Investigating the compatibility of polymerase chain reaction and oligonucleotide ligation assay for one step detection of point mutations

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ABSTRACT

We present a systematic optimization of the components of polymerase chain reaction (PCR) and oligonucleotide ligation assay (OLA) to combine them into a one-pot reaction. Such a one-pot reaction would enable the simultaneous amplification of a target nucleic acid sequence via PCR (for species identification) and the detection of a point mutation within that sequence via OLA (for the determination of antimicrobial resistance). The results can be visualized directly on a lateral flow assay without additional equipment. Specifically, here, we amplify a 163 bp region of the rpoB gene of Mycobacterium tuberculosis and detect the 531 mutation therein. The concentrations of each component of PCR and OLA buffer were optimized to design a new buffer compatible with the combined reaction. An important finding of the study is that the performance of PCR and OLA is highly sensitive to magnesium ion (Mg^{2+}) concentration, and the incompatibility in the Mg^{2+} concentration is preventing the integration of the two reactions in the present study. Using a DNA polymerase that can tolerate high Mg^{2+} concentration might lead to the successful integration of PCR and OLA. Compared to the existing methods of conducting the PCR and OLA reaction sequentially, the one-pot reaction would significantly reduce the reaction time, user intervention, and plausible risk of amplicon contamination arising from opening the PCR tubes.

INTRODUCTION

Point mutations are a common phenomenon through which infectious pathogens evade the immune system and develop antimicrobial resistance (AMR). AMR is a pandemic with an estimation to cause 10 million deaths annually by 2050 if not controlled.[1] Knowledge of AMR helps in designing better courses of treatment and reduces the spread of resistant strains. The gold standard to determine point mutations is whole genome sequencing. Although accurate, the method is infrastructure-intensive and inaccessible at remote locations.

A well-established method for point mutation detection is the oligonucleotide ligation assay (OLA). OLA is based on the hybridization of short oligonucleotides with amplified DNA followed by ligation to generate single-stranded products. The product formation is contingent upon the presence or absence of mutations.[2] A detailed understanding of OLA can be found in our previous publication.[3] OLA products can be coupled with lateral flow assays (LFAs) for faster and low-cost point mutation detection.[4–6] However, all these methods require manual transfer of the amplified product to individual ligation reaction tubes, posing the risk of amplicon contamination. Chen et al. developed a closed tube assay with two-stage thermal cycling that combines PCR and OLA to detect multiple mutations in human and salmon sperm DNA.[7] However, the assay requires real-time PCR to track the fluorescence intensities of dyes, which increases instrumentation costs. It is not currently possible to conduct single-pot PCR and OLA, followed by lateral flow detection.

To overcome this gap, we attempted to combine PCR and OLA into a single reaction that is compatible with end-point lateral flow detection. The reaction aims to simultaneously amplify the DNA (in a thermal cycler) and generate single-stranded bi-labelled OLA products, which can be visualized on lateral flow assays. The assay is demonstrated to detect the wild-type strain

of Mycobacterial tuberculosis with the nucleotide sequence TCG at 531 codon (S531) in the rpoB gene. Note that we previously demonstrated the ability to distinguish between a wild type and mutant sequence at this codon using PCR and OLA.[3] To design the one-pot assay, we studied the compatibility of i) PCR and OLA components r, ii) PCR and OLA enzymes with their respective supplied buffers, and iii) PCR and OLA thermal cycling conditions. A key finding of this study was that the performance of PCR and OLA are very sensitive to magnesium concentrations. If the Mg^{2+} concentration is varied from the precise level, the signal intensity at LFA is reduced. Perhaps using a PCR enzyme that can tolerate high Mg^{2+} concentration will lead to the successful integration of PCR and OLA. Finally, the two reactions were combined in one pot, and the effect of Mg^{2+} and OLA probe concentrations on the signal intensity at LFA was studied. The one-pot assay takes 1.5 hours and only requires a portable thermal cycler. The work provides a first insight into the incompatibility of standard PCR and OLA enzymes at their supplied buffers and a starting point for designing appropriate buffers to combine the assays into a single pot. The work provides a fundamental understanding of PCR and OLA components and explores the possibilities of designing one-pot reactions for point mutation detection.

EXPERIMENTAL SECTION

PCR, OLA, and LFA were performed as described previously.[3] Sequences of primers, probes and competing oligomers are provided in Supporting Information Table 1. LFA strips were imaged on a Canon LiDE 220 flatbed scanner, and the acquired images were analyzed using ImageJ software.

