PAPER IN FOREFRONT

Multiplex detection of clinical pathogen nucleic acids via a three-way junction structure-based nucleic acid circuit

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Abstract

Nucleic acid testing technology has made considerable progress in the last few years. However, there are still many challenges in the clinical application of multiple nucleic acid assays, such as how to ensure accurate results, increase speed and decrease cost. Herein, a three-way junction structure has been introduced to specifically translate analytes of loop-mediated isothermal amplification to a catalytic hairpin assembly. For different analyses, a well-optimized nucleic acid circuit can be directly applied to detection, through only one-component replacement, which only not avoids duplicate sequence design but also saves detection cost. Thanks to this design, multiple and logical analysis can be easily realized in a single reaction with ultra-high sensitivity and selectivity. In this paper, *Mycoplasma pneumoniae* and *Streptococcus pneumoniae* can be clearly distinguished from the clinical mixed sample with negative control or one analyte in one tube single fluorescence channel. The fair experimental results of actual clinical samples provide a strong support for the possibility of clinical application of this methodology.

Keywords Catalytic hairpin assembly · Three-way junction · Loop-mediated isothermal amplification · Multiplex detection

Introduction

Respiratory infections are caused by the invasion of pathogens in the human respiratory tract and have the potential to be fatal, especially for children. Respiratory infections are very common, and highly contagious infections such as Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS) and coronavirus disease 2019 (COVID-19) seriously threaten global public health security. Therefore, timely diagnosis of infectious diseases is important to prevent widespread outbreaks. Currently, the main techniques used to detect respiratory infections include immunoassays [1], polymerase chain reaction (PCR) [2, 3], next-generation sequencing (NGS) [4–6] and enzyme-linked immunosorbent assay (ELISA) [7]. However, these methods generally have disadvantages of time-consuming processes, high cost, the need for expensive testing equipment and low accuracy, which makes it difficult to meet the actual needs of clinical diagnosis [8, 9], especially with portable assays or off-the-shelf point-of-care tests (POCT). In addition, respiratory infections usually can be caused by a variety of pathogens, which means that clinical patients may have multiple infections. Furthermore, the symptoms of respiratory pathogens are often similar, such as a cough and fever, making it difficult to differentiate between pathogens by clinical symptoms. Accordingly, an ideal multi-pathogen detection method with high accuracy and low cost, along with rapid speed, is urgently needed in clinical practice to assist portable diagnosis and precise treatment.

In recent years, a series of isothermal amplification techniques which can carry out exponential nucleic acid enrichment at a constant temperature have been considered

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as promising PCR alternatives to shorten or simplify the detection progress, including loop-mediated isothermal amplification (LAMP) [10–14], rolling circle amplification (RCA) [15, 16], nucleic acid sequence-based amplification (NASBA) [17] and recombinase polymerase amplification (RPA) [18]. Among these methods, LAMP can amplify DNA templates upwards of ~ 10^9-fold at 55–65 °C with only one polymerase, thus attracting special attention for its ultra-high sensitivity and simple downstream signal output [19]. Even so, there are still various practical problems, such as false positives, low sensitivity and low signal-to-noise ratio. Multiplex detection of different pathogens is also difficult to achieve. Traditional multiplex detection requires designing different labeled probes according to different detecting target and collecting signals in different emission channels [20–22]. There is thus a significant technical barrier to developing low-cost and portable detection devices or instruments.

To overcome the above disadvantages, we and others have coupled LAMP with strand-exchange nucleic acid circuits [23] such as catalytic hairpin assembly (CHA) [24] and hybridization chain reaction (HCR) [25]. For example, a typical CHA reaction uses a single-stranded DNA (ssDNA) catalyst to trigger two successive toehold-mediated strand-exchange reactions and induce two hairpin structures (H1 and H2) self-assembling into a stable duplex product (H1:H2). This process can recognize the catalyst with ultra-high sequence specificity and generate 50–100-folds amplification within a few hours [26–30]. Therefore, when a piece of loop sequence within LAMP amplicons is designed as a CHA catalyst, CHA can provide ultra-sequence specificity and high signal-to-background ratio in addition to the ultra-sensitivity [31]. In a recent advance, we have proven that the ssDNA catalyst can be replaced by a partial duplex DNA (three-way catalyst) which can trigger the CHA reaction by forming a three-way (3W) junction structure with H1 [32, 33]. The major advantage of this improvement, called 3W-CHA, is that for different targeting sequence detection, only the sequences of domain α* of CHA-H1 and domain β* of TP must be changed according to the new targets. This means that different targets have respective H1 but share the same H2 and reporter sequences, avoiding complicated sequence rebuilding for new targets. In addition, we propose a new mode of multiplex assay, in which 3W-CHA can realize "one-tube one-channel multiple assay" by reading different concentrations of consumed H1s that correspond to different fluorescence values. This has shown great potential in solving the problem of multi-tube analysis and multi-channel analysis for multiplex detection. However, this proof-of-concept proposal has not been verified using any clinical application.

In this paper, we validate for the first time the above advantage of 3W-CHA (i.e. high universality and intelligence in multiple assays) by coupling LAMP reactions from clinical samples infected by pediatric respiratory pathogens. Pediatric respiratory diseases are the most common type of disease in children and are prone to causing pneumonia, showing an increasing trend year by year, and pneumonia has become one of the leading causes of death in children. *Mycoplasma pneumoniae* (MP) and *Streptococcus pneumoniae* (SP) were selected as detection targets in this work because they are the most common pathogens of clinical respiratory diseases in children. They have similar clinical symptoms, and the patients are likely to have both infectious diseases. Therefore, the multiplex testing for MP and SP facilitates precise treatment and symptomatic use of drugs. The method, called LAMP-3W-CHA, can detect as low as 200 copies/μL of both MP and SP DNA with high specificity and signal-to-background ratio. And all four situations for the existence of MP and SP can be clearly identified with a single fluorescence probe (FAM) and in one tube, which has been well-proven with 23 clinical samples. The actual samples include seven alveolar lavage fluid samples and 16 throat swab samples.

