

Novel fluorescence enhancement IgE assay using a DNA aptamer

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In this paper, we demonstrate a fluorescence immunoglobulin E (IgE) assay probe based on a DNA aptamer. A Texas red-labeled short DNA strand (T-DNA) complementary with part of the IgE aptamer sequence was used to produce the fluorescence enhancement effected upon the binding of IgE to the aptamer. Another short DNA strand labeled with dabcyI quencher (Q-DNA) complementary with part of the aptamer sequence nearby the T-DNA location was used to lower the background fluorescence. The IgE can be detected in the concentration range from 9.2×10^{-11} to 3.7×10^{-8} mol L⁻¹ with a detection limit of 5.7×10^{-11} mol L⁻¹.

1. Introduction

Nucleic acid-based target protein detection is a promising field in molecular biotechnology useful for the rapid and accurate identification of biospecies as well as the diagnosis of infections.^{1–3} The specificity of short synthetic DNA probes raises the hope of distinguishing small differences in sequence, ultimately achieving single nucleotide resolution. It is possible to select DNAs from a large, random pool that bind to a ligand of interest with SELEX (systematic evolution of ligands by exponential amplification) technology. Aptamers, the synthetic DNA/RNA oligonucleotides isolated through SELEX for their ability to selectively bind to interested biomolecules, possess properties that rival those of antibodies in both therapeutic and diagnostic applications.^{4,5} When binding takes place, the aptamer undergoes a conformational transition from an unfolded structure to a folded one. In most diagnosis applications, the specific molecular recognition of proteins is often accomplished by antibodies. Compared to enzymes or antibodies, the aptamers have some advantages, such as their facile synthesis and modification, similar sensitivities, and specificities and low cost. Furthermore, while antibodies are sensitive to temperature and denatured easily upon contact with surfaces, leading to limited shelf-lives, aptamers are stable during long-term storage. Proteins often suffer from issues related to control of orientation and denaturation during and after covalent immobilization,⁶ while modification of aptamers could be performed to conjugate functional groups to allow covalent binding to occur.⁷ Aptamers have already been employed in a range of analytical techniques,^{8–15} including fluorescence.¹⁶ Fluorescence spectroscopic methods offer high sensitivity and selectivity and applicability in non-separation detection and *in situ* monitoring.^{17,18} Fang *et al.*¹⁹ have applied a simple method based on a fluorescein-labeled aptamer for the detection of platelet-derived growth factor (PDGF) in homogeneous solutions. By means of fluorescamine, a donor fluor reacting with aliphatic amines, Merino and Weeks²⁰ synthesized a Texas red-labeled ATP aptamer containing a single

2'-amine substituted cytidine. Addition of ATP reduces the fluorescence intensity due to resonance energy transfer as the 2'-amine is constrained to a low reactivity state, thereby disfavoring formation of the fluorescamine. Another ATP aptamer developed by Rupcich *et al.*²¹ undergoes a structural switch in the presence of ATP to release a dabcyI (4-(4-dimethylaminophenylazo)benzoic acid)-labeled oligonucleotide strand, which in turn relieves the quenching of a fluorescein label that is covalently tethered to the aptamer rather than bound to a short complementary DNA strand.

With the increasing application of nucleic acid strategies for disease studies, the detection of immunoreaction-related proteins, especially immunoglobulins in serum, attracts increasing attention in biomedical research. Immunoglobulin E (IgE), found only in mammals, plays a key role in allergic responses. IgE is capable of triggering the most powerful immune reactions.²² Most of our knowledge of IgE has resulted from the research into the mechanisms of type-1 hypersensitivity.²³ Rapid detection of IgE is of great interest in dealing with patients with allergy-mediated disorders.^{24,25} It significantly increases in patients afflicted with allergic asthma, atopic dermatitis,²⁶ and other immune deficiency-related diseases, such as AIDS.²⁷

