Supporting Information

Polymerase Chain Reaction With “V” Shape Thermal Cycling Program

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1.Material and Apparatus

Primers used in this study were all PAGE-purified by Sangon Biotech Co., Ltd. (Shanghai, China). *EasyTaq* DNA polymerase, *Taq* buffer, and dNTPs were purchased from TransGen Biotech (Beijing, China). KAPA2G Robust DNA Polymerase was purchased from Kapa Biosystems. Ex *Taq* DNA Polymerase was purchased from Takara Biomedical Technology (Beijing) Co., Ltd.Q5 High-Fidelity DNA Polymerase was purchased from New England Biolabs, *Taq* DNA polymerase was purchased from TIANGEN Biotech(BEIJING) Co., Ltd. The template λ-DNA was purchased from Takara Biomedical Technology (Beijing) Co., Ltd. The DNA isolation kit was purchased from Foregene Co., Ltd. (Chengdu, China). SYBR Green I was purchased from probes.invitrogen.com. All the PCR amplifications (including ordinary PCR and Real-time PCR) and VPCR reactions were performed in a Thermo Scientific PikoReal Real-Time PCR System unless otherwise noted.

2.Methods and Results

2.1 Hypothesis validation

In the hypothesis validation part, both the KAPA2G Robust DNA Polymerase and the *Taq* DNA polymerase were tried to finish the amplification. Figure S1 shows that VPCR can work in the two systems. It takes 16min 51s for the KAPA2G Robust DNA polymerase to achieve the amplification while it takes about 32min for *Taq* DNA polymerase to generate a similar amount of PCR products.

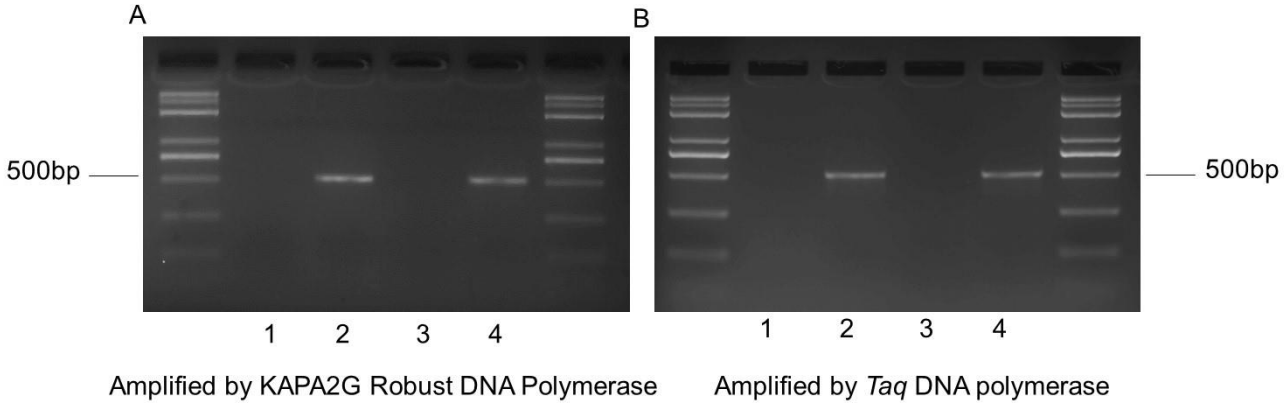

Figure.S1A. Amplification results obtained by KAPA2G Robust DNA Polymerase. Lane 1 and lane 2 were the amplification results of conventional PCR; Lane 3 and lane 4 were the amplification results of VPCR. Lane 1-4: No template control, λ DNA (final concentration:10pg/μl), No template control, λ DNA (final concentration:10pg/μl). Reactions were performed in a final volume of 10 μl mixture containing 1x KAPA2G Buffer A, additional 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of KAPA2G Robust DNA Polymerase, 0.4 μmol/L of LD/LR500, 10pg/μl of λ-DNA. Conventional PCR amplifications were conducted under the following cycling conditions: 94 °C for 30 s, 60°C for 30 s, 72°C for 30 s, 30 cycles. VPCR amplifications were conducted as follows: 94 °C for 0 s, 60°C for 0 s,30 cycles, thermal ramp rate 1.7 °C/s. B. Amplification results obtained by *Taq* DNA Polymerase. Lane 1 and lane 2 were the amplification results of conventional PCR; Lane 3 and lane 4 were the amplification results of VPCR. Lane 1-4: No template control, λ DNA (final concentration:10pg/μl), No template control, λ DNA (final concentration:10pg/μl).. Reactions were carried out in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of LD/LR500, 10pg/μl of λ-DNA. Conventional PCR amplifications were conducted under the following cycling conditions: 94 °C for 30 s, 60°C for 30 s, 72°C for 30 s, 30 cycles. VPCR amplifications were conducted as follows: 94 °C for 0 s, 60°C for 0 s,30 cycles, thermal ramp rate 0.8°C/s.

