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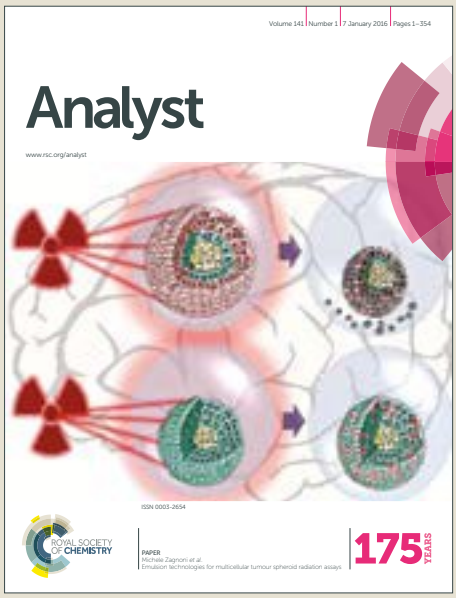

Journal Name

View Article Online
DOI: 10.1039/C9AN00195F

ARTICLE

Ultrasensitive and simple fluorescence biosensor for detection of the *Kras* wild type by using the three-way DNA junction-driven catalyzed hairpin assembly strategy

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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In this work, a label-free fluorescence biosensor was proposed for simple detection of the *Kras* wild type by using the three way DNA junction-driven catalyzed hairpin assembly strategy. In this system, a three-way DNA junction probr (JP) and two hairpin probes (H1 and H2) were designed. In the presence of the *Kras* wild type, an autocatalytic DNA machine can be activated. This leads to the generation of numerous free G-rich sequences, which can associate with a fluorescent dye N-methylmesoporphyrin IX (NMM) to yield an amplified fluorescence signal for the target detection. This sensing platform showed a high sensitivity towards the *Kras* wild type with a detection limit as low as 2.7 fM without any labelling, immobilization, or washing steps. The designed sensing system also exhibits an excellent selectivity for the *Kras* wild type compared with other interference DNA sequences. Furthermore, the presented biosensor is robust and has been successfully applied for the detection of the *Kras* wild type in a real biological sample with satisfactory results, suggesting that this method is promising for simple and early clinical diagnosis of the genetic diseases. Thanks to its simplicity, cost-effectiveness, and ultrasensitivity, our proposed sensing strategy provides a universal platform for the detection of the other genetic diseases by substituting the target-recognition element.

Introduction

The Kirsten rat sarcoma viral oncogene (*Kras*), encoding a 21 kD Ras protein, is one of the important genetic markers for confirming the tumor targeted therapy drugs and a key downstream regulation factor in the epidermal growth factor receptor (EGFR) signal transduction pathway which is frequently activated in colorectal cancer (CRC).[1,2] Previous studies have revealed that only those CRC patients with wild-type *Kras* may benefit from anti-EGFR treatment and a poor therapeutic response to anti-EGFR antibody drugs occurs in CRC upon *Kras* mutations.[3-5] However, the *Kras* is mutated in 35 to 40% of CRC, which is the third most common cancer in the world.[6] Therefore, the development of methods for the rapid qualitative and quantitative detection of the *Kras* mutations is essential in prognostics and diagnostics for individualized patients' therapeutic strategies in CRC.[7,8]

Conventional methods for the detection of the *Kras* wild type include real-time PCR,[9,10] pyrosequencing,[11,12] dideoxy sequencing[13], and high resolution melting curve analysis.[14,15] Even though these methods could give accurate results, they are expensive, time-consuming, and require fussy pre-treatment. Thus, much work is still required towards the development of convenient, simple, and low-cost detection approach for the *Kras* wild type analysis.

As alternatives to conventional methods, biosensors have been widely used for the cancer detection,[16-18] such as colorimetric biosensors,[19-21] electrochemical biosensors[22,23] and fluorescence biosensors.[24,25] However, most of them exhibit relativity poor sensitivity and require the labelling and immobilization, or washing steps, which inhibits the application of these technologies. To overcome these limitations, the label-free and non-enzymatic catalyzed hairpin assembly (CHA) strategy has been applied.[26-30]

Herein, a simple, facile, highly sensitive and selective detection method for the *Kras* wild type was developed by using the three-way DNA junction-driven catalyzed hairpin assembly and non-enzymatic target recycling amplification assay. In this system, the *Kras* wild type could bind with the DNA strand 1 (S1) and the DNA strand 2 (S2) to formed as a novel DNA three-way junction probe (JP), which could activate the CHA, resulting the reuse of the three-way junction and the production of plentiful G-quadruplex. Ultimately, the increasing G-quadruplex/NMM complex leads to a dramatically enhancement in the fluorescence intensity. Taking advantage of the signal amplification method, the designed biosensor could achieve a high sensitivity for the *Kras* wild type detection. Owning to its cost-effectiveness, easy operation, and flexibility, the sensing system could be a promising platform for the early point-of-care diagnosis of the genetic diseases.

