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Interference reduction isothermal nucleic acid amplification strategy for COVID-19 variant detection

Guodong Li, Chung-Nga Ko, Zikang Wang, Feng Chen, Wanhe Wang, Dik-Lung Ma, Chung-Hang Leung

Abstract

Common reference methods for COVID-19 variant diagnosis include viral sequencing and PCR-based methods. However, sequencing is tedious, expensive, and time-consuming, while PCR-based methods have high risk of insensitive detection in variant-prone regions and are susceptible to potential background signal interference in biological samples. Here, we report a loop-mediated interference reduction isothermal nucleic acid amplification (LM-IR-INA) strategy for highly sensitive single-base mutation detection in viral variants. This strategy exploits the advantages of nicking endonuclease-mediated isothermal amplification, luminescent iridium(III) probes, and time-resolved emission spectroscopy (TRES). Using the LM-IR-INA strategy, we established a luminescence platform for diagnosing COVID-19 D796Y single-base substitution detection with a detection limit of 2.01 × 10^5 copies/μL in a linear range of 6.01 × 10^5 to 3.76 × 10^8 copies/μL and an excellent specificity with a variant/wild-type ratio of significantly less than 0.0625%. The developed TRES-based method was also successfully applied to detect D796Y single-base substitution sequence in complicated biological samples, including throat and blood, and was a superior to steady-state technique. LM-IR-INA was also demonstrated for detecting the single-base substitution D614G as well as the multiple-base mutation H69/V70del without mutual interference, indicating that this approach has the potential to be used as a universal viral variant detection strategy.

Keywords:
Viral variant
Detection
Single-base mutation

1. Introduction

Coronavirus disease 2019 (COVID-19) variants with increased transmissibility or the ability to evade immunity will likely continue to arise in the future due to frequent error-prone replication[1]. The currently most prevalent COVID-19 variant, Omicron, possesses a combination of previous mutations (e.g. D614G, H69/V70del, H655Y, N679K, and P681H), new receptor-binding domain mutations (e.g. N440K, G466S, S477N, Q493K, G496S, Q498R, and Y505H), new N-terminal domain mutations (e.g. Δ143–145, A67V, T95I, L212I, and Δ211), and new mutations spike fusion domain mutations (e.g. D796Y) [2–4].

Currently, the most commonly available detection methods for viral variant diagnosis are viral sequencing and polymerase chain reaction (PCR)-based methods. Viral sequencing (e.g. whole genome sequencing or partial S-gene sequencing) is the standard method for viral variant diagnosis[5]. Despite the importance of this technique for the identification of new variants, it is often tedious (requiring multiple steps, including overlapping primer design, amplicon sequencing, reading merging, sorting reads by amplicon, read clustering, assembly, etc.)(6),

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The mRNA-21 sequences, the luminescence method still showed some limitations in their ability to specifically detect base mutations, especially for single-base mutation. For example, the one- or two-base mutant miRNA-21 sequences, the luminescence signal was monitored using a QuantStudio™ 7 Flex System (Applied Biosystems, Foster City, CA, USA).

2.4. Detection of mutation sequence using steady-state and TRES modes

The NEMA-based amplification production was diluted to a final volume of 250 μL in 50 mM Tris–HCl buffer (pH = 7.0, 20 mM Tris, 50 mM KCl) with 2.0 μM of probe V_1 at room temperature. The steady-state signal of the mixture solution was recorded using a SpectraMax® iDS System (Molecular Devices, Sunnyvale, CA, USA). The TRES signals of the mixture solution with 350 ns delay time were recorded with the excitation at 370 nm on a Fluorolog-3 spectrophotometer (Horiba Jobin Yvon Inc., France).

3. Results and discussion

3.1. Recognition of D796Y single-base substitution sequence in LM-IR-INA strategy

D796Y single-base (G-to-T) mutation (Fig. 1A), a special variant site in the COVID-19 Omicron spike protein[2], was used as a model for viral single-base substitution detection. The recognition principle of the D796Y detection is illustrated in Fig. 1B. A padlock for D796Y (padlock_D796Y) was firstly designed, which contains one G-quadruplex DNA PS2.M complementary sequence (green region), two cut sites of Nb.BbvCI nicking endonuclease (5’-CCTCAGC-3’, yellow regions), and a D796Y complementary sequence that is divided into two separate regions (grey regions) at the 5’- and 3’-termini of the padlock. In presence of the D796Y substitution sequence, the 5’- and 3’-termini of the padlock_D796Y are brought together hybridization, thus facilitating the ligation and cyclization of the padlock_D796Y with the assistance of T4 DNA ligase. The target D796Y sequence is then cleaved by Exo I/III, leaving behind the circular loop_D796Y. In contrast, with the D796 WT sequence, the mismatch of the nucleotide bases prevents the hybridization of D796 WT with the padlock_D796Y and the two ends of the padlock sequence from coming into close proximity. Then, all open-chain sequences, including both the padlock_D796Y and the D796 WT sequences, are cleaved by Exo I/III.