2.2 Comparison between the threshold cycle (Ct) value of VPCR and conventional PCR

40 cycles of Real-time VPCR using the VPCR cycling protocol (94°C for 0 s, 60°C for 0 s) was finished within 24min 10s, while 40 cycles of conventional Real-time PCR (94°C for 30 s, 60°C for 30 s and 72°C for 30 s) took more than 86min. It is found that the threshold cycle (Ct) value of VPCR is similar to that of conventional PCR (Figure S2: △Ct=1), implying little decrease in amplification efficiency of each VPCR cycle compared to ordinary PCR.

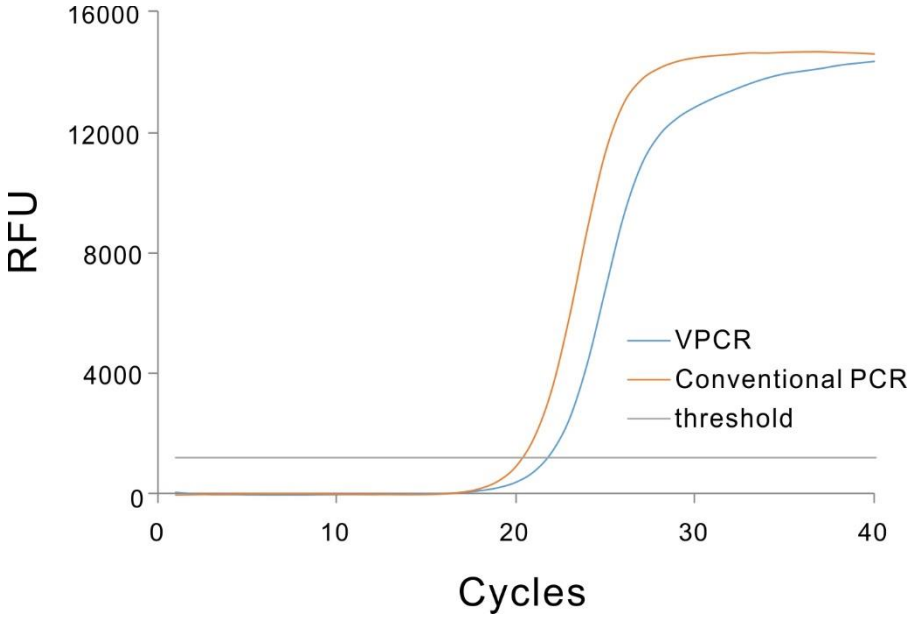

Figure.S2. Fluorescence vs Ct values for 40 cycles of VPCR (blue) and conventional PCR (orange). Reactions were performed in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer LA/LRA, 0.4 x SYBR green dyes,0.5pg/μl of λ-DNA.

2.3 Optimal low temperature (T_L) of different primers.

Different primers with the length from17 to 46 and melting temperature ranged from 61°C to 80°C were designed to study the optimal T_L of each primer. The T_L for each primer was optimized by Real-time monitoring.