Tris-HCl (89781), potassium chloride (84984), magnesium chloride (31196), potassium acetate, magnesium acetate, β-Nicotinamide adenine dinucleotide hydrate (NAD; 87253),

Dithiothreitol (DTT; 17315), Triton-X were procured from Sisco Private Limited (SRL), India. DNA rTaq polymerase was acquired from Takara (R001A). The corresponding 10X rTaq buffer (henceforth called PCR buffer) consists of 100 mM Tris-HCl (pH 8.9 at 25ºC), 500 mM potassium chloride, and 15 mM magnesium chloride. Taq DNA ligase was acquired from New England Biolabs (NEB; M0208S). The corresponding 10X ligase buffer (henceforth called OLA buffer) consists of 200 mM Tris-HCl, 250 mM potassium acetate, 100 mM magnesium acetate, 10 mM NAD 1, 100 mM DTT, and 1% Triton® X-100 at pH 7.3 at 25ºC.

RESULTS AND DISCUSSIONS

Detecting point mutation using OLA and LFA

To identify the pathogen genotype using OLA followed by LFA, 2 µL PCR amplified product comprising the mutation of interest is mixed with an OLA reaction mixture. The mixture consists of three probes specific for each mutation of interest: wild (WT) probe tagged with FITC at 5' end, mutant (MUT) probe tagged with Digoxigenin (DIG) at 5' end, and common (COM) probe tagged with biotin at 3' end and phosphorylated at 5' end, ligation buffer, and a DNA ligase enzyme (Fig. 1A). The mixture is subjected to ligation cycle consisting of 40 cycles of 95°C for 30s and 47°C for 1min. During the reaction, all probes hybridize with the complementary DNA strain. However, the ligase enzyme ligates (forms a covalent bond) only between the probes that are entirely complementary to the target DNA. That is, for the WT target, the ligase enzyme ligates only the WT and COM probe, forming the FITC-biotin bi-labelled product and for the MUT target the ligase enzyme ligate only the MUT and COM probe forming the DIG-biotin bi-labelled product (Fig. 1A). At the end of ligation reaction, the mixture consists of ligated bi-labelled probes and un-ligated probes, both hybridized to the target DNA. The ligation product is detected on LFA. LFA consists of WT and MUT test lines and a control (CTRL) line. The WT test line consists of an anti-FITC antibody to detect the

FITC-biotin bi-labelled complex, and the MUT test line consists of an anti-DIG antibody to detect the DIG-biotin bi-labelled complex. The CTRL line consists of bovine serum albumin to capture streptavidin-coated gold nanoparticles. When the ligation products with WT target were directly added for LFA, both WT and MUT test line signals were observed (Fig. 1B(i)). Thus, unligated probes must be separated prior to LFA to generate the specific signal. This was achieved by using a high concentration of an un-labelled oligomer (known as competing oligomer, CO), which has the same sequence as the ligated probe.[3,6] CO replaces the ligated and un-ligated probes from the complementary DNA strand separating the un-ligated probes. Thus, a WT target produces only a WT signal (Fig. 1B(ii)), and a MUT target produces only a MUT signal (Fig. 1B(iii)).