The aptamers are able to block the IgE regulated release of important mediators in allergic responses. A SELEX-based high-affinity D17.4 DNA aptamer has been isolated by Wiegand *et al.*²⁸ In order to stabilize the structure of the aptamer, Liss *et al.*¹³ extended both the 5'- and 3'-ends of D17.4 by adding 5'-GCGC-3' sequences, allowing the formation of a longer stem and, consequently, a more stable tertiary structure. The aptamer was subsequently improved by the rational design and as a result not only displayed equal sensitivity and selectivity compared to the antibody, but also retained the linear measurement range and reusability of the first aptamer. A label-free protein biosensor based on aptamer-modified carbon nanotube field-effect transistors (CNT-FETs) has been fabricated for the detection of IgE.²⁹ Gokulrangan *et al.*³⁰ developed an aptamer-based fluorescence polarization method designed to detect IgE levels in the range of 1–100 nM with a detection limit of 350 pM. These authors have chosen a Texas red conjugated with an 8-atom dye linker, where the aptamer was covalently linked to the dye molecule. They pointed out that the binding affinity of such

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labeled aptamers was not altered significantly as a result of the labeling process. It is desirable that the detection limit should be further lowered, as IgE is the least abundant of the immunoglobulins. In clinical samples, the concentration of IgE is found to be in the sub-nanomolar range (about 0.800 nM) in the blood serum of normal people.³¹ This is five orders of magnitude lower than the most abundant immunoglobulin, IgG, which is normally present at about 100 μ M in human serum.²⁶

Aiming to further decrease the detection limit of the IgE assay, we intended to use a short DNA strand labeling Texas red (T-DNA) complementary with part of the aptamer sequence comparing to the direct covalent linkage with the aptamer. The hybridization between the short T-DNA and the aptamer sequence should be more flexible. This construct might be beneficial for retaining the bioactivity of the aptamer when the secondary structure and the location binding IgE of the aptamer are not known exactly. Besides the major effect of binding between IgE and the aptamer on the fluorescence enhancement as practised by Gokulrangan *et al.*,³⁰ we tried to introduce another short DNA strand labeling dabcyI quencher (Q-DNA) complementary to another part of the aptamer sequence. It was thought that the hybridization of the Q-DNA with the aptamer in close proximity to the T-DNA would decrease the fluorescence background in the absence of IgE. In this way, besides the fluorescence enhancing effect of binding IgE, we expected to introduce an additional 'molecular beacon'-type effect in the design of a novel aptamer-based fluorescence probe for IgE with a substantially improved detection limit. In this paper, we report the results of the mentioned research previously. A picomole range detection limit for IgE was achieved which makes it possible to detect IgE levels 8 times lower than the IgE concentration in normal human serum without interference.

2. Results and discussion

2.1 Experimental principle

Fig. 1 demonstrates the concept of the duplex-to-complex structural transition coupled with fluorescence enhancement. Our assay consists of two separate steps: (1) in the presence of IgE (the ellipse), the structure of the aptamer is transformed into the IgE-aptamer duplex assembly; (2) a mixture of T-DNAs

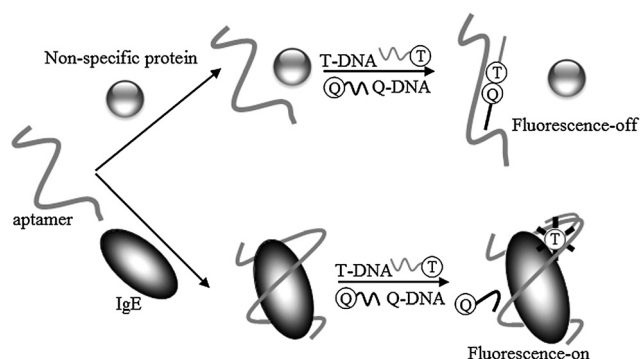


Fig. 1 Schematic of the interaction between IgE (the ellipse) and an unmodified aptamer (the long wavy bond) together with a Texas red-containing DNA (T-DNA, short oligonucleotide tagged with T) and a dabcyI-containing DNA (Q-DNA, short oligonucleotide tagged with Q).

(short oligonucleotide tagged with T) and Q-DNAs (short oligonucleotide tagged with Q) was added to the resulting solution. The unmodified aptamer (Fig. 1, the long wavy bond) contains a T-DNA-binding sequence and a Q-DNA-binding sequence. The T-DNA would hybridize with the corresponding complementary sections of the aptamer producing a strong concentration-dependent fluorescent signal. When the non-specific protein (Fig. 1, the sphere) was introduced in the blank experiment, the aptamer naturally binds to the T-DNA and Q-DNA, and the hybridization was followed by the fluorescence quenching of the Texas red.