Experimental

Materials

All the oligonucleotides, including isotheral amplification primers, modified sequences and gene sequences were synthesized by Sangon Biotech (Shanghai, China), and the sequences are shown in Table S1. The concentrations of the sequences were determined by measuring the absorbance at 260 nm using a DeNovix DS-11 + FX spectrophotometer (DeNovix Inc., Wilmington, DE, USA). The nucleic acid extraction kits used to extract nucleic acids from actual samples were purchased from Tiangen Biotech (Beijing, China). The Mycoplasma Pneumoniae Detection Kit (which uses real-time PCR) was purchased from Daan Gene Co., Ltd (Guangzhou, China). All the oligonucleotides were stored in 1× TE buffer (pH 7.5) or H_2O at -20 °C. The 10× isothermal buffer (10× Iso) and Bst 2.0 DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). Buffers used here were 1× Iso (20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 4 mM $MgSO_4$, 0.1% Tween 20, pH 8.8) and 1× TNaK (20 mM Tris–HCl, 140 mM NaCl, 5 mM KCl, pH 7.5, 5 mM Mg^{2+}). All reagents used were of analytical grade.

Method for the validation of LAMP reaction

LAMP reaction was prepared for MP p1 gene (M21519.1) and SP cpsA gene (MK606437.1). The primer mixtures were prepared in advance, and contained 5 μM F3, 5 μM B3, 20 μM FIP and 20 μM BIP. The volume of each reaction system was 25 μL of 1× isothermal buffer (20 mM

Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH = 8.8), including 3 µL of different copies of the synthetic target genes, 2 µL of primer mixture, 2 µL of 4 mM dNTPs, 0.5 µL of 100 mM $MgCl_2$ and 10 µL of 5 M betaine. The reaction mixtures were heated at 95 °C for 2 min, followed by chilling on ice for 2 min. Then 1.5 µL (8U) of Bst 2.0 DNA polymerase was added to mixtures above to trigger the LAMP reactions and 1.5 µL 20× Eva-Green was added to monitor DNA amplification. Twenty microliters of each reaction solution was put into a Light-Cycler 96 instrument (Roche Diagnostics GmbH, Germany) for 1 h and recorded every 1 min at 60 °C. The results of the LAMP reaction are shown in Figure S3.

Standard LAMP reaction

This experiment refers to the experimental method in the article [33]. The LAMP reaction was prepared for the MP p1 gene (M21519.1) and SP cpsA gene (MK606437.1). The MP and SP LAMP reaction primer mixtures were prepared in advance, both containing 5 µM F3, 5 µM B3, 20 µM FIP and 20 µM BIP. The volume of each reaction system was 50 µL of 1× Iso buffer, including 6 µL of different copies of the synthetic target genes, 4 µL of primer mixture, 4 µL of 4 mM dNTPs, 1 µL of 100 mM $MgCl_2$ and 10 µL of 5 M betaine. The reaction mixtures were heated at 95 °C for 2 min, followed by chilling on ice for 2 min. Then 3 µL (8U) of Bst 2.0 DNA polymerase was added to the mixtures to trigger the LAMP reactions. The reactions were incubated at 60 °C for 90 min, followed by heating to 80 °C for 20 min to denature the DNA polymerase. Afterwards, some of the products were kept at 4 °C until they were analyzed by electrophoresis on 1% BBI LE agarose gel. Each well was loaded with 5 mL of the LAMP product and an additional 1 µL of 6× orange loading dye (40% glycerol, 0.25% Orange G). The electrophoresis gel was developed at 120 V for 30 min. The rest of the products were kept an -20 °C until the next experiments [33].

Standard 3W-CHA detection

This experiment refers to the experimental method in the article [33]. In standard 3W-CHA detection, targets at different concentrations were hybridized with 200 nM TP in 1× TNak buffer via a standard annealing process, in which mixtures were heated at 95 °C for 5 min and cooled down to room temperature at 0.1 °C s^{-1}. This annealing process can be applied to all other annealing steps mentioned in the subsequent experiments. Then the mixtures were further mixed with 200 nM H1, 800 nM H2 and 200 nM reporter duplex (containing 200 nM F and 400 nM Q, and annealed before) in 1× TNak buffer in equal volume. H1 and H2 were pre-annealed to be refolded. The final reaction concentrations were 50 nM H1, 200 nM H2, 50 nM F, 100 nM Q and 50 nM TP. In order to prevent the loss caused by plastic adsorption and ensure accurate fluorescence quantification, we added 2 µM $(dT)_{21}$ in all reaction solutions. Seventeen microliters of each reaction solution was transferred to a Nunc 384 low-volume black microplate and recorded every 1 min at 55 °C on a Cytation™ 5 imaging multi-mode plate reader (Biotek, Winooski, VT, USA).

Multiplex target detection with 3W-CHA

The procedure of multiplex target detection at 55 °C was similar to that for standard 3W-CHA detection with a little modification. Different targets were hybridized with different TPs (T_{MP} with TP_{MP} and T_{SP} with TP_{SP}) in the same tube in 1× TNak buffer via a standard annealing process. Then the mixtures were further mixed with H1 solution (containing 400 nM $H1_{MP}$ and 200 nM $H1_{SP}$), 600 nM H2 and 600 nM reporter duplex (containing 600 nM F and 1.2 µM Q) in 1× TNak buffer in equal volume. The final concentrations of TP_{MP} and TP_{SP} were both 50 nM. In order to prevent the loss caused by plastic adsorption and ensure the accurate fluorescence quantification, we added 2 µM $(dT)_{21}$ in all reaction solutions. Seventeen microliters of each reaction solution was transferred to a Nunc 384 low-volume black microplate and recorded every 1 min at 55 °C on a Cytation™ 5 imaging multi-mode plate reader (Biotek, Winooski, VT, USA).

LAMP detection with 3W-CHA

The method for the validation of the LAMP reaction is shown in the supporting information. The end-point LAMP reaction detected with 3W-CHA was performed in the same manner as standard 3W-CHA detection for oligonucleotide, except that the oligonucleotide target is replaced by LAMP product.

Multiplex detection with LAMP-3W-CHA

Multiplex LAMP product detection was conducted by the LAMP reaction product we made earlier. For this purpose, 2.5 µL MP LAMP product and 3 µL SP LAMP product were annealed with 400 nM TP_{MP} and 200 nM TP_{SP} in a total volume of 25 µL of 1× TNak buffer. All of the above mixtures were further mixed with CHA components ($H1_{MP}$, $H1_{SP}$, H2 and reporter duplex F:Q) in 1× TNak buffer. The final reaction contained 100 nM $H1_{MP}$, 50 nM $H1_{SP}$, 150 nM H2 and 150 nM reporter duplex (containing 150 nM F and 300 nM Q). In order to prevent the loss caused by plastic adsorption and ensure the accurate fluorescence quantification, we added 2 µM $(dT)_{21}$ in all reaction solutions. Seventeen microliters of each reaction solution was transferred to a Nunc 384 low-volume black microplate and recorded every

1 min at 55 °C on a Cytation™ 5 imaging multi-mode plate reader (Biotek, Winooski, VT, USA).

