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# Hybridization Chain Reaction-based Assay for The Detection of Influenza A Virus*

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**Abstract** Rapid point-of-care (POC) detection of influenza A virus is critical for timely and effective flu prevention and control. In this study, we developed a hybridization chain reaction (HCR)-based assay, through coupling HCR reaction with fluorescence quenching by graphene oxide (GO), for the rapid detection of influenza A virus. The target is detected as it triggers HCR, which results in the extension of short DNA chains, protecting the 6-carboxy-fluorescein amidite (FAM) group from GO quenching. The results demonstrate that it could specifically recognize target nucleic acid fragment of influenza A virus from other pathogen, and even from single-base mismatched oligonucleotides. A good linear correlation between fluorescence intensity and the target concentrations ranging from 10 to 40 nmol/L was achieved, with a detection limit of 5 nmol/L. Its detection performance was verified on nasopharyngeal swab samples, which is the first clinical application of HCR-based influenza assay. This HCR-based method shows many advantages including the enzyme-free amplification of nucleic acids, simple reaction system and convenient protocols, suggesting its availability for POC detection

**Key words** influenza A, hybridization chain reaction, graphene oxide, point-of-care (POC), enzyme-free detection
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Influenza viruses are RNA viruses that cause influenza in humans, dogs, horses, pigs, birds, and other organisms. These viruses belong to the Orthomyxoviridae family of viruses, which can cause acute upper respiratory tract infections, spread rapidly through air, and often cause periodic pandemics worldwide [1–2]. During these seasonal pandemics, approximately 600 million people are infected each year, resulting in 250 – 500 thousand deaths and 3 – 5 million serious cases[3]. Among these viruses, influenza A virus represents the main infective agent, leading to death of infected individuals. This virus is highly susceptible to environmental and other factors and has a high frequency of mutations. Some of the mutated virus strains can easily infect humans and lead to a global flu outbreak [4–5]. Therefore, improving the efficiency of rapid point-of-care (POC) detection of influenza A virus is important for the diagnosis, treatment guidance, and rapid prevention and control of epidemics.

Influenza A viruses can be further subtyped according to the differences in the antigens expressed on the surface of the particles, hemagglutinin (HA, 18 subtypes) and neuraminidase (NA, 11 subtypes) [6–7]. Since both of these antigens can undergo antigen drift, generating new subtypes, general population is more susceptible to the infection by influenza A virus, making it more likely to cause pandemic, and even global flu epidemic [8–10]. Currently, influenza A virus detection methods include isolation and culture,

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immunological detection, and molecular biology assays[11–13]. Isolation and culturing require high quality specimens and are very time-consuming, and, due to these characteristics, this method does not meet the needs of rapid testing. Although the immunological method is relatively simple, it depends on a specific antigen-antibody reaction, which is cost-consuming. Molecular detection techniques, such as RT-PCR and qPCR, are simple to perform, with high accuracy and good availability for quantitative analysis which have almost become the golden standard for virus detection in laboratories. However, these methods are based on the use of expensive and large instruments, with complex experimental processes demanding more experienced operators, which does not meet POC testing requirements[14]. Therefore, the development of a simple molecular technique for the rapid detection of influenza A virus is crucial for the early screening, diagnosis, and treatment of influenza A cases, which will benefit the prevention and managing of influenza A outbreaks.

In recent years, due to its high sensitivity, low equipment requirements, fast procedures, and ease of use, isothermal amplification has been widely applied for the POC testing. Commonly used isothermal amplification techniques, such as loop-mediated isothermal amplification (LAMP), rely on the use of enzymes, which is costly and demands specific reagent storage conditions. In 2004, Dirks *et al.*[15] developed an enzyme-free isothermal amplification method called hybridization chain reaction (HCR). In HCR reaction solution, two hairpin short DNA chains with sticky ends are designed to contain the partial completerary sequences to each other and initiator sequence. The two hairpin chains can stably coexist until the presence of initiator sequence[16–17]. The initiator can trigger the HCR reaction through the following procedures: it first opens one of the hairpin short chain and exposes the complementary sequence to the other hairpin chain, which will then be opened[18]. The secondly opened hairpin DNA chain has the same sequence with the initiator sequence, so that these two hairpin short chains can alternatively open each other, and finally form long dsDNA chains[19–20]. This procedure can be applied at room temperatures, without enzymes. Fewer reagents are necessary, and reaction is controllable and flexible. Combing HCR with nanomaterials for the development of rapid POC detection techniques has been reported[18–19]. Although HCR-based detection methods have been described, these studies are restricted to methodological research at laboratory stage, and specific pathogen target sequences have rarely been investigated.