2.2 Fluorescence enhancement of IgE binding

As shown in Fig. 2, the sample containing T-DNA in the absence (Fig. 2, curve a) or presence (curve b) of Q-DNA shows rather low fluorescence intensity with an emission peak around 610 nm. A significant fluorescence enhancement was observed when the aptamer was added into a solution containing T-DNA. It can be interpreted as that the binding of aptamer with T-DNA will bring a significant microenvironment change, resulting in detectable variations in the fluorescence values.¹⁹ Interestingly, the fluorescence value of the T-DNA/aptamer/Q-DNA complex (curve c) is lower than that of the T-DNA/aptamer duplex (curve e). In the former configuration, the T-DNA and Q-DNA are presumably located in close proximity to produce extensive quenching of the fluorescent probe. Compared with the sample containing T-DNAs, aptamer and Q-DNAs (curve c), the introduction of IgE sharply induced the increase of the fluorescence intensity (curve d). The analyte IgE itself does not contribute to the fluorescence. Namely, IgE might induce a change in the fluorescence signal using the present system which

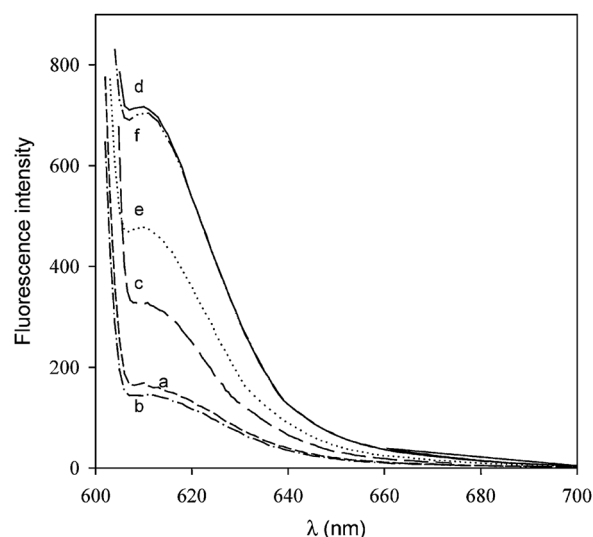


Fig. 2 Fluorescence spectra of solutions containing T-DNA (curve a), a mixture of T-DNAs and Q-DNAs (curve b), a complex of T-DNAs/aptamer/Q-DNAs (curve c), the mixture of T-DNAs, aptamer, IgE and Q-DNA (curve d), a duplex assembly of T-DNAs/aptamer (curve e), and a complex of T-DNAs/aptamer/IgE (curve f). The relative standard deviations obtained by five repeated measurements were not more than 5%.

seemed to be associated with the change of microenvironment around the fluorophore involved.¹⁹

As a control experiment, the fluorescence enhancement of the T-DNAs/aptamer duplex assembly (Fig. 2, curve e) and the T-DNAs/aptamer/IgE complex (curve f) was investigated. The hybridization reactions were carried out and the fluorescence spectra were recorded as described in the Experimental section. Shown in Fig. 2, although an obvious change of fluorescence intensity was triggered by IgE in the absence of Q-DNA, a higher fluorescence signal was obtained upon addition of the mixture of Q-DNA and T-DNA into the solution containing aptamer and IgE. Therefore, it is reasonable that the assay system in the presence of Q-DNA was utilized to evaluate the analytical characteristics of the proposed detection strategy due to its higher signal-to-background ratio than that in the absence of Q-DNA.

2.3 Experimental conditions of target detection

The order of the addition of DNA and IgE solution was investigated. The T-DNA/Q-DNA/aptamer solution was mixed with the IgE solution at 5 °C and incubated for 60 min. The fluorescence spectrum of the resulting solution was shown as curve a in Fig. 3. Curve b of Fig. 3 was the fluorescence spectrum of another sample prepared by mixing IgE/aptamer solution with T-DNA/Q-DNA solution under the identical conditions. One can see that the fluorescence intensity of curve b is slightly higher than curve a, indicating that the single aptamer exhibited more excellent bioactivity on binding IgE in comparison with the complex of T-DNA/aptamer/Q-DNA. The protocol by mixing IgE/aptamer solution with T-DNA/Q-DNA solution can offer a better response signal.