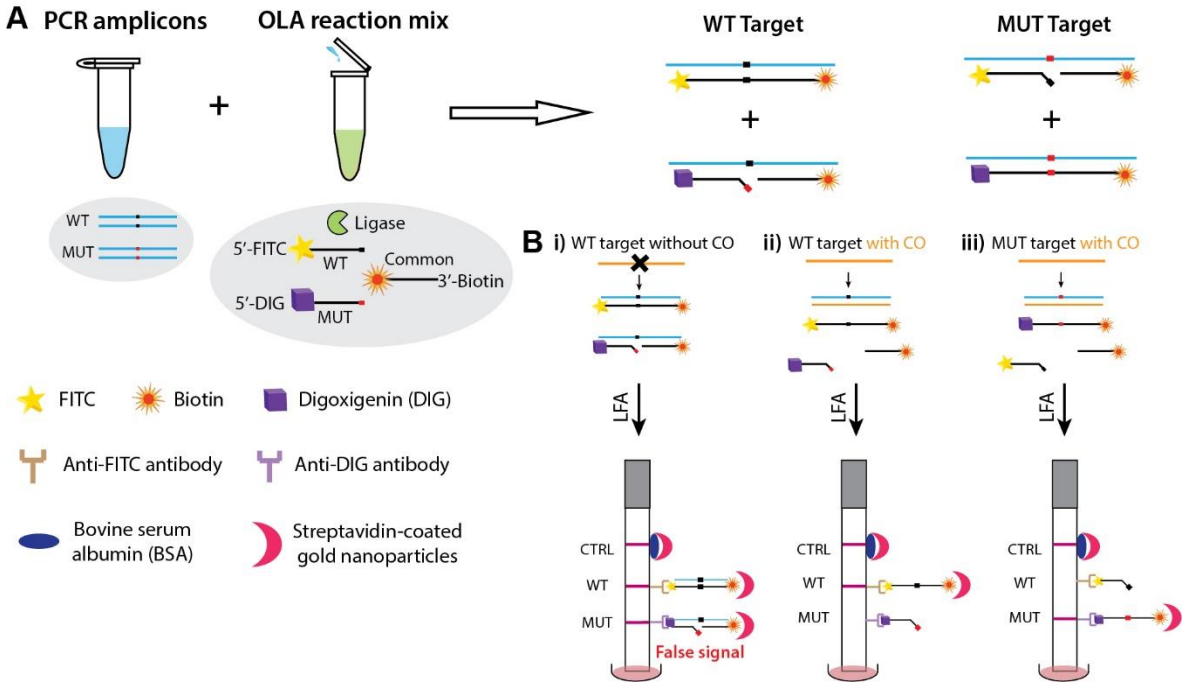

Figure 1. Point mutation detection using OLA followed by LFA. (A) Molecular mechanism of OLA. Adding PCR amplicons to OLA reaction mix and subjecting to ligation cycle forms bi-labelled product depending on the genotype. For WT target, FITC-biotin bi-labelled product is formed and for MUT target, DIG-biotin bi-labelled product is formed. **(B)** Role of competing oligomer (CO) in eliminating false signal on LFA. (i) Running the ligation product directly through LFA produces false MUT signal for a WT target. (ii, iii) CO replaces ligated and un-ligated probes from the target DNA, separating the un- ligated probes and ensuring only the ligated probes are detected on LFA.

Investigating compatibility of PCR and OLA components

The first step in combining PCR and OLA was to investigate the compatibility of individual reaction components. First, we studied the effect of PCR components on OLA. OLA reactions were spiked with different PCR components, resulting in four different reaction mixtures consisting of either - i) 1X PCR buffer, ii) 0.4 µM of IF and IB primers, iii) 2 units of rTaq enzyme, or iv) an additional 8 µL of WT PCR amplified product. dNTPs were excluded as they are unlikely to affect the ligation reaction. As rTaq has the maximum efficiency at 72ºC, OLA spiked with rTaq was incubated at 72ºC for 7 mins prior to ligation. It was observed that the signal intensities for all PCR component-spiked OLA reactions were similar to the OLA reaction with no PCR components (Fig. 2A; None), indicating that none of the PCR components inhibits the ligation reaction. Adding the PCR buffer in OLA results in the following buffer composition in the mixture- 30 mM Tris-HCl, 75 mM K^+, and 11.5 mM Mg^{2+}. The signal produced by this mixture (Fig. 2A; PCR buffer) has a similar intensity as that of OLA with no PCR components (Fig. 2A; None), indicating that OLA can operate at high salt concentrations.

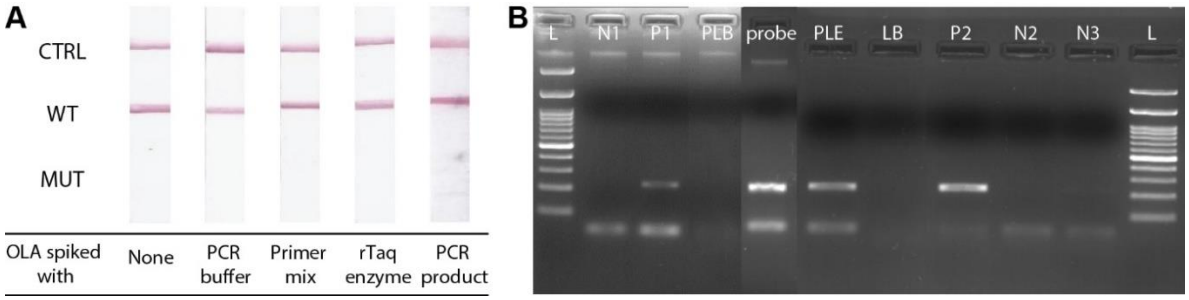

Figure 2. Investigating the compatibility of PCR and OLA reaction components. (A) Effect of PCR components on OLA. None of the PCR components was affecting the ligation efficiency of OLA. **(B)** Effect of OLA components on PCR. OLA probes (probe) and ligase enzyme (PLE) do not affect PCR amplification. But reactions with PCR and ligation buffer (PLB) and only ligation buffer (LB) do not show amplification, indicating incompatibility of PCR with OLA buffer. N1, N2, N3: no template control, P1, P2: PCR without OLA components, L: 100 bp DNA ladder