Nucleic acid extraction with nucleic acid extraction kit

Two hundred microliters of the actual sample was put into a 2 mL centrifuge tube, 20 µL of Proteinase K solution was added, and the solution was vortexed to mix well. Then, 200 µL of Buffer GB was added and mixed the solution well. The solution was incubated at 56 °C for 10 min with occasional shaking of the sample and brief centrifugation to remove droplets from the inside of the tube cap. Then, 200 µL of ethanol (96–100%) was added to the solution, which was gently inverted, and the sample was mixed and left at room temperature for 5 min. The solution obtained in the previous step was added to a CR2 column, centrifuged at 12,000 rpm (~ 13,400×g) for 30 s, then the waste solution was discarded and the CR2 column was returned to the collection tube. Next, 500 µL of GD buffer was added to the CR2 column and centrifuged at 12,000 rpm (~ 13,400×g) for 30 s, after which the waste solution was discarded and the CR2 column was returned to the collection tube. Subsequently, 600 µL of PW rinse solution was added, the mixture was added to the CR2 column and centrifuged at 12,000 rpm (~ 13,400×g) for 30 s, and the waste solution was discarded and the CR2 column returned to the collection tube. Again, 600 µL of PW rinse solution was added and centrifuged at 12,000 rpm (~ 13,400×g) for 30 s, after which the waste solution was discarded and the CR2 column was returned to the collection tube and centrifuged at 12,000 rpm (~ 13,400×g) for 2 min, and the waste solution was poured off. Finally, the CR2 column was transferred to a clean centrifuge tube, 20–50 µL of TB elution buffer was added dropwise to the middle of the adsorbent membrane and centrifuged at 12,000 rpm (~ 13,400×g) for 2–5 min at room temperature, and the solution was collected in a centrifuge tube.

Actual sample detection with LAMP-3W-CHA

The nucleic acids were extracted from actual samples using a nucleic acid extraction kit before LAMP reaction (SI). The primer mixtures of the LAMP reaction were prepared as before. The volume of each reaction system was 25 µL of 1× Iso buffer, including 3 µL of the extracted nucleic acid, 2 µL of primer mixture, 2 µL of 4 mM dNTPs, 0.5 µL of 100 mM MgCl$_2$ and 5 µL of 5 M betaine. The reaction mixtures were heated at 95 °C for 2 min, followed by chilling on ice for 2 min. Then 1.5 µL (8U) of Bst 2.0 DNA polymerase was added to the mixtures above to trigger the LAMP reactions. The reactions were incubated at 60 °C for 90 min, followed by heating to 80 °C for 20 min to denature the DNA polymerase. Finally, the LAMP reaction product was used to initiate the 3W-CHA reaction in the same manner as multiplex LAMP product detection with 3W-CHA.

Validation of PCR reaction with PCR kits

The MP PCR reaction with PCR kits was detected with the Mycoplasma Pneumoniae Detection Kit (real-time PCR method) purchased from Daan Gene Co., Ltd (Guangzhou, China). Two microliters of nucleic acid extracted using the kit from actual samples was added to the tube containing 40 µL PCR reaction solution and 3 µL Taq enzyme. Then, 20 µL of each reaction solution was put into the LightCycler 96 instrument (Roche Diagnostics GmbH, Germany). The run editor of the instrument is shown in Table S2.

Results and discussion

The principle of target mimic gene detection using three-way junction CHA

The CHA circuit is a nucleic acid circuit which can also achieve 100-fold signal amplification. In the traditional CHA circuit (Figure S1), a single-stranded DNA oligonucleotide is repeatedly used to initialize the CHA reaction without being consumed, like a catalyst [24, 34]. To detect different input targets, we make an important modification on the trigger and obtain the nucleic acid circuit, called three-way junction-based CHA (3W-CHA) (Fig. 1). When T (defined as α–β) and TP (defined as β*-γ) are linked together by β-β* hybridization, the T:TP duplex replaces the single-stranded DNA oligonucleotide in the CHA reaction and acts as a trigger to initiate the toehold-mediated chain displacement reaction, which can open the hairpin, called H1. Meanwhile, T:TP:H1 formed a three-way junction structure. The exposed segment I of H1 hybridizes with a segment I* of another hairpin, called H2, thus exposing its segment III and forming the H1:H2 duplex. Then the exposed segment III of H2 can replace the 3′ quencher-labeled strand (Q) and combine with 5′ FAM-labeled strand (F). Finally, the fluorescent groups are excited to generate fluorescent signals, and the fluorescence intensity depends on the amount of H1 being released. The T:TP duplex can be recycled into the next cycle of the 3W-CHA reaction without being consumed, just like a catalyst. The concentration of released FAM (represented by the final fluorescence enhancement) is theoretically equivalent to that of H1. In addition, two specific reactions, namely T:TP recognition obtained by β-β* hybridization and T:TP-H1 recognition obtained by α-α* hybridization, provide a double guarantee for allele recognition. As a result, the 3W-CHA circuit can not only detect the target mimic gene sensitively and quickly, but can also realizes 100-fold signal amplification.
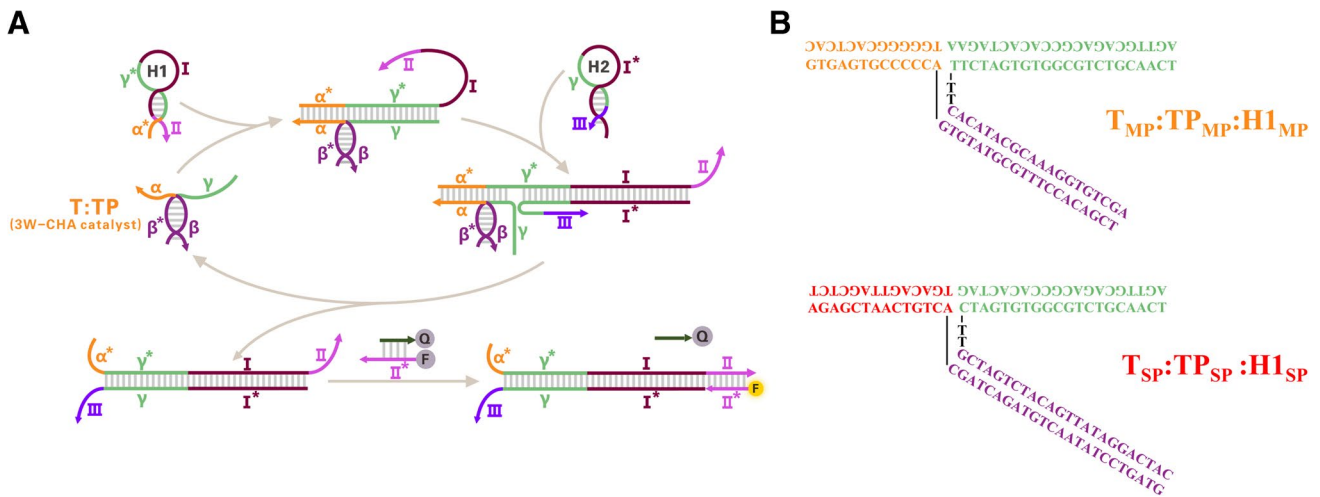
Fig. 1 **A** Scheme for a detector based on 3W-CHA. Asterisks (*) are used to represent complementary sequences. **B** The sequence design of the three-way junction structures $T_{MP}:TP_{MP}:H1_{MP}$ and $T_{SP}:TP_{SP}:H1_{SP}$