The dependence of the fluorescence signal on the incubation temperature was investigated, and the results are shown in Fig. 4. In the absence of IgE (Fig. 4, white bars), the assay showed a low

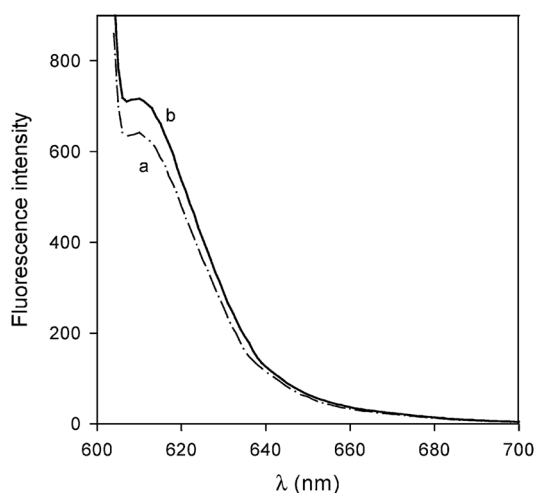


Fig. 3 Fluorescence spectra recorded with different order of the addition of DNA or IgE solution. IgE solution was added to the mixture of T-DNA, Q-DNA and aptamer (curve a); T-DNA/Q-DNA solution was added to the mixture of aptamer and IgE (curve b). The relative standard deviations obtained by five repeated measurements were not more than 5%.

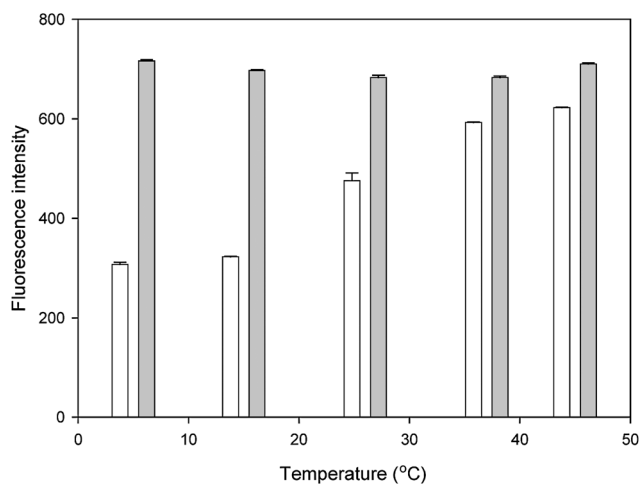


Fig. 4 Signaling profile. The signaling DNA aptamer mixture was incubated at 5, 15, 26, 37 and 45 °C with (grey bars) and without (white bars) $1.8 \times 10^{-8} \text{ mol L}^{-1}$ IgE. Each target–aptamer mixture was incubated for 60 minutes after the solution mixing. The standard deviations obtained by five repeated measurements were shown as the error bars.

and stable fluorescence intensity at 5 °C. When the temperature was raised from 5 °C to 15, 26, 37, or 45 °C, the fluorescence intensity of the solution increased in a manner that was indicative of heat denaturation of the DNA duplex assembly. A possible reason was that a high incubation temperature led to the dissociation of more Q-DNA from the T-DNA/aptamer/Q-DNA duplex assemblies, resulting in the increase in fluorescence intensity. However, in the presence of IgE (Fig. 4, grey bars), the fluorescence peak intensity of the spectra remained nearly unchanged when the temperature increased. T-DNA was possibly difficult to dissociate from the T-DNA/aptamer/IgE complex due to the spatial steric hindrance in the three-dimensional structure at a slightly higher temperature. This phenomenon indicated that the optimum reaction temperature was 5 °C.

2.4 Analytical performance of the aptamer-based system

To decrease the fluorescence background, the IgE detection was carried out under the optimized conditions found. As shown in Fig. 5, the fluorescence intensity increased with increasing concentration of IgE. The relative fluorescence intensity was defined as the ratio of ΔF (the difference between fluorescence intensity (F_{target}) in the presence of analyte and that ($F_{\text{no target}}$) in the absence of analyte) to the fluorescence intensity in the absence of analyte:

$$\text{Relative fluorescence intensity} = \frac{\Delta F}{F_{\text{no target}}} \times 100\%$$