The iridium(III) complex probe V_1 is a highly selective PS2.M G-quadruplex probe with enhanced luminescence (Fig. 1C) as described in our previous work[18,25]. In order to optimize the V_1-recognized sequence of padlock_D796Y to detect viral variants, the three PS2.M G-quadruplex-containing sequences (ON1, ON2, and ON3) with different nucleic acid lengths was firstly designed and synthesized (Table S1). These sequences contain a PS2.M G-quadruplex, parts of the Nb.BbvCI nicking endonuclease cut sites, and random sequences. PS2.M G-quadruplex can fold into G-quadruplex structures in the presence of K+ and obtain the circular loops.

2.2. NEMA-based amplification

The mixture of the circular loop and primer 1 were firstly heated to 95 °C for 3 min and cooled down to room temperature to generate the circular loop/primer 1 duplex. The NEMA-based amplification reaction was performed followed by the addition of phi29 polymerase (10 U/μL), phi29 polymerase buffer, dNTPs (500 μM), CurstSmart buffer, and 1 μg/mL of BSA, and Nb.BbvCI (1 μL/μL). Then, the NEMA-based amplification products were obtained by incubating at 37 °C for 25 min and inactivating by heating the reaction solution at 80 °C for 5 min. Finally, the NEMA-based amplification products were stored at –20 °C.

2.3. The fluorescence signal monitoring of NEMA-based amplification products

NEMA-based amplification was monitored by using the SYBR Green II probe following the previously reported methods. The fluorescence signal was monitored using a QuantStudio™ 7 Flex System (Applied Biosystems, Foster City, CA, USA).

2. Experimental

2.1. Recognition of mutation sequence

The 5’-phosphorylated padlock_D796Y (1 μM) mixed with the target mutation sequences were heated to 95 °C for 5 min, followed by cooling to ambient temperature and further incubation at 25 °C for 30 min with T4 DNA ligase (1 U/μL) and 65 °C for 5 min. Then, 10 U/μL of exonuclease I (Exo I) and 1 U/μL of exonuclease III (Exo III) were used for the digestion of the linear nucleic acid by incubating at 37 °C for 40 min, followed by inactivating the enzymes by heating at 70 °C for 5 min to

expensive (about US$87 per COVID-19 genome)[7,8], and time-consuming (at least three to five days)[9]. PCR-based methods (e.g., reverse transcriptase PCR and double-mismatch allele-specific real-time reverse transcription–polymerase chain reaction (RT-PCR)) have been used to detect COVID-19 mutations[10,11]. However, PCR-based methods require expensive equipment, the use of enzymes (including reverse transcriptase and DNA polymerase), repeated heating and cooling cycles, and may be affected by interfering substances from biological samples. Moreover, whilst RT-PCR achieves its highest clinical sensitivity for COVID-19 when targeting the conserved regions of the viral genome, this technique is less able to identify variant-prone regions, such as in the S gene[11,12].

In recent decades, several isothermal amplification-based methods for the detection of base mutation have been reported, such as rolling circle amplification (RCA)[13], recombinase polymerase amplification (RPA), loop-mediated isothermal amplification (LAMP)[14], and nicking endonuclease-mediated isothermal amplification (NEMA) [15–18]. Nicking endonuclease-mediated rolling circle amplification (NEM-RCA), which combines NEMA with RCA, offers comparatively simpler experimental operation and reaction components, as well as lower amplification temperature. However, these methods are still limited by high background signal interference, and weak probe-induced emission signal [19], hampering the development of the NEM-RCA strategy.