Next, we studied the effect of OLA components on PCR. Three 20 µL PCR mix tubes were spiked with different OLA components such that the final mixture in each tube consisted of either i) 1X Taq ligase buffer, ii) 50 nM each of WT, MUT and COM probes, or iii) 32 units of Taq ligase enzyme. A PCR assay without PCR buffer and with 2 µL of 10X ligation buffer was also prepared. The reactions were subjected to a PCR cycle consisting of initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 20 s, and a final extension at 72 °C for 7 min. The amplified products were visualized in a UV cabinet after gel electrophoresis (Fig. 2B). No amplification was observed for the PCR assay spiked with the ligation buffer (Fig. 2B; PLB), indicating that PCR is incompatible with the OLA buffer. PCR performed with OLA probes (Fig. 2B; probe) and ligase enzyme (Fig. 2B; PLE) did not affect the signal intensity. PCR performed with ligation buffer instead of PCR buffer (Fig. 2B; LB) did not show amplification. This is because compared to the PCR buffer, the ligation buffer has i) higher Tris-HCl, ii) lower K^+, iii) higher Mg^{2+}, iv) NAD/DTT/Triton-X, v) lower pH, and vi) acetate as the anion. Any combination of the above factors could be responsible for no amplification. Multiple buffer compositions containing different concentrations of the above components were prepared to find the reason for the loss of signal, and the buffer compatibility was tested with PCR and OLA.

PCR with varying buffer compositions

PCR was performed with various in-house prepared buffers. The 10X buffer compositions are mentioned in Supporting Information Table 2. P1 buffer has the same composition as commercial rTaq buffer and was prepared at two different pH: 7 and 8.9. PCR with P1 buffer at pH 7 did not show a visible band (Fig. 3A; $P1_7$), while PCR with P1 buffer at pH 8.9 buffer

produced a visible band (Fig. 3A; $P1_{8.9}$). Note that for PCR with P1 buffer, the unreacted primer band was also not visible, suggesting the degradation of primers at neutral pH. Henceforth, the pH of all subsequent buffers was maintained at 8.9. P2 buffer has half the concentration of KCl (25 mM) than P1 buffer to match the K+ concentration in the ligase buffer. P3 buffer has 10 times higher MgCl2 (15 mM) than P1 buffer to check if PCR can work at high Mg2+ concentration. No band was observed for reaction with P3 (Fig. 3A; P3) and P4 (Fig. 3A; P4) buffers, indicating that PCR is inhibited when i) K^+ concentration was reduced from 50 mM to 25 mM and ii) Mg^{2+} concentration was increased from 1.5 to 15 mM.

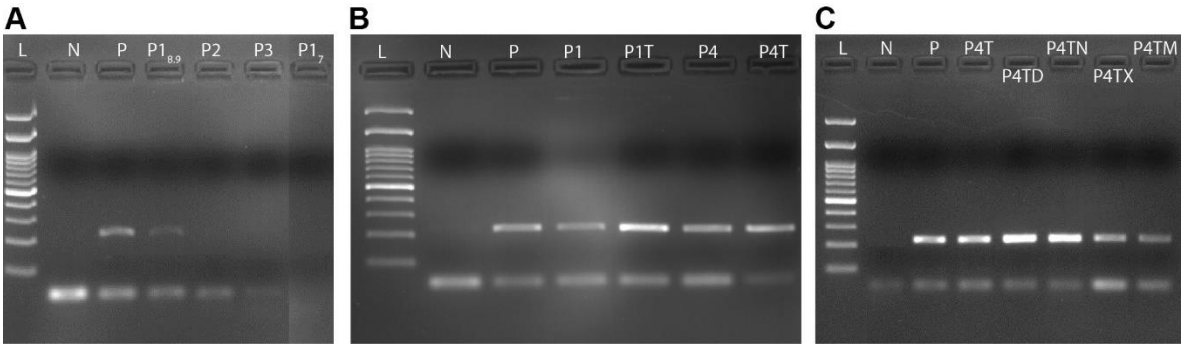

Figure 3. PCR with varying buffer compositions. (A) PCR works with in-house prepared rTaq buffer, P1 at pH 8.9. Decreasing KCl concentration to 25 mM (P2) or increasing $MgCl_2$ to 15 mM (P3) inhibits PCR. **(B)** Increasing Tris-HCl concentration to 20 mM (P1T, P4T) increases the signal intensity. Replacing chloride salts with acetate salts does not affect the signal intensity (P4). **(C)** Presence of DTT (P4TD), NAD (P4TN), Triton-X (P4TX) and their combination (P4TM) in P4T buffer do not affect the signal intensity. L: 100bp ladder, N: no template control, P: PCR with commercial rTaq buffer.