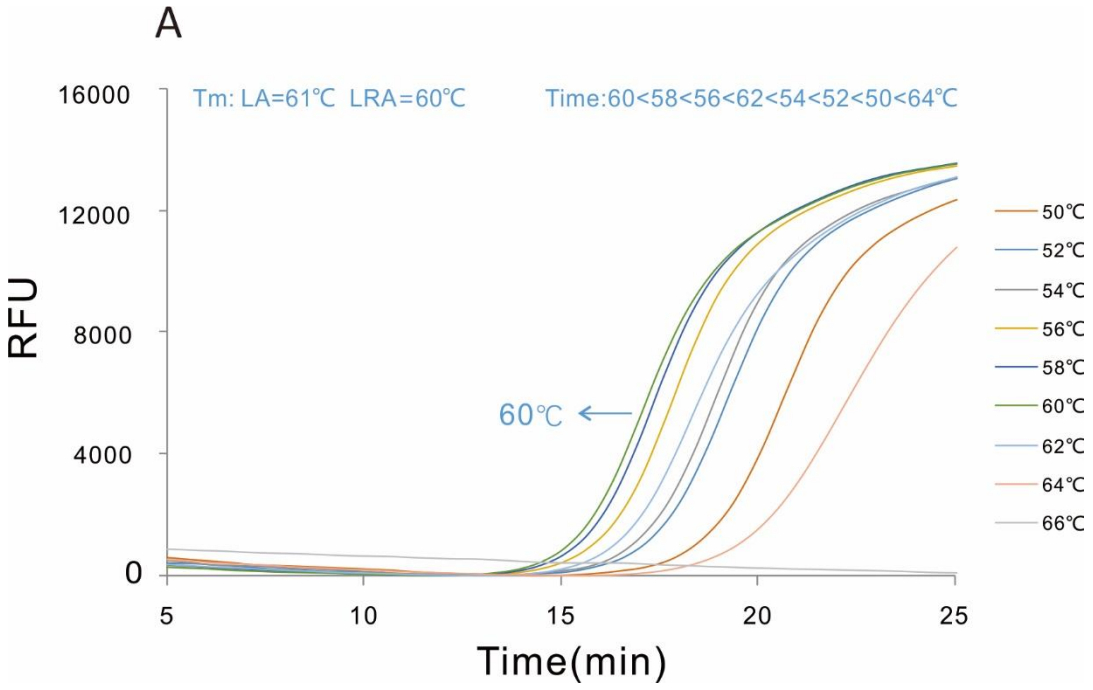

Figure S3. A Optimal T_L of LA/LRA for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 50°C to 66°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 60°C, which is equal to the calculated Tm value of the primers. Reactions were performed in triplicate in a final volume of 10 µl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/µL of *EasyTaq* DNA polymerase, 0.4 µmol/L of each primer, 0.4 x SYBR green dyes, 0.5pg/µl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 50-66°C (set as shown in the Figure S3. A) for 0 s,50 cycles.

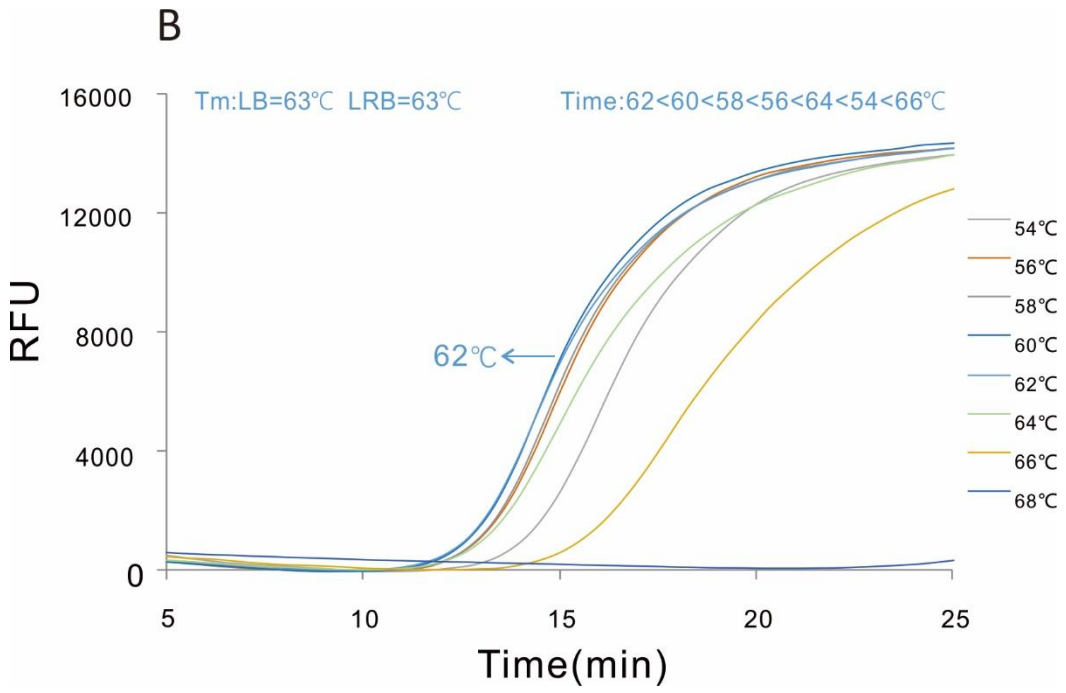

Figure S3. B Optimal T_L of LB/LRB for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 54°C to 68°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 62°C, which is very close to the calculated Tm value of the primers (Tm: LB=63°C LRB=63°C). Reactions were performed in triplicate in a final volume of 10 µl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/µL of *EasyTaq* DNA polymerase, 0.4 µmol/L of each primer, 0.4 x SYBR green dyes, 0.5pg/µl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 54-68°C (set as shown in the Figure S3. B) for 0 s,50 cycles.