Experimental

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†Electronic Supplementary Information (ESI) available: Fig. S1-S3 and Table S1. See DOI: 10.1039/x0xx00000x

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DOI: 10.1039/C9AN00195F

Materials and reagents

Dimethyl sulfoxide (DMSO) and tris-(hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich (St. Louis, Mo). Human serums were provided by the 4th Affiliated Hospital of Guangzhou Medical University (Guangzhou, China). Note: informed consent was obtained from all human subjects for the collection of human serums required for performing this experiment. NMM was purchased from Frontier Scientific Inc. (Logan, Utah, USA). A 5 mM NMM stock solution was prepared in DMSO and stored at -20 °C before use. All other reagents and materials were of analytical grade and used without purification. All of the solution were prepared with ultrapure water (18.2 MΩ/cm) from a Millipore Milli-Q water purification system (Billerica MA).

All of the DNA oligonucleotides were ULTRAPAGE-purified and were purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) and their sequences were listed as follows:

Target (*Kras* wild type): 5'-GGTAGTTGGAGCTGGTGGCGTAGGCA AGAGTG-3'

DNA strand 1 (S1): 5'-CGACATCTAACCTCACCAGCTCCAACTACC-3'

DNA strand 2 (S2): 5'-CACTCTTGCCTACGCAGCTGACT-3'

Hairpin probe 1 (H1): 5'-AGTCAGCTAGGTTAGATGTCGCTGACTGGG TAGGGCCGACATCTAACCTAG-3'

Hairpin probe 2 (H2): 5'-ATGTCGGCCCTACCCAGTCAGCGACATCTAA CCTAGCTGACTGGGTAGGGCGGGTTGGG-3'

M1: 5'-GGTAGTTGGAGCTGGTGGCGTAGGCAACAGTG-3'

M2: 5'-GGTAGTTGGAGCTGGTGGCGTAGCCAACAGTG-3'

M3: 5'-GGTAGTTGGAGCTGGTGGCCTAGCCAACAGTG-3'

NC: 5'-GCTATACATTCTTACTATTTTATTTAATCCCA-3'

Underline letters represent the mismatched bases.

Assay procedure

At first, the DNA oligonucleotides S1, S2, H1 and H2 were prepared in 20 mM Tris-HCl buffer (pH = 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 15 mM KCl), respectively. Then, the individual DNA solutions were incubated at 95 °C for 7 min and then gradually cooled to room temperature. The obtained DNA solution was stored at 4 °C for further use.

Various concentrations of the *Kras* wild type were then incubated with 100 nM S1, 100 nM S2, 300 nM H1, and 400 nM H2 with a reaction volume of 50 μL for 90 min at room temperature. Next, 1 μM NMM was introduced to the above solution, and this was further incubated at room temperature for 30 min. Finally, the resulting solutions were diluted to 200 μL. The fluorescence spectra were recorded using a SpectraMax i3x (Molecular Devices, the U. S.) at room temperature. The scanning wavelength ranged from 570 to 640 nm (λ_{ex} = 399 nm, λ_{em} = 610 nm). The fluorescence intensity at 610 nm was used to estimate the performance of the proposed biosensor. The slit widths of both the excitation and emission were 10 nm, and the PMT detector voltage was 700 V. All measurements were performed in 20 mM Tris-HCl buffer (pH = 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 15 mM KCl).

Gel electrophoresis

To prepare the hydrogel, 5 mL of 30% gel solution (29:1), 1 mL TBE buffer (10×), 100 μL APS, 4 μL TEMED, and 3.8 mL deionized water were mixed together. This mixture contained a final gel percentage of 15%. The gel was polymerized for 1.5 h at room temperature and then soaked in a 1× TBE buffer prior to use. 5 μL of each reacted sample was mixed with 1 μL of 6 × loading buffer and was subject to the 15% non-denaturing polyacrylamide gel electrophoresis (PAGE). The PAGE was carried out in 1×TBE at a constant voltage of 70 V for about 60 min at room temperature. After staining in diluted SYBR gold solution, the gels were scanned using a Gel Doc XR documentation system (Bio-RAD Laboratories Inc. USA).