In order to detect MP and SP. Two 3W-CHA reactions were designed according to previous publication [33]. Single-stranded nucleic acid measuring 33 and 38 nucleotides (nt) in length within MP and SP genes were selected as the targeting sequence mimics, termed T_{MP} and T_{SP}, respectively. As the sequence design in Fig. 1B shows, TP_{MP} with $H1_{MP}$ and TP_{SP} with $H1_{SP}$ were designed according to sequences of T_{MP} and T_{SP}, respectively. The α^* and β^* domains were only different between different TPs and H1s, and the two 3W-CHA reactions used the same H2, F and Q sequences. As shown, both $T_{MP}:TP_{MP}$ (Fig. 2A) and $T_{SP}:TP_{SP}$ (Fig. 2B) duplexes could successfully trigger the

3W-CHA reaction with high signal-to-noise ratio and efficiency. Note that the reaction rates of the two reactions are slightly different because of the different sequences being detected. The different CG ratio and Gibbs free energy have a relatively large effect on the efficiency of the reaction.

Simultaneous 3W-CHA detection of T_{MP} and T_{SP} in one tube

As derived from the principle of 3W-CHA, we only need to change the base sequence of α^* (on H1) and β^* (on TP) to realize the detection of different target genes, while other
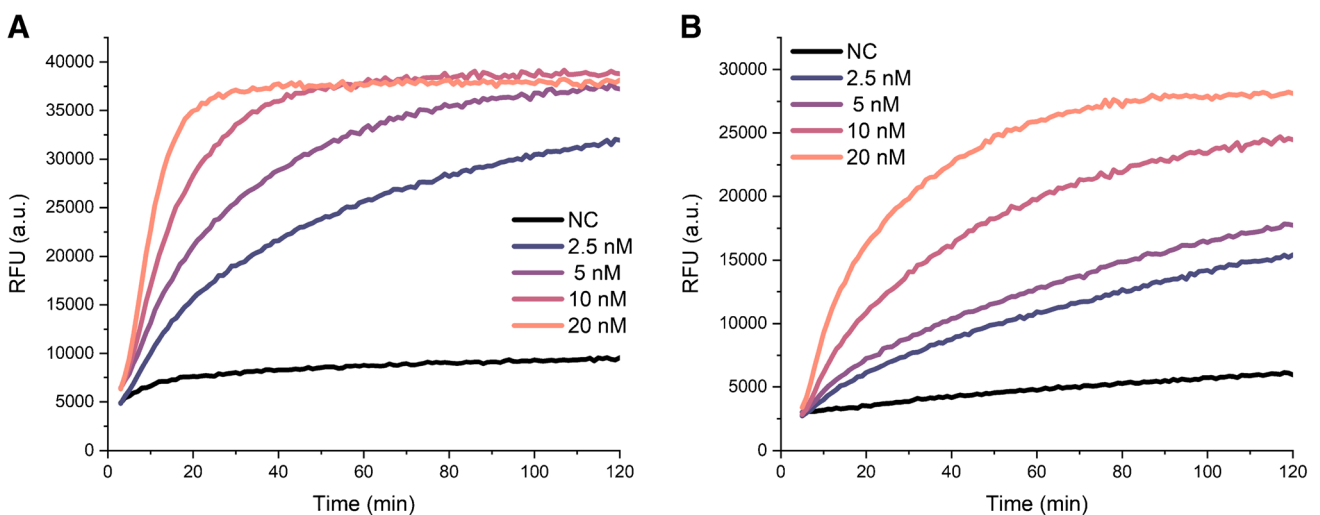
Fig. 2 The 3W-CHA reaction of T_{MP} and T_{SP}. **A** Detection of different concentrations of T_{MP}. $[H1_{MP}] = 1/4 [H2] = [F] = 1/2[Q] = 50$ nM, $[TP_{MP}] = 50$ nM. **B** Detection of different concentrations of T_{SP}. $[H1_{SP}] = 1/4[H2] = [F] = 1/2[Q] = 50$ nM, $[TP_{SP}] = 50$ nM. The concentrations of the target (T_{MP} or T_{SP}) were 0, 2.5 nM, 5 nM, 10 nM and 20 nM

sequences (H2, F and Q) that play a key role do not need to be changed. Making use of this advantage, we can realize the multiple detection of TP_{MP} and TP_{SP} in one tube and with a single fluorescence channel. TPs and all CHA components were the same as above. The mixtures were added into a probe mixture containing 100 nM $H1_{MP}$ and 50 nM $H1_{SP}$, which allowed T_{MP} and T_{SP} to release up to 50 nM and 100 nM of fluorescent FAM reporter, respectively, when the reaction was terminated at the end. For example, when ≥ 10 nM T_{MP} or T_{SP} was present, about 2 h was enough for the reactions to be completed. The results of simultaneous detection are shown in Fig. 3B. Based on the fluorescence signal platform, it is reasonable to determine whether the sample contains the target gene and the specific type of the target gene. To identify the presence of T_{SP}, the fluorescence readout is set above the NC line but below the 50 nM line. Similarly, to identify the presence of T_{MP}, the fluorescence readout should be above the 50 nM line but below the 100 nM line, while to identify the presence of both T_{MP} and T_{SP}, the fluorescence readout should be significantly above the 100 nM line and infinitely close to the 150 nM line. Please note that there is still a slowly increasing trend

for the fluorescence of NC and T_{SP}. This is normal because of the background reaction induced by the high concentration of $H1_{MP}$, but it will not affect the result if we control the reaction time to less than 2 h.