Luminescent metal complexes, especially iridium(III) complexes, have been employed to detect a range of species due to their high photostability, large Stokes shift, selective luminescence enhancement in the presence of G-quadruplex DNA, and long-lived emission that allows discrimination from fluorescence noise in biological matrices by use of time-resolved techniques[20–24]. Our previous studies have demonstrated that iridium(III) complex-based interference reduction biosensing strategies, such as nicking endonuclease-mediated interference reduction rolling circle amplification (NEM-IR-RCA) and interference reduction nucleic acid amplification strategy (IR-NAAS), are suitable for the highly specific detection of target nucleic acid sequences, such as miRNAs and COVID-19 RNAs[18,25]. However, these methods still showed some limitations in their ability to specifically detect base mutations, especially for single-base mutation. For example, for the one- or two-base mutant miRNA-21 sequences, the luminescence intensity of the NEM-IR-RCA platform was only about threefold lower compared to the intensity induced by the wild-type sequence. In this study, a simple-to-operate platform termed loop-mediated interference reduction isothermal nucleic acid amplification (LM-IR-INA) strategy was designed for highly sensitive single-base mutation detection in viral variants. This strategy combines NEMA, luminescent iridium(III) probes, and time-resolved emission spectroscopy (TRES) to increase sensitivity. Notably, this strategy exhibits excellent specificity in discriminating between viral wild-type (WT) and variant sequences, such as D796Y, D614G, and H69/V70del, thereby providing an efficient platform to identify the COVID-19 Omicron variant with high fidelity. Importantly, the TRES-based LM-IR-INA strategy was successfully applied to detect viral variants with high specificity in complicated biological samples with extensive background fluorescence, including throat and blood samples, which is an advantage compared to previously reported biosensors for COVID-19 variant detection.
Fig. 1. Recognition of D796Y single-base substitution sequence in the LM-IR-INA strategy. (A) Illustration of D796Y substitution and D796 WT sequences. (B) Schematic illustration of the D796Y identification stage of LM-IR-INA using a D796Y single-base substitution sequence as a mimic target. Upper: Identification of highly sensitive D796Y using a D796Y single-base substitution sequence as a mimic target. Lower: Identification of a conservative nucleic acid sequence detection using a D796 sequence as mimic control. (C) Schematic illustration of the luminescent "turn-on" sensing based on the V_1 probe using the steady-state mode. (D) Steady-state response of the probe V_1 (1 µM) with or without the PS2-M-based sequences ON1, ON2, and ON3. The ratio of luminescent intensity (I/I0) between probe control group V_1 and V_1 + ON1, V_1 + ON2, V_1 + ON3 at 580 nm was quantified with the excitation at 370 nm. P values were calculated using a one-way ANOVA with Tukey’s multiple comparison test. ** P < 0.01 vs. V_1 + ON1 group. (E) Native gel electrophoresis (15%) analysis of the cyclization of Padlock_D796Y.
be detected by the G-quadruplex-selective probe $V_1$ [18,25]. As shown in Fig. 1D, probe $V_1$ in presence of ON1 showed the highest luminescent intensity and possessed a ca. 6.00-fold enhancement of the I/I₀ ratio at maximum emission wavelength in LM-IR-INA reaction buffer in steady-state mode. Thus, ON1 was selected for further padlock design of LM-IR-INA strategy.

The mechanism of this assay was verified by native gel electrophoresis (Fig. 1E). The D796 (lane 1) and D796Y (lane 2) target sequences appear as single bands with high mobility, while the longer padlock_D796Y sequence appears as a slower-moving band (band II, lane 3). When the target D796Y sequence is added to the padlock, it forms a hybridized structure with lower mobility (band I, lane 8). Subsequently, addition of ligase and Exo I/III results in the cleavage of single-stranded DNA (ssDNA) fragments, leaving behind the circular loop_D796Y with intermediate ability (band III, lane 6). Consistent with the mechanism, the addition of Exo I/III without ligase results in complete digestion of nucleic acid material as the circular loop_D796Y is not formed (lane 7). In contrast, the D796 WT sequence is unable to hybridize effectively with the padlock and hence a slower-moving band is not observed (lane 4). As a result, digestion cannot be performed and the entire genetic material is digested upon addition of Exo I/III (lane 5). The successful preparation of the circular loop_D796Y firstly validates the feasibility of the LM-IR-INA strategy towards the recognition of the D796Y single-base substitution sequence.

3.2. Amplification of D796Y single-base substitution sequence

After Loop_D796Y is liberated, the RCA reaction is initiated with the addition of phi29 DNA polymerase (Fig. 2A). In order to improve the sensitivity of the strategy, the nicking endonuclease Nb.BbvCI was also introduced to assist an exponential nucleic acid amplification [18]. In the NEM-RCA mechanism, the double-stranded DNA (dsDNA) RCA product can be cleaved by Nb.BbvCI at the two recognition sites, and the released ssDNA fragments can hybridize with unreacted circular padlock probe to trigger the start of a new circular RCA reaction. Consequently, exponential amplification is achieved. Importantly, the reaction can be performed under isothermal conditions, allowing for easy experimental operation for viral variant detection.