Results and discussion

Strategy for the *Kras* wild type detection

The strategy for the amplified detection of the *Kras* wild type on the basis of the CHA signal amplification strategy is schematically demonstrated in Scheme 1. The biosensor consisted of two DNA strands (S1 and S2) and two hairpin probes (H1 and H2). Significantly, the S1 and the S2 can activate the CHA when they are linked together. The S1 and S2 can co-recognize the target gene to form a three-way DNA junction probe (JP), which make the toehold and branch-migration regions (domain II, domain III and domain IV) of this CHA together. The H1 and the H2 are well-designed according to the domains of II, III and IV and can partially hybridize to each other. Domain V and domain VI of the H2 are able to form G-quadruplex. When the H2 in its hairpin structure, the G-rich sequence is caged in the stem of the H2. In the absence of the target, the S1 and the S2 can't link together and the self-assembly between the H1 and H2 can't be started. Thus, the system only yields a relatively low fluorescence background. In the presence of the target, it can bind with the domain I_1 of the S1 and domain I_2 of the S2 through base pair, resulting the formation of the JP. Subsequently, the JP could trigger the signal amplification process of the proposed DNA machine. The detail is that the JP bind the toehold (domain II^*) of the H1, potentially initiating a branch migration (the toehold-mediated stand displacement reaction) and expose domain IV, V of the H1, leading the formation of a JP/H1 complex where another toehold (domain IV of the H1) for the H2 is expose. Then, the domain IV^* of the H2 can hybridize to the newly exposed toehold (domain II^*) of the H1, again initiating a branch migration. As a result, the domain IV^*, V^* and II^* of the H2 are fully combine with the domain IV, V and II of the H1, and form the JP/H1/H2 complex. Finally, the domain IV, III and II of the H2 can displace the JP, coincidently with the self-assembly of the H1/H2 complex and formation of the G-quadruplex (domain V and domain VI of the H2). Importantly, the released JP can act as a catalyst to trigger the catalytic assembly of another pairs of the H1 and the H2, achieving an amplified output signal for the *Kras* wild type. Finally, N-methyl mesoporphyrin IX (NMM) is added to the mixture to produce the fluorescence signal. Most importantly, the sensing system using G-quadruplex/NMM as the signal reporter, avoids the use of any labels or modification procedures.

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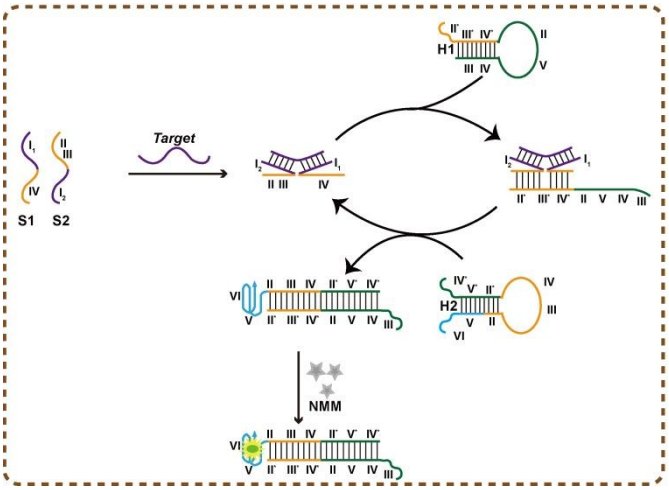

Scheme 1 Schematic representation of the design strategy for the amplified detection of the *Kras* wild type on the basis of the three-way DNA junction-driven catalyzed hairpin assembly and non-enzymatic target recycling amplification strategy.

Feasibility study

To verify the ability of the designed autocatalytic DNA machine for the target detection, the fluorescence spectra was investigated under different conditions. As shown in Fig. 1a, when the H2 alone in the solution (black curve), it exhibited a weak fluorescence emission peak at 610 nm, implying that when the G-rich sequence was blocked in the H2, the NMM can't interact with the G-rich sequence. When both the H2 and the H1 were present (blue curve), the fluorescence intensity was slight enhanced compared with the fluorescence signal of the H2. In the absence of the target (purple curve), the solution exhibited a very weak fluorescence as with the black and blue curve. However, after the system added the target, the fluorescence intensity improved sharply (red curve), suggesting that the CHA were finished. The G-rich sequence was released and then interacted with the fluorescence dye NMM. These results gave strongly evidence that our designed sensing system can feasibly provide an amplified fluorescence signal for the detection of the target *Kras* wild type.