In this study, we designed a HCR procedure specifically for the detection of influenza A virus. Here, the target nucleic acid is detected as the trigger of HCR, which results in the extension of 6-carboxy-fluorescein amidite (FAM) modified short DNA chains into long chains, to reduce the GO quenching. We verified its detection performance on nasopharyngeal swab samples, which is the first clinical application of HCR-based influenza assay to our best knowledge.

## 1 Experimental

### 1.1 Materials

All primers used in the experiments were synthesized by Sangon Biotech (the sequences are listed in Table 1). We purchased PBS (Life technologies), GO (XFNANO), agarose gel, nucleic acid extraction kit, One-step RT-qPCR kit, and FastQuant RT kit from Tiangen Biotech. All types of EP tubes, centrifuge tubes, and pipet tips were all purchased from Axygen. Plates (96-well) were purchased from Corning.

Viral strains and nasopharyngeal swab samples. Nasopharyngeal swab samples were collected from patients suspected to be infected with influenza A virus. Strains of influenza A H1 virus, influenza B virus, and adenovirus were isolated and stored. H5 subtype lysate and H7 subtype viral plasmid vector were stored or generated in the laboratory.

### 1.2 Methods

HCR. Primers used in our experiments were designed based on the highly conserved influenza A M protein sequence and modified with the FAM group. Since the dsDNA chain is negatively charged, electrostatic repulsion protects the FAM group from quenching. Fluorescent signal of the FAM groups on the H1 and H2 sequences that did not find target sequences is quenched by GO, allowing the detection of the target sequences. Relevant sequences are presented in Table 1.

Specifically, H1 and H2 primers were dissolved and heated for 2 min at 95℃ in metal bath (Sangon Biotech $H_2O_2$-PROI) before cooling for 1 h at room temperature (approximately 25℃). H1 and H2 concentrations were 1 μmol/L each, while the

**Table 1 Primer sequences used in HCR analyses**

| | 5′→3′ |
|---|---|
| $I_{target}$ | GACCAATCCTGTCACCTCTGAC |
| H1 | TCCTGTCACCTCTGACCTAAGTGTCAGAGGTGACAGGATTGGTC |
| H2 | ACTTAGGTCAGAGGTGACAGGAGACCAATCCTGTCACCTCTGAC |
| H1-FAM | FAM−TCCTGTCACCTCTGACCTAAGTGTCAGAGGTGACAGGATTGGTC |
| H2-FAM | ACTTAGGTCAGAGGTGACAGGAGACCAATCCTGTCACCTCTGAC−FAM |
| $I_{mismatch}$ | GACCAATC*G*TGTCACCTCTGAC |

Note: Underlined sequences represent the sticky ends and their complementary sequences; double-underlined sequences are loop structures and their complementary sequences; and italicized letter represents the mutated base.

concentrations of the target sequence ($I_{target}$) differed (0, 100 nmol/L, 500 nmol/L, 1 μmol/L, and 3 μmol/L). After incubating the samples for 4 h at room temperature, agarose gel electrophoresis was performed by electrophoresis tank (Beijing 61 biotechnology DYY-6C) (gel concentration: 2% ; voltage: 120 V; time: 30 min), and the results were recorded (Bio-Rad Gel Doc XR+ System).