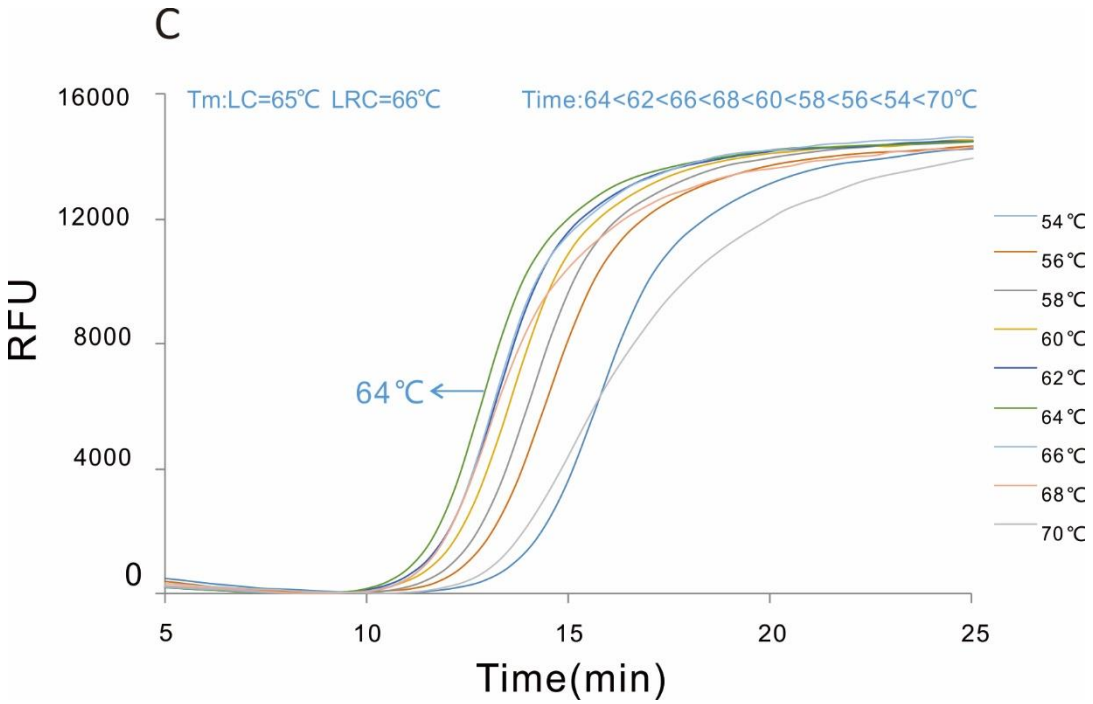

Figure S3. C Optimal T_L of LC/LRC for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 54°C to 70°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 64°C, which is very close to the calculated Tm value of the primers (Tm: LC=65°C LRC=66°C). Reactions were performed in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer, 0.4 x SYBR green dyes, 0.5pg/μl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 54-70°C (set as shown in the Figure S3.C) for 0 s,50 cycles.