As seen in the inset to Fig. 5, the logarithm of IgE concentration was proportional to the relative fluorescence intensity in the range of 9.2×10^{-11} to $3.7 \times 10^{-8} \text{ mol L}^{-1}$ with the detection limit ($S/N = 3$) of $5.7 \times 10^{-11} \text{ mol L}^{-1}$ (11.2 ng/mL). The fluorescence intensity changed slightly after the introduction of IgE at a concentration beyond the linear range. The linear regression equation was expressed as Relative fluorescence

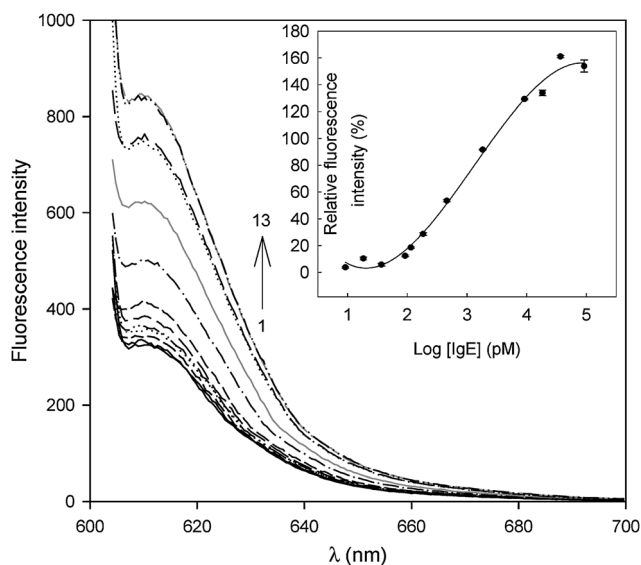


Fig. 5 Fluorescence spectra of mixtures in the presence of analyte at different concentrations. Inset shows the linear responses to analyte concentrations as follows: (1) 0 mol L⁻¹; (2) 9.2×10^{-12} mol L⁻¹; (3) 1.8×10^{-11} mol L⁻¹; (4) 3.7×10^{-11} mol L⁻¹; (5) 9.2×10^{-11} mol L⁻¹; (6) 1.1×10^{-10} mol L⁻¹; (7) 1.8×10^{-10} mol L⁻¹; (8) 4.6×10^{-10} mol L⁻¹; (9) 1.8×10^{-9} mol L⁻¹; (10) 9.2×10^{-9} mol L⁻¹; (11) 1.8×10^{-8} mol L⁻¹; (12) 3.7×10^{-8} mol L⁻¹; (13) 9.2×10^{-8} mol L⁻¹. The illustrated error bars represent the standard deviation of three measurements obtained at each IgE concentration.

intensity (%) = $55.96 \log [\text{IgE}] - 96.32$, with a correlation coefficient of $r = 0.9948$.

2.5 Specificity and recovery of the IgE assay

Some protein compounds commonly existing in biological samples were chosen as interferences to investigate the selectivity of the proposed assay. As shown in Fig. 6, the signal induced by IgE at 1.8×10^{-8} mol L⁻¹ was more than thirty-fold than the

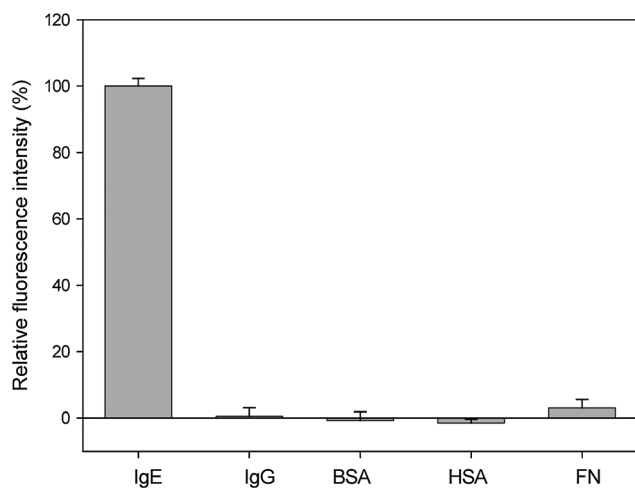


Fig. 6 Reporting specificity. Signaling profiles were obtained for a series of signaling aptamer solutions containing different targets. Error bars were estimated from at least three independent measurements.