Fluorescence quenching by GO. Different concentrations of GO were added to the reaction mixtures, to a final volume of 200 μl, with the final concentrations of 50 nmol/L of H1 and H2. Fluorescence spectra at different concentrations (excitation wavelength: 480 nm, emission wavelength detection range: 510 – 600 nm) using a multimode detection platform (Molecular Devices, SpectraMax i3x) were determined after 30 min incubation. Fitted curve, showing fluorescence intensities in relation to each analyzed concentration at 520 nm, was generated.

Detection of target sequences using HCR-based method. The FAM-modified H1 and H2 primers were heated in a solution at 95℃ for 2 min in metal bath (Sangon Biotech $H_2O_2$-PROI) before cooling the sample at room temperature for 1 h. H1, H2 (final concentrations: 50 nmol/L), and different concentrations of target sequences $I_{target}$ were mixed and incubated for 2.5 h at room temperature. Afterward, GO (final concentration: 20 mg/L) was added, to the final volume of 200 μl. These solutions were incubated at room temperature for 30 min, which was followed by excitation at 480 nm and fluorescence intensity detection at 520 nm using a multimode detection platform (Molecular Devices, SpectraMax i3x).

For the viral sample analyses, total nucleic acids were extracted from the viral strain or nasopharyngeal swab samples, using a commercial kit (Tiangen Biotech). Following the reverse transcription reaction, target detection was performed as described, using the HCR-based system.

## 2 Results and discussion

### 2.1 HCR reaction gel electrophoresis results

The influenza A M1 protein virus is involved in its replication, it can interact with the HA and NA proteins[21–22]. When the target sequence $I_{target}$ is absent from the solution, two DNA hairpin sequences were shown to stably coexist in the solution as short-chain dsDNAs. However, after the addition of the target sequence, the H1 and H2 sequences are alternatively opened and hybridized to form long-chain dsDNA products with sticky ends. To validate the sequence design, different concentrations of $I_{target}$ were added to the solutions containing H1 and H2 sequences. Agarose gel electrophoresis results were consistent with those previously reported: in the absence of $I_{target}$, HCR does not occur, and bands at smaller sizes were observed. However, in the presence of $I_{target}$, HCR is triggered, and dsDNAs are generated (Figure 2). With the increase in the $I_{target}$ concentrations, dsDNA fragments become shorter.

### 2.2 Optimization of HCR-based detection condition

Optimization of GO-induced fluorescence quenching conditions. GO is a new type of two-dimensional nanocarbon material, surface-rich in oxygen-containing functional groups [23–24]. These surface carbon atoms are linked by sp2 hybridization to form a unique π-conjugated plane[25–26]. Free-moving delocalized π electrons generated at the π-conjugated