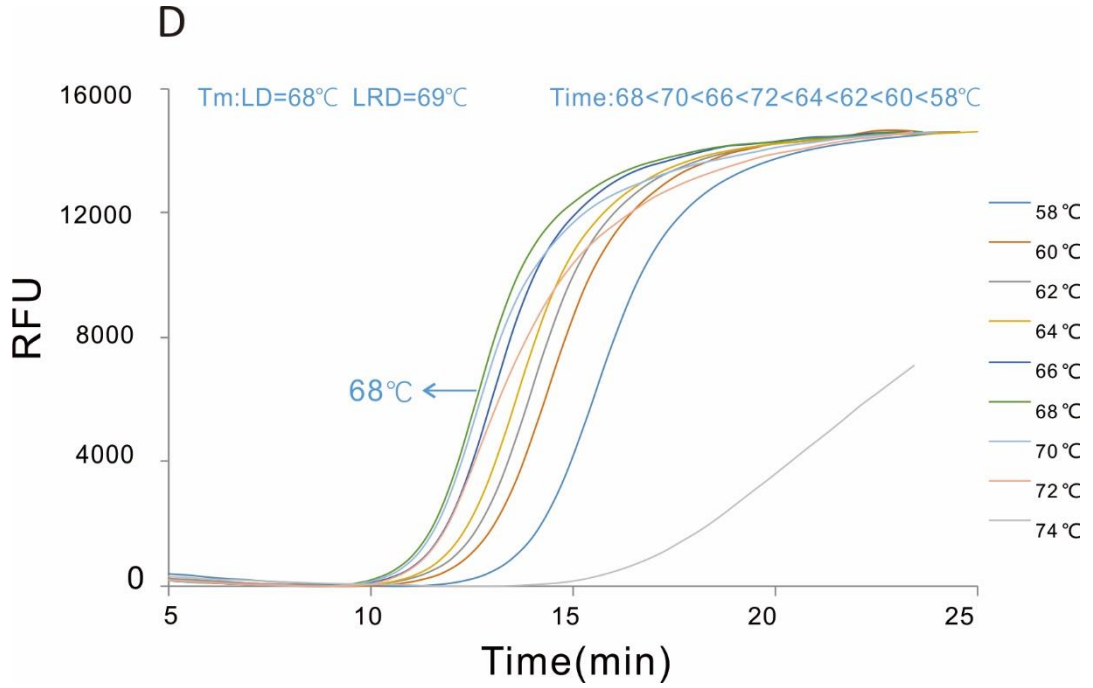

Figure S3. D Optimal T_L of LD/LRD for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 58°C to 74°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 68°C, which is equal to the calculated Tm value of the primers (Tm: LD=68°C LRD=69°C). Reactions were performed in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer,0.4 x SYBR green dyes,0.5pg/μl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 58-74°C (set as shown in the Figure S3. D) for 0 s,50 cycles.

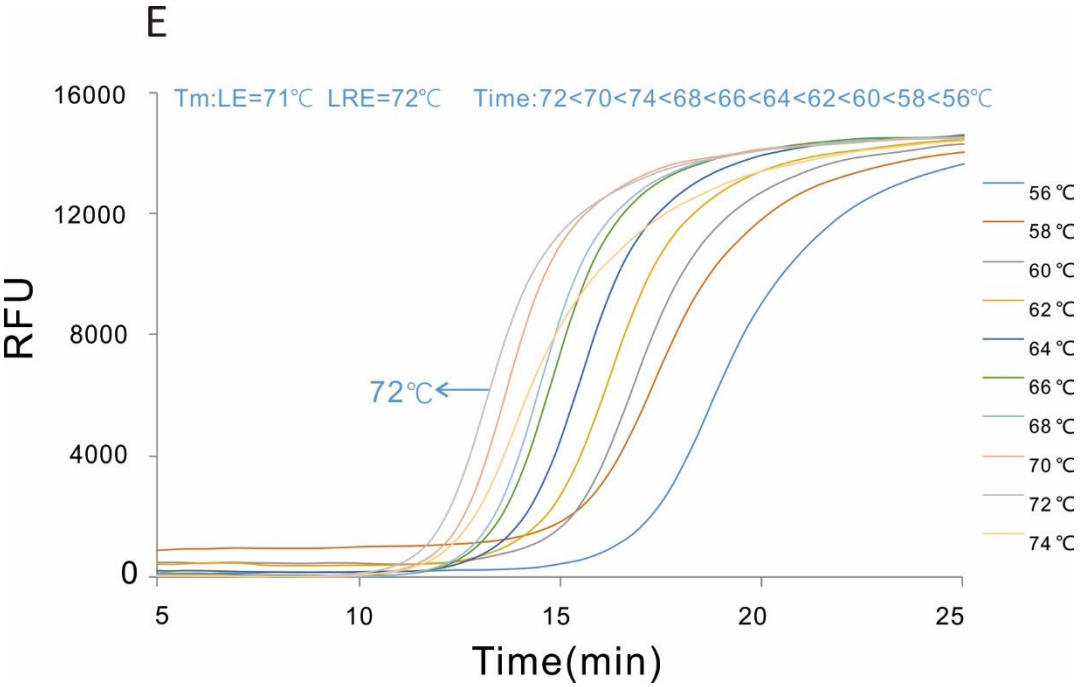

Figure S3. E Optimal T_L of LE/LRE for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 56°C to 74°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 72°C, which is very equal to the calculated Tm value of the primers (Tm: LE=71°C LRE=72°C). Reactions were performed in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer, 0.4 x SYBR green dyes,0.5pg/μl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 56-74°C (set as shown in the Figure S3. E) for 0 s,55 cycles.