To further verify the feasibility of this study, the PAGE was used to confirm the results of the fluorescence assay. The electrophoresis results are shown in Fig. 1b. Obviously, when mixed the H1 and the H2 together (lane 2), there was no H1/H2 complex formed, indicating that the H1 and the H2 could coexist in the solution. When the S1, the S2, the H1 and the H2 were present (lane 3), there are also no H1/H2 complex formed, suggesting that the CHA haven't been activated when the S1 and the S2 can't link together. However, after the *Kras* wild type added, nearly most the H1 and the H2 converted to the H1/H2 complex with the obvious bands of released the S1/S2/*Kras* complex, demonstrating that the target make the S1 and the S2 together and the CHA have been activated (lane 4). The above results further indicate that our designed detecting system can be utilized for the amplified detection of the target *Kras* wild type.

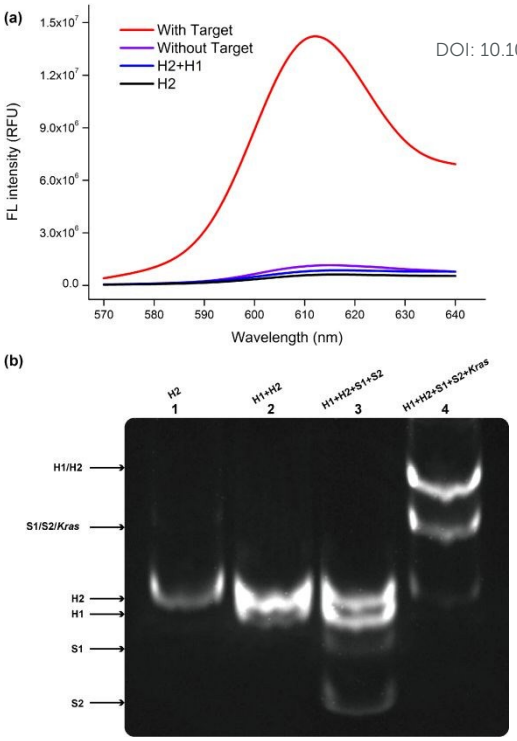

Fig. 1 (a) Fluorescence spectra of the system under different conditions. S1: 100 nM; S2: 100 nM; H1: 300 nM; H2: 400 nM; NMM: 1 μM; (b) the PAGE images of lane 1: H2; lane 2: H1+ H2; lane 3: S1 + S2 + H1 + H2; lane 4: S1 + S2 + H1 + H2 + *Kras*. S1: 1 μM; S2: 1 μM; H1: 1 μM; H2: 1 μM; The experiments were performed at room temperature (25 °C).

Optimization of this assay

The experimental parameters (such as the reaction temperature, the concentration of the H2, and the reaction time) that could affect the analytical performance of the designed sensing system were optimized. The optimizations were performed by varying one experimental condition and keeping the conditions of the other parameters constant. In this work, the reaction temperature plays an important role in the sensing process. It not only effects the stability of the H1 and the H2, but also the binding kinetics among the H1 and the H2, the target *Kras* wild type, the S1 and the S2. The effect of reaction temperature on the response of the fluorescence sensor was investigated by detecting 10 nM of the *Kras* wild type at different temperatures (4 °C, 25 °C, 37 °C, and 45 °C). The blank sample (in the absence of the *Kras* wild type) at each temperature was tested under the same conditions. As shown in Fig. S1 (ESI†), the fluorescence signal increased with the increase of the temperature in the range from 4 to 25 °C. Further increase the temperature will results in a decrease of the signal (green histogram in Fig. S1, ESI†). Also, the higher temperature may change the conformation of the H2, which increases the background signal (blue histogram in Fig. S1, ESI†).[31] In order to obtain the best signal-to-background ratio (S/N) (red line in Fig. S1, ESI†), 25 °C was considered to be the optimum reaction temperature.