3W-CHA detection coupled with LAMP amplification (LAMP-3W-CHA) for ultrasensitive detection

The above experiments confirm that 3W-CHA can effectively detect the target mimic gene with high efficiency and universality. But the nM level sensitivity for the TP will not be enough to meet the ultra-high requirement for pathogen gene detection. In order to enhance the sensitivity, LAMP reaction was imported, in which the sequences of TP_{MP} and TP_{SP} were, respectively, designed as the loop sequences within the LAMP products. In this way (shown in Fig. 4A), the detection of TP_{MP} or TP_{SP} was replaced by the detection of the LAMP products for the MP or SP gene template. This method is shortened as LAMP-3W-CHA. Electrophoresis was used to verify that the self-designed LAMP reactions were very effective (Figure S3). We then carried out the LAMP-3W-CHA for MP and SP gene templates,
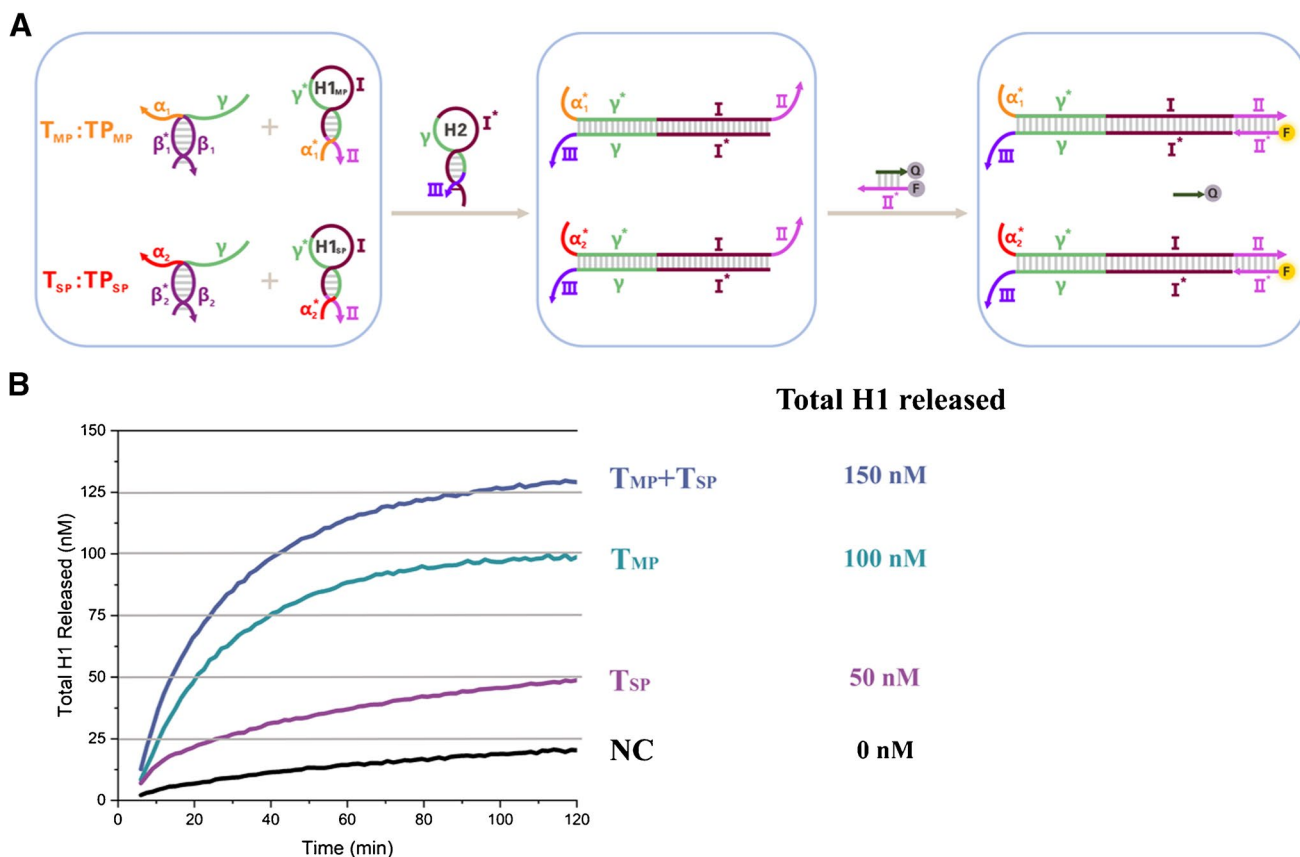
Fig. 3 Simultaneous 3W-CHA detection of T_{MP} and T_{SP} in one tube. **A** Sequence design schematic of the two 3W-CHAs for T_{MP} and T_{SP}. **B** The results of the simultaneous detection for T_{MP} and T_{SP}. $[H1_{SP}] = 1/2[H1_{MP}] = 1/3[H2] = 1/3[F] = 1/6[Q] = 50$ nM, $[TP_{MP}] = [TP_{SP}] = 50$ nM. Once present, $[T_{MP}] = [T_{SP}] = 10$ nM
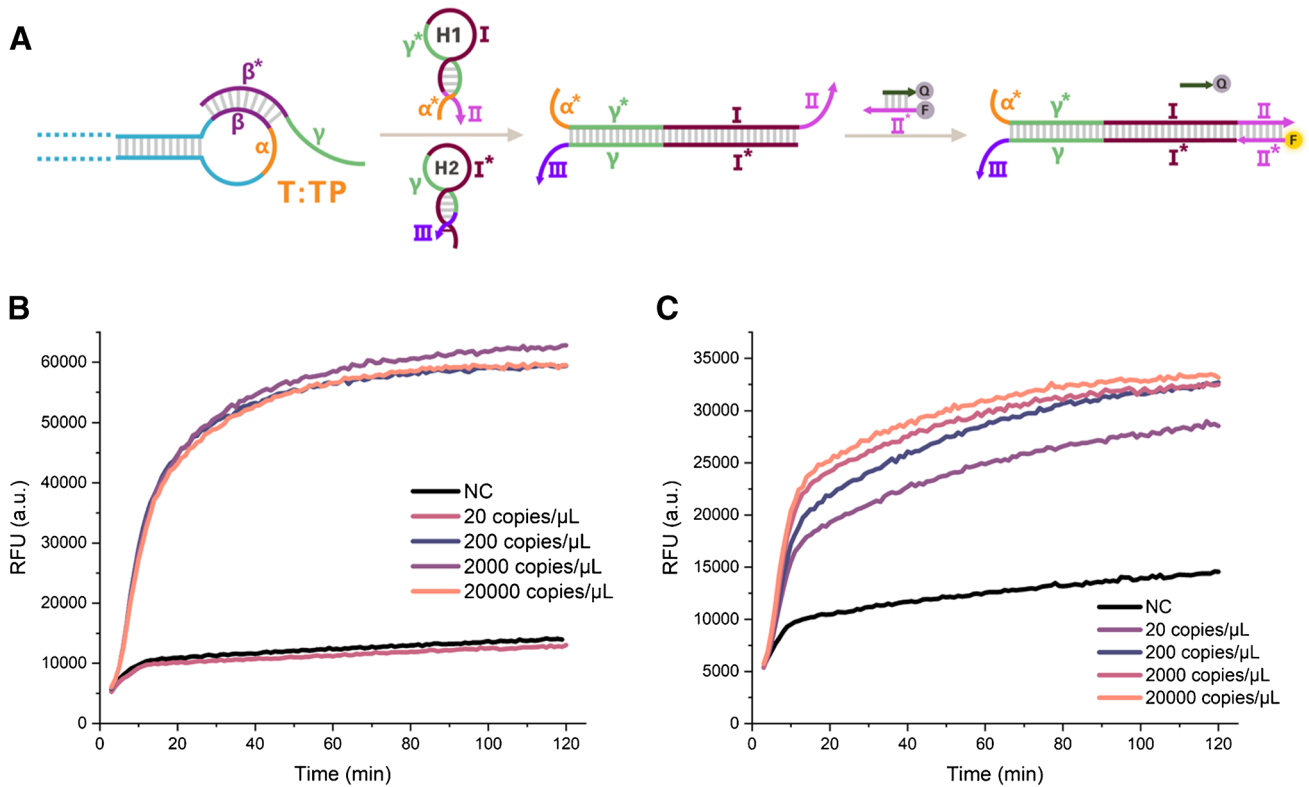
Fig. 4 3W-CHA detection of LAMP amplicons. **A** Schematic of using 3W-CHA to detect loop amplicons of the LAMP reaction. **B** Responses of 3W-CHA to single MP LAMP ampli-cons. $[H1_{MP}] = 1/4[H2] = [F] = 1/2[Q] = 50$ nM, $[TP_{MP}] = 50$ nM. **C** Responses of 3W-CHA to single SP LAMP amplicons. $[H1_{SP}] = 1/4[H2] = [F] = 1/2[Q] = 50$ nM, $[TP_{SP}] = 50$ nM