Table 1 Recovery of IgE detection at different concentrations

Sample	Added (mol L ⁻¹)	Found (mol L ⁻¹)	Recovery (%)	RSD (%)
1	3.7×10^{-10}	3.9×10^{-10}	106	3.2
2	1.6×10^{-9}	1.5×10^{-9}	94	8.4
3	3.7×10^{-9}	3.6×10^{-9}	97	3.8
4	9.2×10^{-9}	9.8×10^{-9}	107	6.3
5	1.5×10^{-8}	1.4×10^{-8}	95	4.9

value induced by 6.7×10^{-8} mol L⁻¹ immunoglobulin G (IgG), 3.0×10^{-8} mol L⁻¹ bovine serum albumin (BSA), 3.0×10^{-8} mol L⁻¹ human serum albumin (HSA) or 4.6×10^{-8} mol L⁻¹ fibronectin (FN) under the same experimental conditions. The experimental results indicated that the developed assay could exhibit a high degree of selectivity for the IgE detection, as expected.

The recovery experiment of different IgE concentrations was carried out to evaluate the applicability and reliability of the proposed sensing system. The assays were performed with five samples at different concentrations, with each sample assayed three times, and the results are shown in Table 1. It was seen that the recoveries of IgE in the added samples and the relative standard derivations were in the range of 94–107% and 3.2–8.4%, respectively. The experimental results illustrated the reliability of the results of the developed sensing system.

3. Experimental

The IgE aptamer with a sequence of 5'-GGGG CACG TTTA TCCG TCCC TCCT AGTG GCGT GCCCC was used without any base modifications for the probe design. The sequence of the Texas red-labeled oligonucleotide and dabcyl-labeled oligonucleotide were 5'-Texas red-GGGACGG (T-DNA) and 3'-dabcyl-AGGATCA (Q-DNA), respectively. The aptamer and labeled oligonucleotides were customer-designed and synthesized by TaKaRa Biotechnology (Dalian, China). High-concentration DNA stock solutions were prepared with appropriate buffer medium and diluted prior to use. IgE was purchased from Bio-design (USA) and used without further purification. The IgE solutions and prepared probes were left on ice when not used to minimize any denaturation. Before assay, they were dissolved in the buffer solution containing 8.1 mM Na₂HPO₄, 1.1 mM KH₂PO₄, 1 mM MgCl₂, 2.7 mM KCl, and 138 mM NaCl and the pH was adjusted to 7.4. The incubation solution involved during detection was the same as the buffer solution except for a higher concentration of NaCl (350 mM).

If not otherwise specified, the following concentrations of oligonucleotides were used for fluorescence measurements: 120 nM for T-DNAs, 120 nM for the aptamers and 240 nM for the Q-DNAs. To perform the fluorescence experiments, 200 μL IgE solution (1.8×10^{-8} mol L⁻¹) was added to 40 μL aptamer solution. After about 30 min, it was added to the mixture of 40 μL of T-DNAs solution and 40 μL of Q-DNAs solution. All solutions could be stored at 5 °C. Typically, fluorescence spectra of the hybridization solutions were recorded after incubation for 60 min. When the temperature-dependent process was monitored, the fluorescence spectra of the hybridization solutions were collected after an equilibration period of 60 min at a certain temperature

(5–45 °C), and the relative fluorescence intensity was plotted as the function of temperature at selected temperature intervals.

Fluorescence spectra were measured using a Hitachi F-4500 fluorescence spectrometer (Hitachi Ltd., Japan) controlled by FL Solution software to fit a curve and determine peak height. The detector is an R928F red-sensitive photomultiplier, and the light is a 150 W Xe lamp. The fluorescence intensity of the aptamer was monitored by exciting the sample at 596 nm and measuring from 600 to 700 nm. All excitation and emission slits were set at 5 nm. Unless state otherwise, all experiments were carried out at 5 ± 2 °C.

4. Conclusion

In summary, it has been shown that the newly designed aptamer-based IgE assay probe with two short complementary DNA strands, one bearing fluorophore the other bearing the quencher group, can effectively lower the detection limit, which makes it possible to detect IgE at relatively low concentration levels as required under real clinical conditions. This approach requires very little information about the aptamer, which needs to have a local conformation change upon binding to its ligand. Therefore, this simple assay with extremely high sensitivity will make it widely applicable and highly useful for sensing of systems with IgE.

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