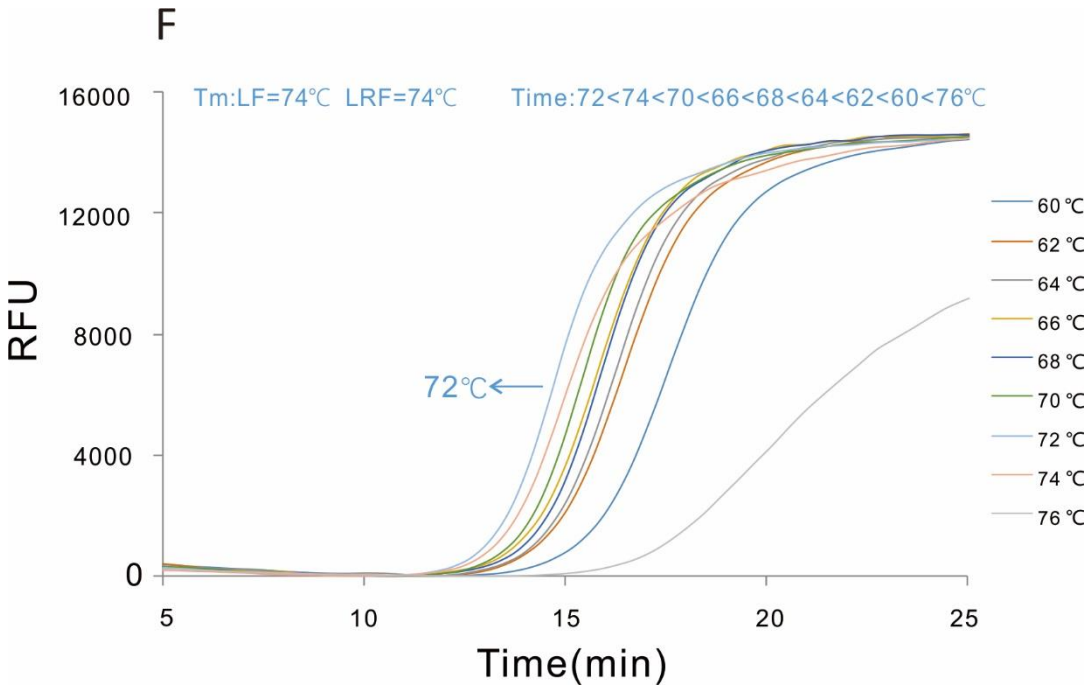

Figure S3. F Optimal T_L of LF/LRF for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 60°C to 76°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 72°C, which is very close to the calculated Tm value of the primers (Tm: LF=74°C LRF=74°C). Reactions were performed in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer, 0.4 x SYBR green dyes,0.5pg/μl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 60-76°C (set as shown in the Figure S3. F) for 0 s,55 cycles.