1X OLA buffer consists of 25 mM potassium acetate. Fig. 2A shows that OLA can operate at K+ concentrations as high as 75 mM (Fig. 2A, PCR buffer). This means that OLA can work with a 50 mM K^+ concentration, which is the K^+ concentration in 1X PCR buffer. Therefore, the K^+ concentration in the subsequent buffers was maintained at 50 mM. Tris-HCl concentration in 1X PCR buffer is 10 mM, while the Tris-HCl concentration in 1X OLA buffer is 20 mM. To investigate the effect of higher Tris-HCl on amplification, PCR was performed

with an additional 10 mM in P1 buffer. It was observed that increasing the Tris-HCl concentration increased the intensity of the amplified band (Fig. 3B; P1T). PCR buffer has chloride salts of potassium and magnesium, while OLA buffer has the same acetate. To investigate the effect of acetate salts, PCR with a new buffer P4, having acetate salts of potassium and magnesium was performed. Replacing the chloride salts with acetate salts did not affect the amplification (Fig. 3B; P4), and increasing Tris-HCl concentration in the P4 buffer increased the intensity of the amplified band (Fig. 3B; P4T). Next, we investigated the effect of DTT, NAD, and Triton-X. PCR tubes with P4T (P4 plus 10 mM Tris-HCl) buffer were spiked with 2 µL each of 100 mM DTT (Fig. 3C; P4TD), 10 mM NAD (Fig. 3C; P4TN), and 1% Triton-X (Fig. 3C; P4TX) individually and all three components combined (Fig. 3C; P4TM). PCR with individually spiked components and their combinations produced a visible signal, indicating that none of the three components inhibit PCR. Combining the results from varying PCR buffer compositions, we designed a new buffer called P5 that has all the components of OLA and is compatible with PCR. The 10X P5 buffer (pH 8.9) consists of 200 mM Tris-HCl, 500 mM K(CH_3COO), 15 mM Mg(CH_3COO)$_2$, 100 mM DTT, 10 mM NAD, and 1% Triton-X.

Effect of Mg^{2+} concentration on OLA and PCR

OLA with 531 probes and WT amplified target was performed with P5 buffer and the ligated products were detected on LFA after denaturation with competing oligomers (Fig. 4A). A faint WT test signal was observed (Fig. 4A; P5). The signal intensity is reduced because P5 buffer has less Mg^{2+} concentration (1.5 mM) compared to commercial Taq ligase buffer (10 mM, Fig. 4A; P). To study the effect of Mg^{2+} concentration on ligation, OLA reactions were spiked with additional Mg(CH_3COO)$_2$. Increasing the Mg^{2+} concentration in OLA increased the WT signal (Fig. 4B). Maximum signal intensity was observed on addition of 4.5 mM and 8.5 mM

$Mg(CH_3COO)_2$, which made the total Mg^{2+} concentration in the reaction of 6 mM and 10 mM respectively. We also increased the volume of ligated products added for LFA to 6 µL (Fig. 4B), but no increase in the signal intensity was observed.