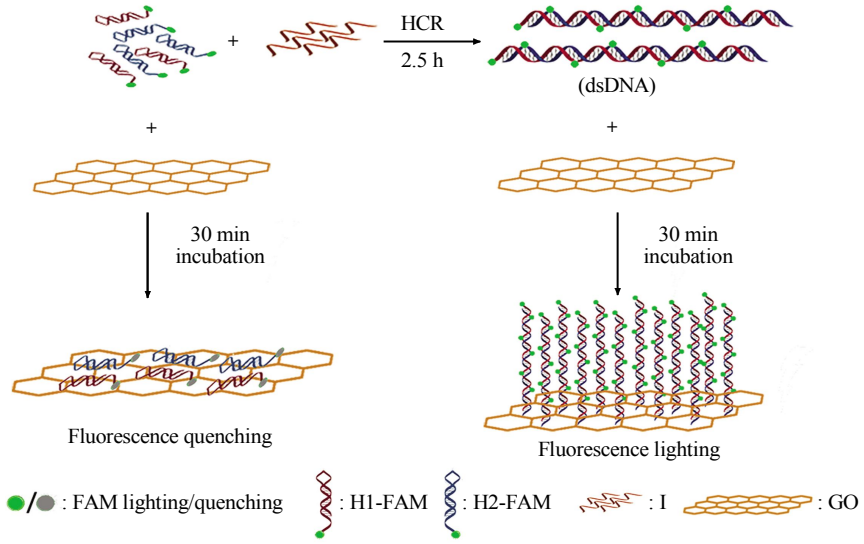


**Fig. 1 Schematic representation of HCR-based detection described here**

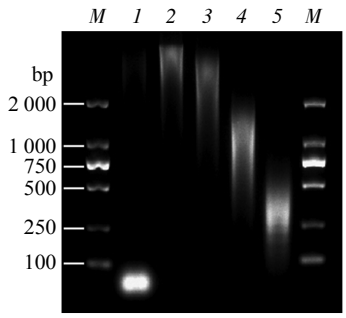


**Fig. 2 HCR-based method followed by gel electrophoresis**

Left to right: Markers 100–2 000 bp; 1–5: $I_{target}$ concentrations 0, 100 nmol/L, 500 nmol/L, 1 μmol/L, and 3 μmol/L, respectively, with 1 μmol/L of H1 and H2; Markers 100–2 000 bp.

plane enable GO-induced fluorescence quenching[27].

The results, showing the GO-induced quenching of FAM-modified H1 and H2 sequences, are presented in Figure 3a. Following the 30 min quenching by GO, the fluorescence of the solution decreased with the increase in GO concentrations, due to the increased probability of contact between GO and the fluorophores on DNA sequences. Fluorescence intensity fitting at 520 nm was shown to lead to a good logarithmic correlation between the concentration of GO and the fluorescence intensity observed in the solution (Figure 3b), indicating good FAM fluorescence-quenching effects of GO. Additionally, our results suggest that the decrease in fluorescence intensity

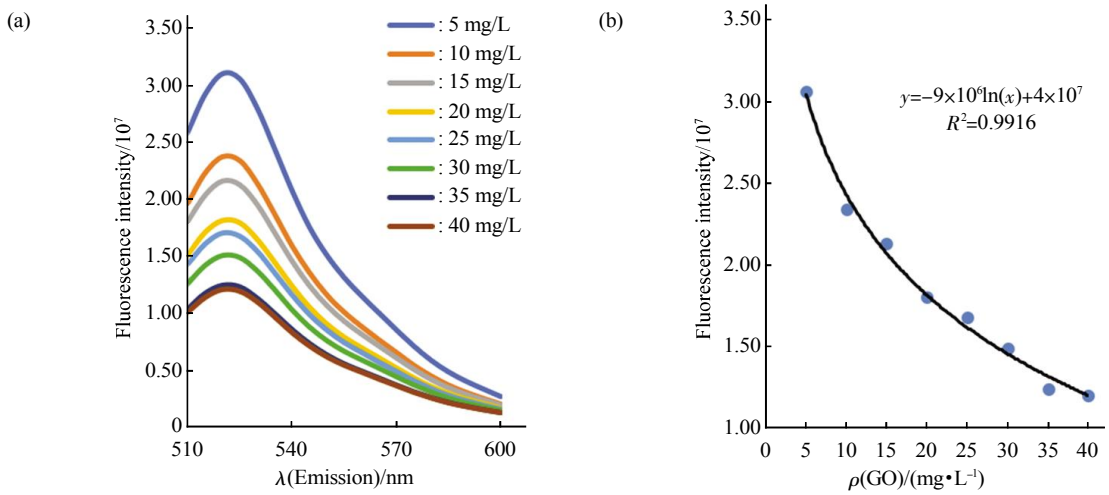


**Fig. 3 Validation of the GO-induced fluorescent signal quenching**

Conditions: H1 and H2 concentrations, 50 nmol/L; reaction duration, 30 min; excitation wavelength, 480 nm; Figure 3a emission wavelength is 510-600 nm and Figure 3b emission wavelength is 520 nm.

deaccelerated with the increase in GO concentrations from 5 mg/L to 40 mg/L, indicating that the saturation with the GO was achieved in the solution.