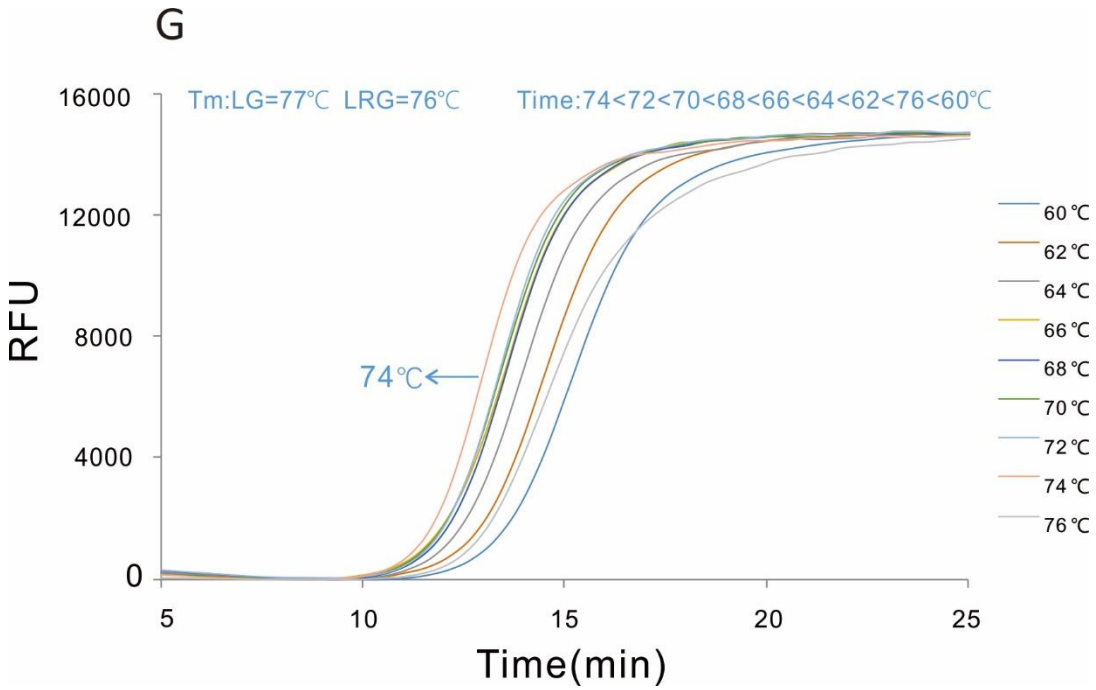

Figure S3. G Optimal T_L of LG/LRG for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 60°C to 76°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 74°C, which is very close to the calculated Tm value of the primers (Tm: LG=77°C LRG=76°C). Reactions were performed in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer, 0.4 x SYBR green dyes,0.5pg/μl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 60-76°C (set as shown in the Figure S3. G) for 0 s,55 cycles.

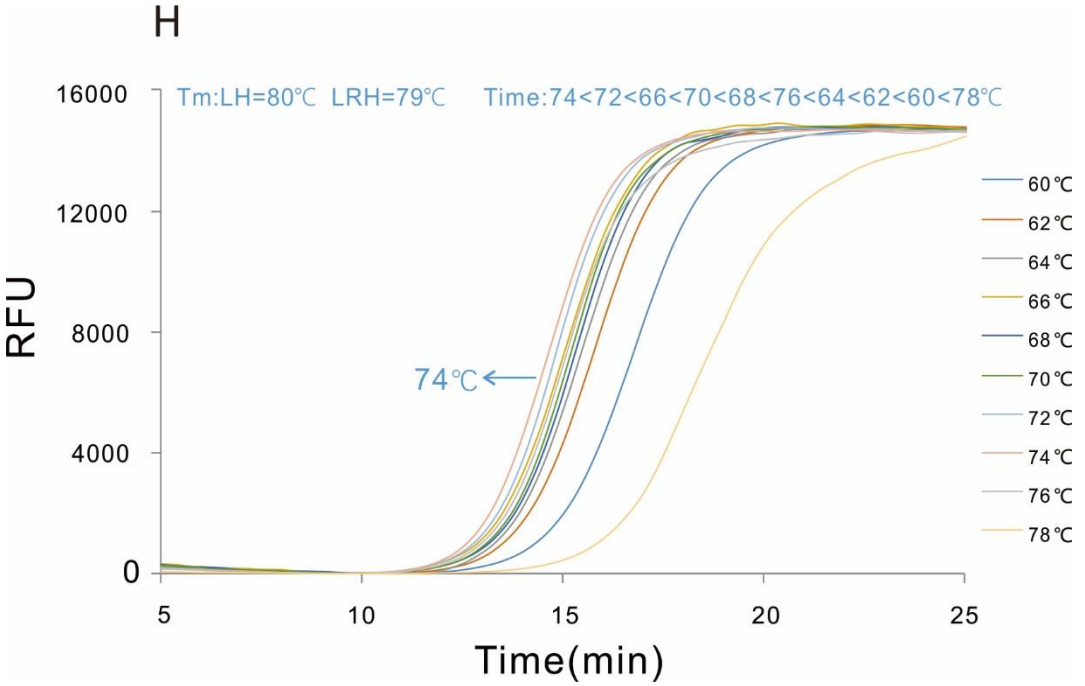

Figure S3. H Optimal T_L of LH/LRH for amplifying the 98bp fragment from λ-DNA is investigated by changing the temperature from 60°C to 78°C. The best result of Real-time VPCR is obtained in terms of time when the T_L is set at 74°C, which is 5°C lower than the Tm of the corresponding primers. That is mainly because the activity of DNA polymerase will decrease when the temperature is higher than 72°C. Reactions were performed in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer, 0.4 x SYBR green dyes,0.5pg/μl of λ-DNA. VPCR were conducted under the following cycling conditions: 94 °C for 0 s, 54-68°C (set as shown in the Figure S3.H) for 0 s,55 cycles.