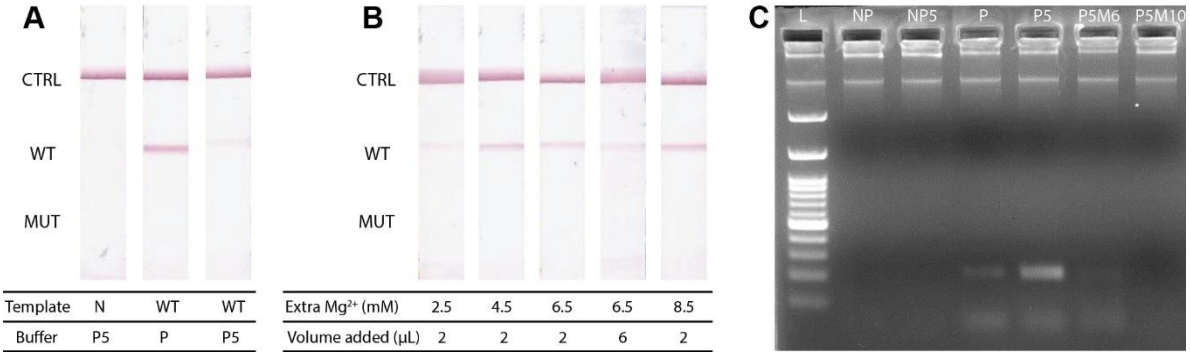

Figure 4. Effect of Mg^{2+} concentration on OLA and PCR. (A) OLA with P5 buffer produced a faint WT signal on LFA. **(B)** OLA with increasing Mg^{2+} concentration. WT signal increases at high Mg^{2+} concentration, indicating that OLA require high Mg^{2+} concentration. **(C)** PCR with increasing Mg^{2+} concentration. PCR is inhibited at higher Mg^{2+} concentration of 6mM (P5M6) and 10 mM (P5M10) indicating that PCR require low Mg^{2+} concentration. N: no template control for OLA, NP: no template control with commercial rTaq PCR buffer, NP5: no template control with P5 buffer; P: positive control with commercial rTaq PCR buffer, P5: positive control with P5 buffer.

We next investigated the effect of Mg^{2+} concentration on PCR. PCR with 6 mM Mg^{2+} produced a very faint band (Fig. 4B; P5M6), while PCR with 10 mM Mg^{2+} produced no band (Fig. 4B; P5M10). Note that at 10 mM Mg^{2+}, the unreacted primers were also not visible. This study concludes that OLA requires a high Mg^{2+} concentration for ligation, while PCR is inhibited at a high Mg^{2+} concentration.

Cycling conditions for one-pot reaction

PCR and OLA have different thermal cycling conditions.[3] To conduct PCR and OLA in one pot, two new cycle conditions were proposed to simultaneously amplify the DNA and generate allele-specific ligated products. The cycles consist of initial denaturation at 95ºC for 5 mins,

40 cycles of 95ºC for 30s and ΔºC for 1 min, and a final extension at 65ºC for 7 mins. For cycle 1, Δ = 60ºC and for cycle 2, Δ = 65ºC.

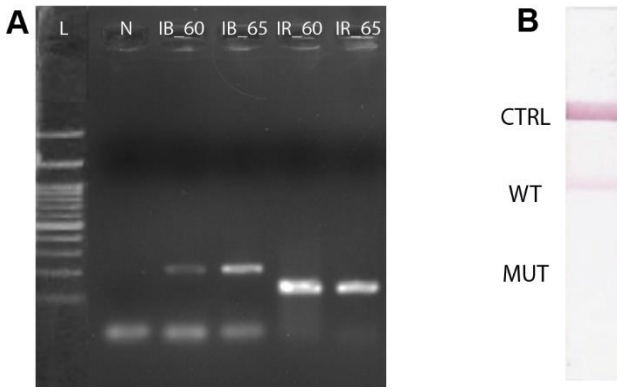

Figure 5. Cycle condition for one pot reaction. (A) PCR with IF-IR primers and 60ºC annealing and extension temperature produced brightest signal (IR_60). **(B)** OLA with IR_60 amplified product produced a visible signal on LFA. N: no template control

New cycle conditions were tested for PCR with P5 buffer and two primer sets: IF-IB and IF-IR. The most intense band was observed for PCR with IF-IR primers and Δ = 60ºC (Fig. 5A). This could be attributed to the higher melting temperature of IR primer than IB primer, which enabled combining the annealing and extension step. 1 µL of IR_60 amplified product was added to the OLA mixture having P5 buffer, and the ligation was performed with new thermal cyclic condition. A WT signal was observed on LFA (Fig. 5B), confirming successful ligation. The low signal intensity is due to the low Mg^{2+} concentration in the P5 buffer.