To confirm the successful amplification in LM-IR-INA, the NEM-RCA reactions were performed in the presence of SYBR Green II probe, a sensitive fluorescent dye that specifically recognizes ssDNA [26,27]. As expected, the reaction with Loop_D796Y (formed in the presence of the D796Y target sequence) showed a gradual fluorescence enhancement as more ssDNA product was produced over time (Fig. 2B). In contrast, since the D796 WT sequence is unable to generate the ligated circular probe, no fluorescence enhancement is observed over time as Phi29 is unable to act on primer 1 alone. These results demonstrate that LM-IR-INA exhibits high amplification efficiency and can discriminate between sequences differing by a single-base.

3.3. Interference-reduction detection of D796Y single-base substitution sequence in LM-IR-INA strategy using TRES

The ssDNA fragments released by Nb.BbvCI-mediated NEM-RCA contain PS2.M G-quadruplex sequences, which can be detected by the G-quadruplex-selective probe $V_1$. In presence of D796Y single-base substitution sequence, probe $V_1$ showed 4.5-fold higher luminescence under steady-state UV illumination after NEM-RCA amplification compared to the control group in absence of D796Y sequence (Fig. 3A). To demonstrate the advantage of interference reduction in the LM-IRINA strategy, luminescence spectra were obtained in the presence of Coumarin460 (Cou460) dye, a model fluorescent interferent. As shown in Fig. 3B, Cou460 clearly interferes with the luminescent emission signal of $V_1$ at 570 – 600 nm under steady-state emission mode.
However, the short-lived fluorescence of Cou460 could be eliminated under TRES mode through employing the time-correlated single photon counting (TCSPC) technique (Fig. 3C). As shown in Fig. 3D, increasing the delay time from 0 ns to 350 ns eliminates the signal of Cou460, allowing the emission peak of probe \( V_1 \) could be clearly observed. Additionally, the discrimination between D796Y and D796 targets generally increased with the delay time (Fig. S1A). These results indicate that the TRES technique in LM-IR-INA strategy can be used increase the robustness of viral variant detection by eliminating the effect of fluorescent interferents in the sample.

To improve the detection sensitivity of the LM-IR-INA protocol, reaction parameters, including Nb.BbvCI nicking endonuclease, Phi29 DNA polymerase, amplification temperature, and pH were optimized. The intensity of the system increased sharply with Nb.BbvCI concentration until 1 U/mL, then increased more gradually up to 100 U/mL (Fig. S1B). Thus, 1 U/mL was chosen as the optimal Nb.BbvCI concentration. Meanwhile, the emission intensity increased with Phi29 DNA polymerase concentration and reached a maximum at 2 µM, before decreasing slightly up to 100 U/mL (Fig. S1C). Thus, 10 U/mL of Phi29 DNA polymerase was selected for further analysis. Furthermore, the manufacturer’s instructions for Nb.BbvCI nicking endonuclease and Phi29 DNA polymerase stated optimal temperature conditions of 30 and 37°C, respectively. Therefore, we investigated the effect of temperature on the efficiency of NEM-RCA in a reaction temperature range of 30–37°C. As shown in Fig. S1D, a temperature of 37°C gave the highest signal intensity. Finally, a pH of 7.0 gave the highest response for LM-IR-INA out of the pH values tested (5.0, 6.0, 7.0, and 8.0) (Fig. S1E). Under these optimized conditions, the LM-IR-INA strategy in presence of D796Y generated a much stronger emission signal compared to D796 WT sequence, demonstrating the ability of the system to detect single-base substitution (Fig. 3E).
3.4. Sensitivity of LM-IR-INA strategy in complicated biological samples

We next evaluated the sensitivity of the LM-IR-INA method for the quantitative analysis of viral variant targets. After amplification, the luminescence intensity of the system increased with increasing concentrations (3.01 \times 10^4 to 3.76 \times 10^{10} copies/\mu L) of the target D796Y in Tris-HCl buffer (Fig. 4A). A linear relationship of between emission intensity at 580 nm and target D796Y concentration was observed in the concentration range of 6.01 \times 10^5 to 3.76 \times 10^8 copies/\mu L, while the limit of detection (LOD) was calculated to be 2.01 \times 10^5 copies/\mu L. (Fig. 4B) on the basis of a signal-to-noise ratio of 3. [28–30] Furthermore, considering the potential application of LM-IR-INA for viral variant detection in complicated biological samples, the assay was performed in spiked mice throat swab (Figs. 4C and 4D) and mice blood (Figs. 4E and 4F) samples. Linear relationships were observed between signal intensity and D796Y concentration from 6.01 \times 10^5 to 3.76 \times 10^8 copies/\mu L, while the LOD values were 2.38 \times 10^5 and 4.27 \times 10^5 copies/\mu L for mice throat swab and blood samples, respectively. In summary, the LM-IR-INA platform allows the quantitation of viral single-base variants in complicated biological samples, suggesting its potential for clinical use.