respectively. After 1 h LAMP reaction, the products from different concentrations of templates were mixed with the corresponding TP and the components of CHA (including H1, H2 and reporter duplex F:Q), and the process of 3W-CHA detection was initialized and monitored in real time using a fluorescence plate reader. As shown in Fig. 4B, LAMP products amplified different concentrations of the templates may generate very similar 3W-CHA signals and consume out all the CHA components within 2 h. There was no concentration dependence because 1 h LAMP reaction may be enough for the templates of different concentrations to generate similarly high amounts of products. The detection limits for the MP template and the SP template are 200 copies/μL and 20 copies/μL, respectively. Both sensitivities meet clinical requirements.

## Simultaneous LAMP-3W-CHA detection of MP and SP gene templates in one tube

On the basis of the above verification of the conception, we carried out the simultaneous detection of the LAMP products amplified from MP and SP gene templates. TPs and 3W-CHA components were the same as above (Fig. 3). The LAMP products (with none, one or two templates) were

added into a probe mixture containing 50 nM TP_{MP}, 50 nM TP_{SP}, 100 nM $H1_{MP}$, 50 nM $H1_{SP}$, 150 nM H2 and 150 nM reporter duplex F:Q. The 3W-CHA results are shown in Fig. 5. The discrimination of the four situations (NC, MP, SP and MP + SP) follows exactly the same method as that of Fig. 3B.