The effect of concentration of the H2 was taken into consideration. The detectable signal of the sensing system mainly

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derives from the H2, which indirectly relies on the concentration of the H2. The higher concentrations of the H2 could cause a relatively high background signal, which restricted the fluorescence response for target *Kras* wild type detection. While the lower concentration could result in a weak fluorescence signal due to the deficient amount of G-sequence for signal readout. As shown in Fig. S2 (ESI†), the S/N level increased gradually with the increasing the concentration of the H2 from 200 to 400 nM, and reached a peak at 400 nM. However, the S/N level decreased when the H2 concentration continued to raise (400-600 nM). Thus, 400 nM of the H2 was chosen for further experiments.

The performance of the signal amplification and detection of the *Kras* wild type was also deeply influenced by the reaction time of the CHA. In order to optimize the reaction time, we control the fluorescence signal response at 25 °C and 400 nM H2. As shown in Fig. S3 (ESI†), the fluorescence intensity of the solution containing 10 nM of the *Kras* wild type increased rapidly when the incubation time ranging from 30 to 90 min and remained at almost a constant level after 90 min, which suggested that the H1/H2 complex was almost completely formed for the formation of the G-quadruplex/NMM complex. To gain the best result, 90 min was selected as the optimum reaction time.

Analytical performance of the biosensor

To investigate the dynamic range and sensitivity of the assay, a series of samples containing different concentrations of the *Kras* wild type were tested under optimal experimental conditions. As shown in Fig. 2a, the fluorescence intensity was enhanced as the concentration of the *Kras* wild type increased (0-100 nM). The relationship between the fluorescence intensity at 610 nm and the concentration of the *Kras* wild type is displayed in Fig. 2b. The resulting calibration curve shows that a linear dependence between the fluorescence intensity and the logarithm of the *Kras* wild type concentration was obtained in the range from 10 fM to 100 nM with a correlation coefficient (R^2) of 0.99 (Fig. 2b, inset). The linear regression equation is $y=2.01\times10^6 lgC+2.56\times10^5$ (y and C represent the fluorescence value and the concentration of the *Kras* wild type, respectively). According to the 3δ rule, the calculated the limit of detection (LOD) is 2.7 fM. Compared with the previously reported method of the *Kras* wild type detection, our sensing platform proposed herein have an acceptable analytical range and a relatively low detection limit (Table S1, ESI†). Such a high sensitivity can be attributed to the fact that the autocatalytic DNA machine can stimulate the formation of numerous G-quadruplex/NMM complexes even at a low concentration of the target *Kras* wild type. Herein, our proposed fluorescence detection strategy holds great potential for monitoring of the *Kras* wild type in real biological samples with high sensitivity.

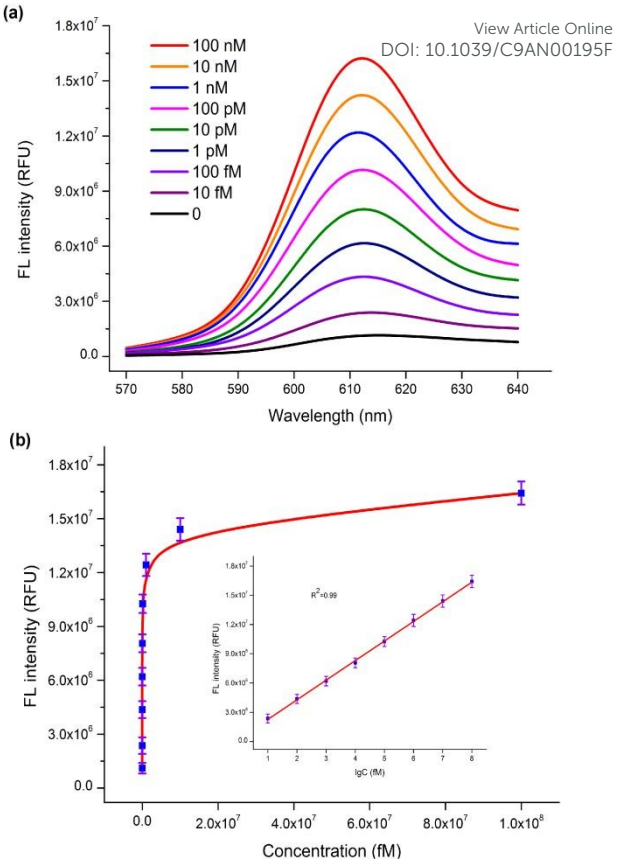

Fig. 2 (a) Fluorescence spectra upon addition of different concentrations of the *Kras* wild type. (b) Plots of the fluorescence intensity at 610 nm as a function of the *Kras* wild type concentration. Inset: Linear relationship between the fluorescence intensity and the logarithm of the *Kras* wild type concentration in the range from 10 fM to 100 nM. The corresponding error bars in (b) represents the standard deviation of three independent measurements obtained at different concentrations of the *Kras* wild type.