To improve the efficiency of the HCR-based detection, GO concentration and the reaction time were optimized (Figure 4). In the control and experimental groups, H1 and H2 concentrations were selected to be 50 nmol/L, while the concentrations of $I_{target}$ were 0 and 5 nmol/L for the control and experimental groups, respectively. As shown in Figure 4, in all groups, after 2.5 h of the incubation at room temperature, fluorescence intensity was quenched by GO at different concentrations (10, 15, 20, 25, and 30 mg/L). With the increase in GO concentrations, fluorescence signal gradually decreased in all the tested solutions, while the signal-to-noise ratio initially increased and then decreased, reaching the maximum at the GO concentration of 20 mg/L. Therefore, we selected 20 mg/L of GO for further analyses.

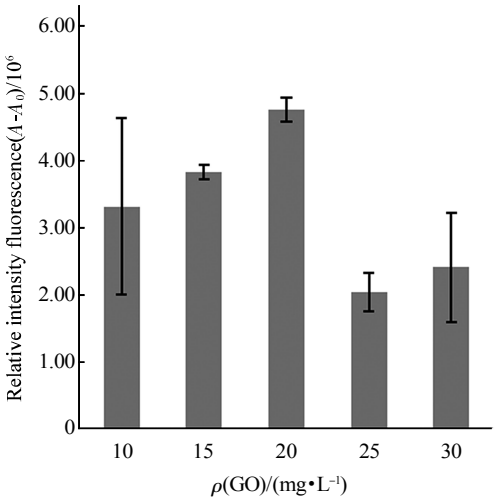


**Fig. 4　Relationship between fluorescence intensity and the applied GO concentrations**

Error bars represent the standard error of three independent experiments.

In all the groups, fluorescence intensity was determined every 5 min for 2.5 h, and 20 mg/L of GO were used to quench the fluorescent signal (Figure 5). The fluorescence intensity in the control rapidly stabilized, after only 5 min of incubation time. In contrast, the fluorescence intensity of the experimental group decreased rapidly at first, until 10 min after the initiation of the experiment, and then gradually

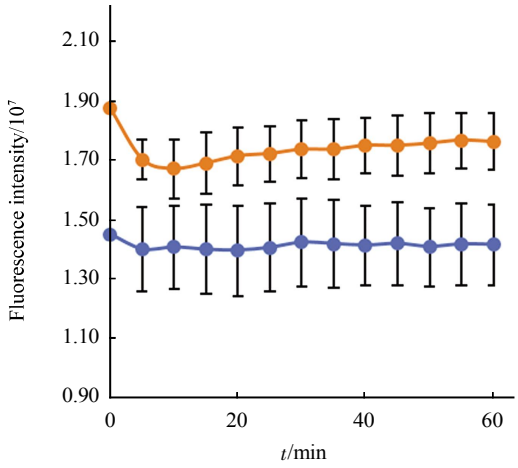


**Fig. 5　Kinetic curve, showing GO-induced fluorescence quenching**

Error bars represent the standard error of three parallel experiments. ●—●: Experimental group; ●—●: Control group.

recovered and stabilized after approximately 25 min from the beginning of the experiment. In the experimental group, the fluorescence intensity decreased rapidly within 10 min after GO addition and gradually recovered with time. This may be explained by the rapid diffusion of GO molecules in the solution, their contact with DNAs, which leads to the rapid quenching of fluorophores on the dsDNAs, and the subsequent partial disassociation of GO from the DNAs due to the electrostatic repulsion, leading to the recovery of fluorescence signal.

Optimization of the HCR duration and specificity evaluation. Furthermore, we optimized the duration of HCR reaction, in order to decrease it, and evaluated the specificity of the HCR-based detection method by measuring the recognition of base-pair mismatch when using this method. To optimize the HCR reaction time, H1 and H2 primers and $I_{target}$ were added at the final concentrations of 50 nmol/L, 50 nmol/L, and 5 nmol/L, respectively, and the HCR reaction was performed for 1, 1.5, 2, 2.5, 3, or 3.5 h. Following the HCR reaction, 20 mg/L of GO were added and the samples were incubated for 30 min to quench the fluorescence of the above HCR reaction solution, after which fluorescence intensity in each sample was determined (Figure 6). In the reactions performed for 1 – 2.5 h, fluorescence intensity was shown to increase after GO quenching

together with the HCR response time. After 2.5 h, fluorescence intensity gradually stabilized, indicating that the HCR approached completion at this time. Therefore, we selected 2.5 h as the optimal HCR response time in this study.