Actual sample detection with LAMP-3W-CHA

To test the feasibility of applying the LAMP-3W-CHA cir-cuit in actual samples, we separately explored the detection of MP and SP in alveolar lavage fluid and throat swabs. We prepared seven alveolar lavage fluid samples and 16 throat swab samples. After the DNA was extracted by the kit, the LAMP-3W-CHA detection procedure was carried out and data treatment methods following the same simultaneous LAMP-3W-CHA protocol established above (Fig. 6). We list the detection results using bar graphs shown in Fig. 6, in which whether a patient is infected by one or two pathogens is clearly presented. The results were then compared with PCR detection. Because there is no dual-detection commer-cial PCR kit for MP and SP, MP and SP detection was car-ried out in two tubes using their respective PCR kits (Figure S5). The results of the MP PCR kit were consistent with the
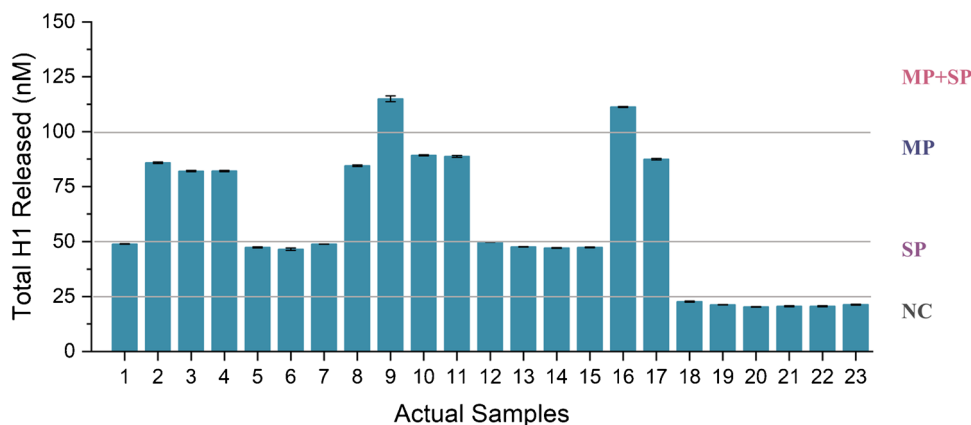
Fig. 5 3W-CHA detection of MP and SP. The result can be logically determined according to the fluorescence signal platform. The final concentrations of each component were as follows: $[H1_{MP}] = 100$ nM, $[H1_{SP}] = 50$ nM, $[H2] = 150$ nM, $[F] = 150$ nM, $[Q] = 300$ nM, $[TP_{MP}] = [TP_{SP}] = 50$ nM
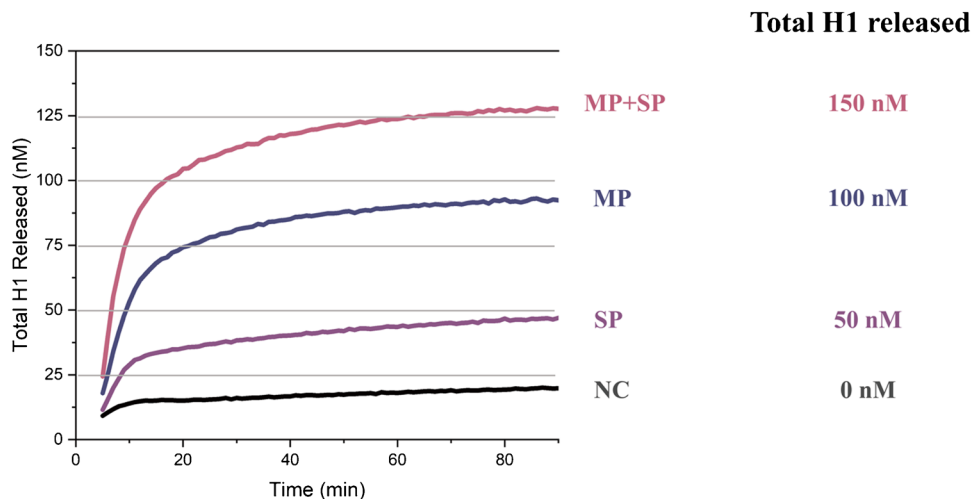
results of our detection, but we could not detect SP using the kit (please note there are few SP PCR kits on the market, so it is hard for us to use another one for comparison). We randomly sent one SP-positive LAMP-3W-CHA (but PCR-negative) sample to WillingMed Technology (Beijing) Co., Ltd. for NGS sequencing, which should be the gold standard method for gene detection. The sequencing result confirmed that the sample is indeed SP-positive, which proves the accuracy of the LAMP-3W-CHA detection. We thus demonstrate LAMP-3W-CHA as a promising clinical method for one-tube multiple detection. It deserves further optimization and instrumentation for even higher practicability. All actual samples were provided by the First Bethune Hospital of Jilin University. The explanation of the routine detection methods for MP and SP in hospital and comparison with LAMP-3W-CHA is shown in as Sect. 2 of the Supporting Information.

Conclusions

In conclusion, we took advantage of the 3W-CHA nucleic acid circuit and placed it downstream of the LAMP reaction to obtain the method called LAMP-3W-CHA. 3W-CHA enables sensitive and rapid detection of target DNA and also enables 100-fold amplification of the signal, with target DNA as low as 2.5 nM unamplified detected by 3W-CHA in 1.5 h. And when we combined 3W-CHA with LAMP reaction, 200 copies/µL of target DNA were able to be detected by LAMP-3W-CHA. Then we realized multiplex detection of MP and SP in one tube and a single fluorescence channel with LAMP-3W-CHA. The results can be logically determined according to the fluorescence signal platform. Finally, we tested the actual samples with LAMP-3W-CHA and compared the results

Fig. 6 Actual sample detection with LAMP-3W-CHA. According to the fluorescence signal platform, it can be logically determined that the actual samples numbered 2, 3, 4, 8, 10, 11, 17 were identified as infected with MP, the actual samples numbered 1, 5, 6, 7, 12, 13, 14, 15 were identified as infected with SP, and the actual samples num-

bered 9, 16 were identified as infected with both MP and SP. The final concentrations of each component were as follows: $[H1_{MP}] = 100$ nM, $[H1_{SP}] = 50$ nM, $[H2] = 150$ nM, $[F] = 150$ nM, $[Q] = 300$ nM, $[TP_{MP}] = [TP_{SP}] = 50$ nM

with those of commercial PCR kits, and the results were matched. The detection of actual clinical samples provides strong support for the possible clinical application of the LAMP-3W-CHA. In addition, this method is simple in design and takes a short time to achieve the detection of new targets, just by changing the sequence α* on H1 and β* on TP. Therefore, we believe that LAMP-3W-CHA can be regarded as a useful tool for nucleic acid detection in clinic diagnostics, and next we will try to combine it with microfluidic chip technology and apply it to point-of-care testing (POCT).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-023-04637-3.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval Families of 23 children signed the patient informed consent form, and the study was approved by the hospital medical ethics committee (AF-IRB-030–06).

References

1. Mertens P, De Vos N, Martiny D, Jassoy C, Mirazimi A, et al. Development and Potential Usefulness of the COVID-19 Ag Respi-Strip Diagnostic Assay in a Pandemic Context. Frontiers in Medicine. 2020;7. https://doi.org/10.3389/fmed.2020.00225

2. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, et al. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol. 1986;51:263–73. https://doi.org/10.1101/sqb.1986.051.01.032.

3. Wang L, Huang Z, Wang R, Liu Y, Qian C, et al. Transition Metal Dichalcogenide Nanosheets for Visual Monitoring PCR Rivaling a Real-Time PCR Instrument. ACS Appl Mater Interfaces. 2018;10:4409–18. https://doi.org/10.1021/acsami.7b15746.

4. Lee H, Martinez-Agosto JA, Rexach J, Fogel BL. Next generation sequencing in clinical diagnosis. The Lancet Neurology. 2019;18:426. https://doi.org/10.1016/S1474-4422(19)30110-3.

5. Kustin T, Ling G, Sharabi S, Ram D, Friedman N, et al. A method to identify respiratory virus infections in clinical samples using next-generation sequencing. Sci Rep. 2019;9:2606. https://doi.org/10.1038/s41598-018-37483-w.

6. O'Flaherty BM, Li Y, Tao Y, Paden CR, Queen K, et al. Comprehensive viral enrichment enables sensitive respiratory virus genomic identification and analysis by next generation sequencing. Genome Res. 2018;28:869–77. https://doi.org/10.1101/gr.226316.117.

7. Lequin RM. Enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA). Clin Chem. 2005;51:2415–8. https://doi.org/10.1373/clinchem.2005.051532.

8. Scohy A, Anantharajah A, Bodéus M, Kabamba-Mukadi B, Verroken A, et al. Low performance of rapid antigen detection test as frontline testing for COVID-19 diagnosis. J Clin Virol. 2020;129:104455. https://doi.org/10.1016/j.jcv.2020.104455.