3.5. Specificity of LM-IR-INA strategy

We then evaluated the ability of the assay to discriminate variant sequences in the presence of the WT sequence. The TRES response of the LM-IR-INA strategy towards up to 1600-fold excess equivalents of the D796 WT sequence compared to the D796Y target sequence was examined (Fig. 5A). Encouragingly, even 1600-times higher concentrations of D796 WT vs. D796Y (i.e., $C_{D796Y}/C_{D796}$ of 0.0625%) only slightly reduced the luminescence signal, indicating that the LM-IR-INA strategy could identify single-base viral variants even in the presence of much higher concentrations of the WT sequence. Apart from the D796Y single-base substitution, a padlock probes for D614G single-base substitution (Fig. S2A) and H69/V70del six-base deletion (Fig. S2B), other two representative COVID-19 Omicron variant sites, were also designed (Table S1). As shown in Figs. S2C, S2D, and 5B, each variant (D796Y, D614G, and H69/V70del) can be selectively detected using the

![Fig. 4.](image-url)
corresponding padlock probe, allowing for multiple confirmations of COVID-19 Omicron variant infection. Moreover, the robustness of LM-IR-INA within the Tris-HCl buffer, throat swab, and blood samples was evaluated by the recovery method. The recovery values for D796Y ranged from 92.91% to 117.46% with a relative standard deviation (RSD) of under 15.16% (Table 1). Taken together, these results suggest that the LM-IR-INA approach has good accuracy and repeatability for detecting single-base variants in complicated samples.

4. Conclusion

In conclusion, we have developed a luminescence TRES-based LM-IR-INA strategy for viral variant detection, including substitution, deletion, etc. The strategy utilizes an NEM-RCA amplification strategy to generate an exponential increase in the number of G-quadruplexes in the presence of the target RNA, while the G-quadruplex-selective iridium (III) complex V_1 acts as a probe to generate a strong luminescence signal in TRES mode. The strategy allowed for the quantitative detection of the viral variant sequence at a detection limit of 2.01 \times 10^5 copies/μL, and a linear range from 6.01 \times 10^5 to 3.76 \times 10^8 copies/μL.

Table S2 presents a comparison of the LM-IR-INA strategy with representative methods reported in the literature. Notably, the variant target could be detected even in the presence of a 1600-fold excess of the WT sequence (i.e., variant ratio of 0.0625%), which is superior to the performance of other reported methods, confirming the excellent specificity of the LM-IR-INA platform.

LM-IR-INA strategy is a universal (including substitution, deletion, etc.), simple (does not require reverse transcription compared with PCR), and highly specific (sufficient specificity to discriminate single-nucleotide variation) method for viral mutation detection. Moreover, compared with steady-state techniques, the TRES-based LM-IR-INA strategy is more robust as it can eliminate the short-lived fluorescence of complicated biological samples. We demonstrated the application of the TRES-based LM-IR-INA strategy for detecting viral variants with high specificity in mouse throat and blood samples. However, it should be noted that target sequences (e.g. D796Y, D614G, and H69/V70del) used in this study are only fragments of the COVID-19 Omicron variant, and cannot fully mimic real viral genetic material. Therefore, further studies are still required in order to validate the effect of the TRES-based LM-IR-INA platform for viral variant detection in clinical practice.

CRediT authorship contribution statement

Guodong Li: Investigation, Funding acquisition, Conceptualization, Writing - original draft. Chung-Nga Ko: Investigation. Zikang Wang: Investigation, Formal analysis. Feng Chen: Investigation. Wanhe Wang: Funding acquisition, Formal analysis, Writing - review & editing. Dik-Lung Ma: Formal analysis, Supervision, Writing - review & editing. Chung-Hang Leung: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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Table 1

| Buffer | Added \(10^8\) copies/μL | Found \(10^8\) copies/μL | Recovery (%) | RSD (%) |
|--------|-----------------|-----------------|-------------|--------|
| Tris-  | 1.00            | 0.93            | 92.91       | 6.19   |
| HCl    | 40.00           | 37.60           | 94.40       | 9.29   |
| Throat | 1.00            | 1.17            | 117.46      | 15.16  |
|        | 40.00           | 27.40           | 93.50       | 5.69   |
| Blood  | 1.00            | 1.01            | 101.17      | 10.58  |
|        | 40.00           | 42.20           | 105.50      | 9.45   |
Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2022.133006.

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