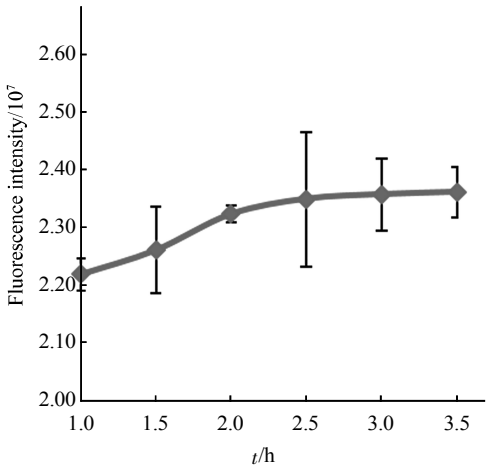


**Fig. 6 Relationship between the fluorescence intensity and HCR response time detected using HCR-based method**

Error bars represent the standard error of three parallel experiments.

Taken together, our results demonstrate the development and optimization of the HCR- and GO-quenching-based detection method. The optimal HCR detection conditions were determined to be as follows: 50 nmol/L of H1 and H2 primers, 2.5 h of HCR time, 20 mg/L of GO, and 30 min fluorescence quenching reaction. Fluorescence intensity, measured at the end of experiment, was determined using the excitation wavelength of 480 nm and emission wavelength of 520 nm.

### 2.3 Evaluation of the HCR detection method potential

Specificity of the HCR detection method. To verify the specificity of the HCR-based detection method, a single-base mutant sequence, $I_{mismatch}$, was used as the target sequence (Figure 7), while the conditions were the same as those described in the previous sections. For the samples containing $I_{target}$, the fluorescence intensity was shown to be significantly higher than that in the other groups ($P < 0.05$). In particular, the fluorescence intensity in the $I_{mismatch}$ group, where a sequence carrying a single-base mismatch was used, was shown to be similar to that of the control groups. We analyzed fluorescence intensity levels in all five groups using the LSD-*t* (least significant difference) test, which demonstrated that a good specificity can be achieved by using our optimized HCR-based method, even when analyzing sequences carrying single-base mismatches.

Quantitative detection of target sequence. Using

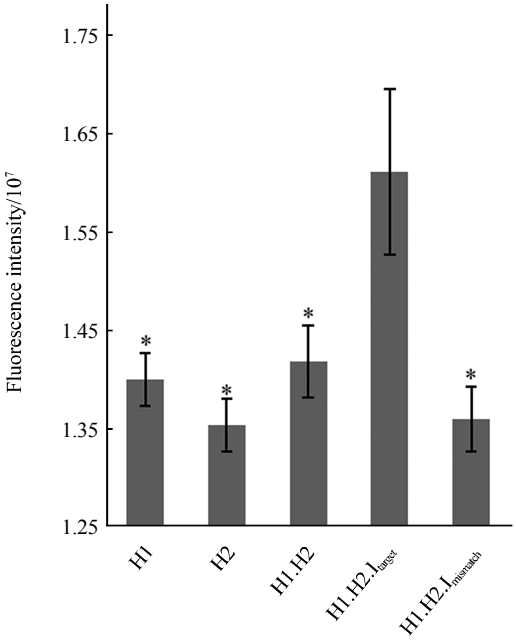


**Fig. 7 Determination of the specificity of the HCR-based method**

H1, only H1 primers at the concentration of 100 nmol/L; H2, only H2 primers at the concentration of 100 nmol/L; H1.H2, both primers at 50 nmol/L; H1.H2. $I_{target}$, 50 nmol/L of both primers and 5 nmol/L of $I_{target}$; H1.H2. $I_{mismatch}$, 50 nmol/L of H1 and H2 and 5 nmol/L of $I_{mismatch}$. All other conditions were as previously described. *$P < 0.05$, H1.H2. $I_{target}$ group *versus* the other groups. Error bars represent the standard error obtained in three independent experiments.