Combining PCR and OLA in one pot

A 20 µL one-pot reaction with all components of PCR and OLA: P5 buffer, dNTP mix, IF-IR primers, rTaq enzyme, WT, MUT and COM probes, Taq ligase enzyme, and 10^3 copies of WT template was prepared. The reaction mixtures were spiked with different $Mg(CH3COO)_2$ concentrations such that the final Mg^{2+} concentration in the mixtures became 1.5 mM, 4 mM, 6 mM, 8 mM, and 10 mM. rTaq has 5'-3' exonuclease activity and 3'-5' proofreading activity;

it is plausible that when dNTP incorporation and probe hybridization co-occur, rTaq hydrolyses and cuts OLA probes, inhibiting ligation. To test the hypothesis, the one-pot reaction was performed with three different probe concentrations: 50 nM, 100 nM and 200 nM. Further high probe concentration was not considered as it produces non-specific signals on LFA.[3]

A total of 15 reactions with five different magnesium concentrations and three different probe concentrations were prepared and subjected to the new thermal cyclic condition. On increasing the Mg^{2+} concentration from 1.5 mM to 4 mM, the band intensity of the amplified product decreased (Fig. 6A(i)). A faint band was observed at 6mM Mg^{2+}, and no amplification was observed at further high Mg^{2+} concentrations (Fig. 6A(ii)). Increasing the probe concentrations did not affect the band intensity of the amplified product. To test the ligation efficiency, the reaction products were visualized on LFA. Reaction with 50 nM probe concentration did not produce a signal with any Mg^{2+} concentration (Fig. 6B(i)). Reactions consisting of 8 mM and 10 mM Mg^{2+} and 100 nM probe did not show a signal (Fig. 6B(ii)), but reactions at the same Mg^{2+} concentrations and 200 nM probe produced a faint WT signal (Fig. 6B(iii)). To confirm that the signal is specific to ligation, 2 μL of the reaction product having 200 nM probe and 10mM $Mg(CH_3COO)_2$ was denatured using 8 M urea, which also produced a faint WT signal on LFA (Fig. 6B(iii); 10U). Two additional control studies were performed to further confirm the signal specificity on LFA. 2 μL of the reaction product having 10 mM Mg^{2+} and 200 nM probe was directly run through LFA without denaturation. The signal was observed on both test lines (Fig. 6B(iv); WD), confirming that the signal after denaturation was due to ligation. Another reaction with the same composition but no ligase enzyme was performed and subjected

to LFA after separating the un-ligated probes with CO. No signal was observed at either test line (Fig. 6B(iv); NL), further confirming that the WT signal is due to ligation.