9. Graf EH, Simmon KE, Tardif KD, Hymas W, Flygare S, et al. Unbiased Detection of Respiratory Viruses by Use of RNA Sequencing-Based Metagenomics: a Systematic Comparison to a Commercial PCR Panel. J Clin Microbiol. 2016;54:1000–7. https://doi.org/10.1128/jcm.03060-15.

10. Fu S, Qu G, Guo S, Ma L, Zhang N, et al. Applications of loop-mediated isothermal DNA amplification. Appl Biochem Biotechnol. 2011;163:845–50. https://doi.org/10.1007/s12010-010-9088-8.

11. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes. 2002;16:223–9. https://doi.org/10.1006/mcpr.2002.0415.

12. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000;28:e63–e63. https://doi.org/10.1093/nar/28.12.e63.

13. Becerer L, Borst N, Bakheit M, Frischmann S, Zengerle R, et al. Loop-mediated isothermal amplification (LAMP) – review and classification of methods for sequence-specific detection. Anal Methods. 2020;12:717–46. https://doi.org/10.1039/C9AY02246E.

14. Zhao Y, Fang X, Yu H, Fu Y, Zhao Y. Universal Exponential Amplification Confers Multilocus Detection of Mutation-Prone Virus. Anal Chem. 2022;94:927–33. https://doi.org/10.1021/acs.analchem.1c03702.

15. Liu D, Daubendiek SL, Zillman MA, Ryan K, Kool ET. Rolling Circle DNA Synthesis: Small Circular Oligonucleotides as Efficient Templates for DNA Polymerases. J Am Chem Soc. 1996;118:1587–94. https://doi.org/10.1021/ja952786k.

16. Ren K, Zhang Y, Zhang X, Liu Y, Yang M, et al. In Situ SiRNA Assembly in Living Cells for Gene Therapy with MicroRNA Triggered Cascade Reactions Templated by Nucleic Acids. ACS Nano. 2018;12:10797–806. https://doi.org/10.1021/acsnano.8b02403.

17. Compton J. Nucleic acid sequence-based amplification. Nature. 1991;350:91–2. https://doi.org/10.1038/350091a0.

18. Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. PLoS Biol. 2006;4:e204. https://doi.org/10.1371/journal.pbio.0040204.

19. Lin Q, Ye X, Huang Z, Yang B, Fang X, et al. Graphene Oxide-Based Suppression of Nonspecificity in Loop-Mediated Isothermal Amplification Enabling the Sensitive Detection of Cyclooxygenase-2 mRNA in Colorectal Cancer. Anal Chem. 2019;91:15694–702. https://doi.org/10.1021/acs.analchem.9b03861.

20. Zhang Y, Chen L, Hsieh K, Wang T-H. Ratiometric Fluorescence Coding for Multiplex Nucleic Acid Amplification Testing. Anal Chem. 2018;90:12180–6. https://doi.org/10.1021/acs.analchem.8b03266.

21. Wang L, Zhang Y, Tian J, Li H, Sun X. Conjugation polymer nanobelts: a novel fluorescent sensing platform for nucleic acid

detection †. Nucleic Acids Res. 2010;39:e37–e37. https://doi.org/10.1093/nar/gkq1294.

22. Nguyen HV, Phan VM, Seo TS. A portable centrifugal genetic analyzer for multiplex detection of feline upper respiratory tract disease pathogens. Biosens Bioelectron. 2021;193:113546. https://doi.org/10.1016/j.bios.2021.113546.

23. Yin P, Choi HMT, Calvert CR, Pierce NA. Programming biomolecular self-assembly pathways. Nature. 2008;451:318–22. https://doi.org/10.1038/nature06451.

24. Li B, Ellington AD, Chen X. Rational, modular adaptation of enzyme-free DNA circuits to multiple detection methods. Nucleic Acids Res. 2011;39:e110–e110. https://doi.org/10.1093/nar/gkr504.

25. Dirks RM, Pierce NA. Triggered amplification by hybridization chain reaction. Proc Natl Acad Sci. 2004;101:15275–8. https://doi.org/10.1073/pnas.0407024101.

26. Su F, Zou M, Wu H, Xiao F, Sun Y, et al. Sensitive detection of hepatitis C virus using a catalytic hairpin assembly coupled with a lateral flow immunoassay test strip. Talanta. 2022;239:123122. https://doi.org/10.1016/j.talanta.2021.123122.

27. Liu J, Zhang Y, Xie H, Zhao L, Zheng L, et al. Applications of Catalytic Hairpin Assembly Reaction in Biosensing. Small. 2019;15:1902989. https://doi.org/10.1002/smll.201902989.

28. Li B, Jiang Y, Chen X, Ellington AD. Probing Spatial Organization of DNA Strands Using Enzyme-Free Hairpin Assembly Circuits. J Am Chem Soc. 2012;134:13918–21. https://doi.org/10.1021/ja300984b.

29. Zhou J, Lin Q, Huang Z, Xiong H, Yang B, et al. Aptamer-Initiated Catalytic Hairpin Assembly Fluorescence Assay for Universal. Sensitive Exosome Detection Analytical Chemistry. 2022;94:5723–8. https://doi.org/10.1021/acs.analchem.2c00231.

30. Wang H, Li C, Liu X, Zhou X, Wang F. Construction of an enzyme-free concatenated DNA circuit for signal amplification and intracellular imaging. Chem Sci. 2018;9:5842–9. https://doi.org/10.1039/C8SC01981A.

31. Tang Y, Zhu Z, Lu B, Li B. Spatial organization based reciprocal switching of enzyme-free nucleic acid circuits. Chem Commun. 2016;52:13043–6. https://doi.org/10.1039/C6CC07153H.

32. Guo L, Lu B, Dong Q, Tang Y, Du Y, et al. One-tube smart genetic testing via coupling isothermal amplification and three-way nucleic acid circuit to glucometers. Anal Chim Acta. 2020;1106:191–8. https://doi.org/10.1016/j.aca.2020.01.068.

33. Tang Y, Lu B, Zhu Z, Li B. Establishment of a universal and rational gene detection strategy through three-way junction-based remote transduction. Chem Sci. 2018;9:760–9. https://doi.org/10.1039/C7SC03190D.

34. Tang Y, Liu Y, Lu B, Guo L, Li B. Adaption and Optimization of Universal Hairpin Transduction in Gene Diagnostics Based on Nucleic Acid Circuits. Chin J Anal Chem. 2018;46:865–74. https://doi.org/10.11895/j.issn.0253-3820.181056.

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