μL^{-1}) and T7 RNA polymerase (1.33 units μL^{-1}, Fermentas, Burlington, Canada). The transcription mixture was then treated with DNase I (three units, Fermentas) in the presence of $CaCl_2$ (0.2 mM) at 37 °C for 15 min. The transcribed RNA was subsequently purified by 10% denaturing PAGE, and quantified by absorbance at 260 nm.

Fluorescence measurements: The signalling mixture contained F_1DNAF_2 (40 nM), extended RNA aptamer (80 nM), Q_1DNA (120 nM) and Q_2DNA (120 nM) in a binding buffer (50 μL total volume) corresponding to each aptamer. Thrombin buffer: Tris·HCl (pH 7.7, 50 mM), NaCl (100 mM), DTT (1 mM) and $MgCl_2$ (1 mM). Theophylline buffer: Tris·HCl (pH 7.5, 50 mM), $MgCl_2$ (20 mM). The mixture was first heated at 65 °C for 2 min, then cooled to room temperature for 10 min and stored at 4 °C until analysis. Fluorescence measurements were taken at a $\lambda_{ex}/\lambda_{em}$ of 645/665 and 495/520 nm every min at 37 °C (unless stated otherwise) on a Cary Eclipse spectrophotometer (Varian). Target-sensing assays were conducted by adding thrombin, theophylline or their structural analogues to signalling mixtures. For degradation assays, the following were introduced: 1) RNase A (0.1 μg μL^{-1}, Fermentas), 2) DNase I (0.06 units μL^{-1}, Fermentas) plus $CaCl_2$ (0.1 mM), 3) NaOH (50 mM) in Tris·HCl (pH 7.5, 50 mM) plus NaCl (300 mM) or 4) no addition (negative control). To test the effects of human serum on the theophylline reporter, the following were added to each signalling mixture: 1) serum (5 μL), 2) filtered serum (10 K filtration column, Sartorius Stedium Biotech), 3) theophylline (60 mM) in serum, 4) filtered theophylline (60 mM) in serum, 5) filtered theophylline (60 mM) or 6) no addition (negative control). To test the effects of human serum on the thrombin reporter, the following were added to each signalling mixture: 1) serum (1 μL), 2) RiboLock RNase inhibitor (4.8 units μL^{-1}) plus thrombin (3 μM) in serum, 3) no addition (negative control). Relative fluorescence (RF) was calculated by using F/F_0, where F_0 and F are the fluorescence intensity before and after addition of target/degrading agent, respectively. To obtain the thermal denaturation profiles, each signalling mixture was incubated at 65 °C for 5 min in the fluorimeter before the temperature was decreased to 20 °C at a rate of 1 °C min^{-1}.

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Keywords: aptamers · biosensors · fluorescence · RNA · structure switching

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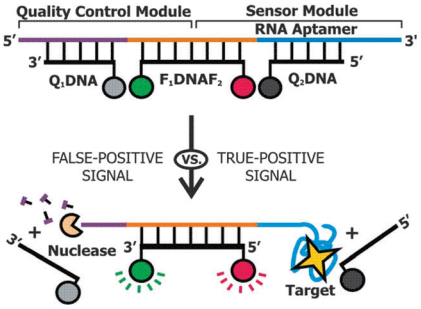

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Quality Control Certification of RNA Aptamer-Based Detection