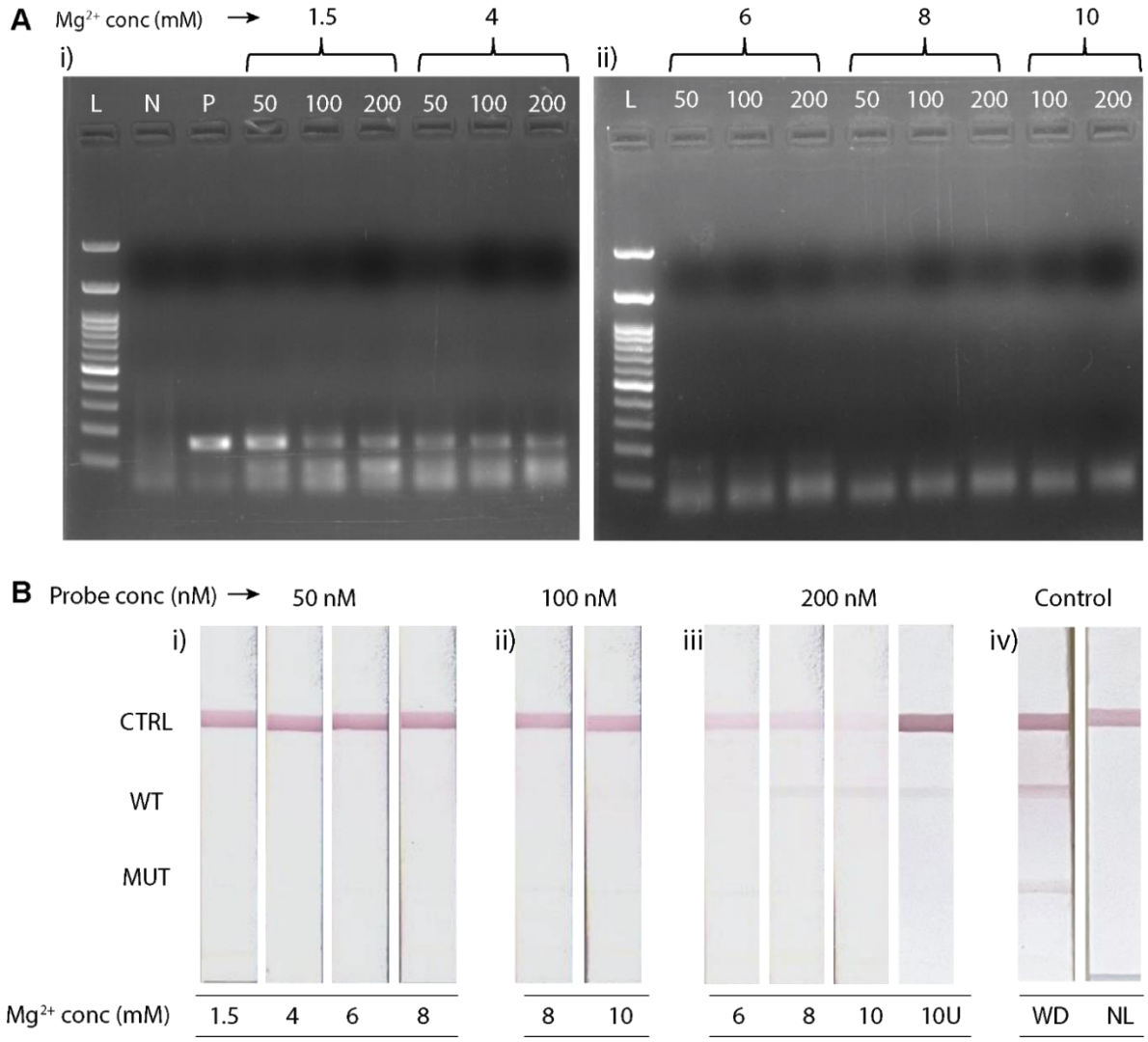

Figure 6. Combining PCR and OLA in one pot. (A) Agarose gel image to visualize the amplified product at (i) 1.5 mM and 4 mM Mg^{2+}concentrations, (ii) 6 mM, 8mM, and 10 mM Mg^{2+} concentrations for 50 nM, 100 nM and 200 nM probe concentrations. The intensity of the amplified product decreases as the Mg^{2+} concentration increases. **(B)** LFA strips to visualize the ligated band. Faint WT signal was observed for reactions with 8mM and 10mM Mg^{2+} and 200 mM probe. WD: without denaturation, NL: no ligase control.

We hypothesize that reactions with 50 nM and 100 nM probes did not generate a signal even at higher Mg^{2+} concentrations because the rTaq enzyme hydrolyzed the probes. At 200 nM probe concentration, some probes remained unhydrolyzed, hybridizing with the

complementary target and producing a faint signal upon ligation. Note that at 8 mM and 10 mM Mg^{2+}, no amplified product was observed on the gel for any probe concentration, while LFA produced a WT signal with 200 nM probe concentration. This is because the sensitivity of agarose gel is less than that of LFA.

We have presented a comprehensive understanding of PCR and OLA to develop a one-pot reaction for point mutation detection. The combined reaction aimed to reduce the detection time, instrument cost, user intervention, and risk of amplicon contamination in the environment. This is the first report that combines PCR and OLA in one pot for point mutation detection and enables direct result visualization using lateral flow assay. The workflow required investigating the compatibility of PCR and OLA components, leading to a new buffer development compatible with both PCR and OLA. The key challenge was to optimize Mg^{2+} concentration as PCR and OLA operate at stringently specific and different Mg^{2+} concentrations.

While we have shown successful integration of PCR and OLA in a single pot, the signal intensity on LFA needs to be improved. The low signal intensity can be due to i) low template amplification due to the reduced activity of rTaq at higher Mg^{2+} concentration and/or ii) probe hydrolysis due to the 5'-3' exonuclease activity of rTaq. Using a DNA polymerase that can tolerate high Mg^{2+} concentration and lacks the 5'-3' exonuclease activity may improve the signal intensity on LFA. Since the current work focused on optimizing the concentrations of components for simultaneous amplification and ligation, the one-pot reaction was demonstrated for detecting only the WT template. However, the assay should also be tested with the MUT template to demonstrate the development of the MUT signal on LFA.

CONCLUSION

The work demonstrates proof-of-principle of combining DNA amplification using PCR and point mutation detection using OLA in a single reaction. The immediate future work in this area is to test the assay with a DNA polymerase which can tolerate high Mg^{2+} concentration. The follow-up work is to test MUT strains and lower copies of the template.

ASSOCIATED CONTENT

Supporting Information. The following Supporting Information is available free of charge: Sequence of oligomers, Buffer composition

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Author Contributions

A.C: Conceptualisation, Methodology, Investigation, Formal analysis, Writing – original draft; B.J.T: Conceptualization, Methodology, Writing – review and editing, Supervision, Funding acquisition.

Notes

The authors declare no conflicts of interest.

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