Selectivity of the biosensor

The selectivity of the sensing platform for the *Kras* wild type to other four kinds of DNA sequences, including a single-base mismatched DNA (M1), a two base mismatched DNA (M2), a three base mismatched DNA (M3), and the non-complementary DNA (NC) at the same concentration of 10 nM, were also analyzed by the present sensing system. As shown in Fig. 3, the fluorescence intensity varies with the target and the four other kinds of DNA. The relative fluorescence intensity for M1, was about 21.9% of the *Kras* wild type. The other three kinds of DNA sequences (M2, M3 and NC) showed almost the same intensity as the blank sample. These results clearly demonstrated the high specificity of our designed sensing system.

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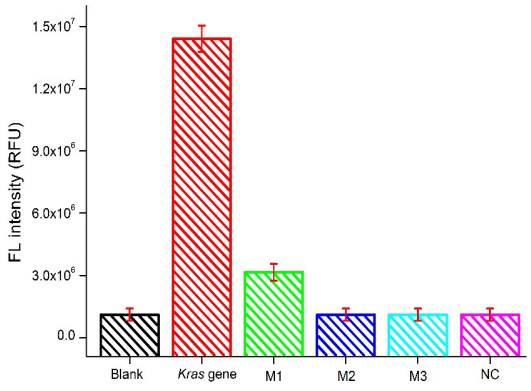

Fig. 3 Selectivity of the sensing system analyzing the *Kras* wild type against other four different kinds of DNA sequences. The concentration was 10 nM for the *Kras* wild type and the four other DNA sequences. The corresponding error bars represent the standard deviation of three independent measurements obtained for different analytes.

Real sample analysis

To investigate whether this fabricated monitoring system could be applied to real samples, a recovery experiment was carried out. The human serum samples, which from healthy people, were filtered through a 0.2 μM membrane to remove the insoluble particles. Aliquots of the serum samples were spiked with different concentrations 0.1, 1, 10 nM of the *Kras* wild type. As shown in Table 1, the recovery of all of the measured samples and the relative standard derivations (RSD) were in the range from 85-102% and 4.6-6.1% (n=5), respectively, which indicated that the possible interference from the human serum sample to the biosensor analysis was negligible. This above data proved that our designed method can be successfully applied for the detection of the *Kras* wild type in real biological samples.

Table 1 Analysis of real samples containing the *Kras* wild type at different concentrations.

Sample	Added (nM)	Found (nM)[a]	Recovery (%)	RSD (%)
1	0.1	0.085	85%	4.6
2	1	0.96	96%	6.1
3	10	10.2	102%	5.7

The data reported in the table represents the average of five measurements.

Conclusions

In conclusion, a simple, facile, highly sensitive and selective detection method for the *Kras* wild type was developed by using the three-way DNA junction-driven catalyzed hairpin assembly and non-enzymatic target recycling amplification assay. The addition of the *Kras* wild type can bind with the S1 and S2 via base pair, resulting the formation of the JP. Subsequently, the JP could trigger the signal amplification process of the proposed DNA machine, which leads to reuse the *Kras* wild type and generate massive G-quadruplex structures. The increasing G-quadruplex interacts with the NMM to form G-quadruplex/NMM complexes, which result in an enhanced fluorescence intensity of the reaction solution for the highly sensitive detection of the *Kras* wild type at concentrations as low as 2.7 fM. Moreover, the designed sensor also exhibits an excellent selectivity toward the *Kras* wild type compared with other interference DNA samples. Furthermore, this method can be applied for the determination of the *Kras* wild type in spiked real biological samples with good recovery and accuracy. Without the use of any labelling, immobilization, washing, or modification steps, the developed method described herein is easy apply for routine monitoring of the *Kras* wild type. Importantly, the designed autocatalytic DNA machine could be easy extended to fabricate versatile and robust sensing platforms for the detection of other genetic diseases by choosing the corresponding recognition elements. With the characteristics described above, this biosensor has great potential for application as a platform for the early clinical diagnosis of the genetic diseases.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Financial support was provided by the Guangdong National Science Funds for Distinguished Young Scholars (2016A030306012), the NSFC (31671933), and the Local Innovative and Research Teams Project of the Guangdong Pearl River Talents Program (2017BT01Z176).

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