the optimal HCR detection conditions, we evaluated the potential of the HCR method for the quantification of $I_{target}$ (Figure 8a). For the $I_{target}$ concentrations ranging from 0 to 40 nmol/L, the fluorescence intensity increased with $I_{target}$ concentration. However, above these concentrations, the fluorescence signal intensity gradually decreased, and finally it stabilized at the concentrations higher than 80 nmol/L. At lower $I_{target}$ concentrations, the HCR chain size and the number of HCR products may increase with the target concentrations, leading to a gradual increase in the fluorescence intensity levels. At 50–70 nmol/L of $I_{target}$,

the number of HCR products increases, but the chain size decreases, leading to the GO-induced FAM quenching and subsequent decrease in fluorescence signal intensity. For the $I_{target}$ concentrations higher than 70 nmol/L, H1 and H2 primers are almost completely consumed, leading to the stabilization of fluorescence signal. As shown in Figure 8b, the fluorescence intensity and $I_{target}$ concentrations were shown to have a good linear relationship ($y$=142728$x$+ 3×10$^6$; $R^2$=0.9858) at the $I_{target}$ concentration range of 10 – 40 nmol/L.

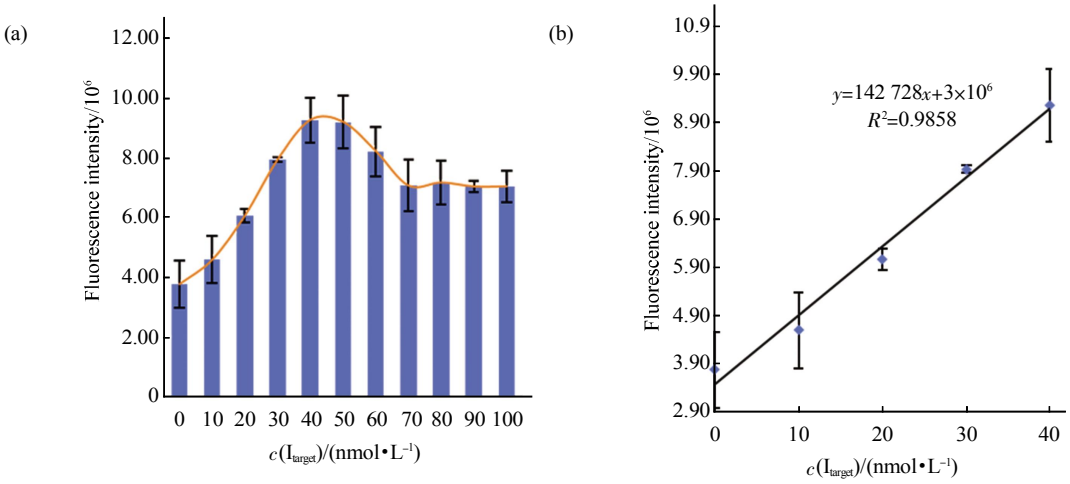


**Fig. 8 Relationship between the fluorescence intensity levels and $I_{target}$ concentration changes**

Error bars represent the standard error from three independent experiments.

HCR applicability in the detection of influenza A virus. We applied the developed HCR-based assay to the detection of influenza A and related viruses. Nucleic acids were extracted from influenza A (H1, H5, H7) virus, influenza B virus, and adenovirus. The optimized detection conditions were used, and the obtained results are presented in Figure 9. Statistical analyses showed that fluorescence signals detected in the samples containing influenza A virus H1, H5, and H7 nucleic acids were significantly higher than those detected in influenza B virus and adenovirus samples. We set the positive threshold value (cut off) according to the fluorescence intensity of 3 times standard deviations over the largest negative signal, as shown in Figure 9 [28]. The result suggests that our optimized method can be applied for the detection of different influenza A subtypes, and false positive results due to the presence of other viruses can be avoided.

Detection performance on nasopharyngeal swab samples. Fifteen nasopharyngeal swab samples of patients suspected to be infected with influenza A virus from an epidemic region were randomly selected from the samples preserved in our laboratory. The extracted

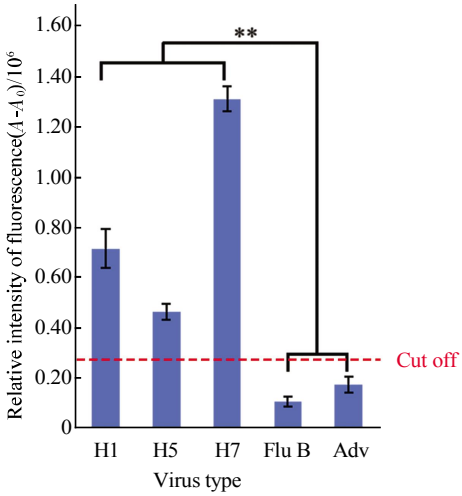


**Fig. 9 Validation of the HCR detection performance using different types of viral strains**

H1, H5 and H7 are influenza A subtypes; influenza B, adenovirus. **$P$ < 0.01, H1, H5, and H7 groups versus other groups (Flu B and Adv represent influenza B and adenovirus, respectively). Error bars represent the standard error from three independent experiments.

nucleic acids were subjected to HCR amplification and RT-PCR (primer sequences: FluA-R, GACCAATCC-TGTCACCTCTGAC; FluA-F, AGCTGAGTGCGAC-CTCCTTAG). The results are presented in Table 2 for comparison. We showed that, of 15 cases analyzed here, three were positive for influenza A virus (No. *2*, *3* and *8*), and HCR test results matched RT-PCR results, indicating that HCR test can be used in clinic and for the POC detection.

**Table 2 Comparison of HCR and RT-PCR detection performances**

| Detection method | Positive samples | Negative samples |
|---|---|---|
| HCR | *2*, *3*, *8* | *1*, *4-7*, *9-15* |
| RT-PCR | *2*, *3*, *8* | *1*, *4-7*, *9-15* |

Since FAM group is prone to fluorescence decay, this may sometimes result in a larger standard deviation of obtained signals. By controlling the difference between different batches of reagents and standardizing the operation, the obtained results are expected to improve and be more stable. Additionally, the time required for the application of HCR-based detection method is expected to be further decreased, by the optimization of detection protocols. Moreover, the portable fluorescence detection devices can be used to meet the requirements for the POC emergency detection of influenza virus.

## 3 Conclusions

We developed an enzyme-free HCR fluorescence detection method for a simple and convenient detection of influenza A virus. Our results showed that the detectable range of the target sequence is 5 – 100 nmol/L, with the linear correlation range of 10 – 40 nmol/L. The method exhibits good specificity even from single-base mismatches and can be used for the identification of a wide variety of influenza A subtypes. Furthermore, the results obtained by analyzing nasopharyngeal swab samples demonstrated a high accuracy of the method developed in this study, and they were shown to agree with the results obtained by using RT-PCR. In sum, we developed and described a simple method for the rapid detection of influenza A virus, which is applicable for POC detection.

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# 基于杂交链式(HCR)反应的甲型流感病毒检测技术研究 *

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**摘要** 甲型流感病毒的现场快速检测对于流感的及时有效防控具有重要意义．本研究基于杂交链式(HCR)反应，利用 GO 对荧光基团的猝灭作用及共同实现了对甲型流感病毒的快速检测．当目标序列存在时，可引发 HCR 反应，使短链 DNA 形成长链，保护 FAM 荧光基团不被猝灭，从而实现目标物的检测．实验结果表明，该方法在 10～40 nmol/L 范围内荧光强度与目标检测物浓度表现出了良好的线性关系，检测范围为 5～100 nmol/L．这种 HCR 等温扩增检测技术具有较好的样本检测能力，具有等温、无酶、反应体系简单、操作步骤简便等优点，表现出良好的现场检测应用前景．

**关键词** 甲型流感，杂交链式反应，氧化石墨烯，现场检测，无酶检测
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