2.4 The effect of primer length on the amplification time.

As the amplification time can be typically reduced when the low temperature (T_L) is set around the Tm of the primers, it is reasonable to expect that the longer primer with higher melting temperature could further shorten the amplification time. Therefore, series of primers with length from17bp to 46bp and melting temperature ranged from 61°C to 80°C were designed. The T_L for each primer was optimized by Real-time monitoring. Figure S3A-H proves that the optimal T_L is near or equal to the Tm of the primers. Then the amplification time of each primer pair is compared under its own optimal T_L (Figure S4). As a result, it shows that the biggest time saver is primer LD at the T_L of 68°C, indicating that the primer should has the appropriate length, rather than the longer, the better.

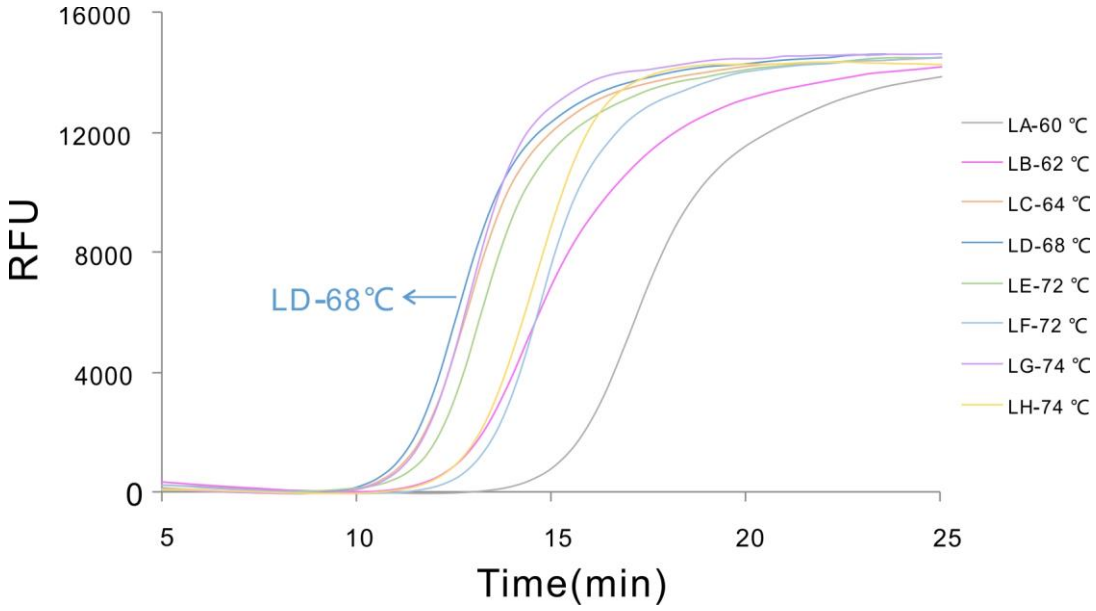

Figure S4. Comparison of the amplification time comsumed by different primers at their own optimal T_L. Reactions were performed in triplicate in a final volume of 10 µl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/µL of *EasyTaq* DNA polymerase, 0.4 µmol/L of each primer, 0.4 x SYBR green dyes, 0.5pg/µl of λ-DNA as template. All corresponding VPCR amplifications were conducted under the following cycling conditions: 94 °C for 0 s, 60-74°C (set as shown in the Figure S4) for 0 s,50-55 cycles.

2.5 TaqMan Real-time VPCR assay

The cleavage-based *Taq*Man Real-time VPCR is tested by analyzing serially diluted *Crocus sativus* genomic DNA using the developed VPCR and conventional PCR. (FigureS5). The corresponding amplification efficiency is 91.53% in VPCR compared to the 90.70% in conventional PCR. The liner relationships are both pretty good. R^2 value of 0.9989 compared to 0.9982 of conventional PCR successfully demonstrates that VPCR is suitable for DNA quantification (Figure S5). However, 40cycles of VPCR only takes 25min32s, while 40cycles of ordinary PCR requires 64min 50s. The amplification efficiency is determined by analyzing calibration curves using the equation: $E(100\%)=[10^{(-1/slope)}-1]\times100$

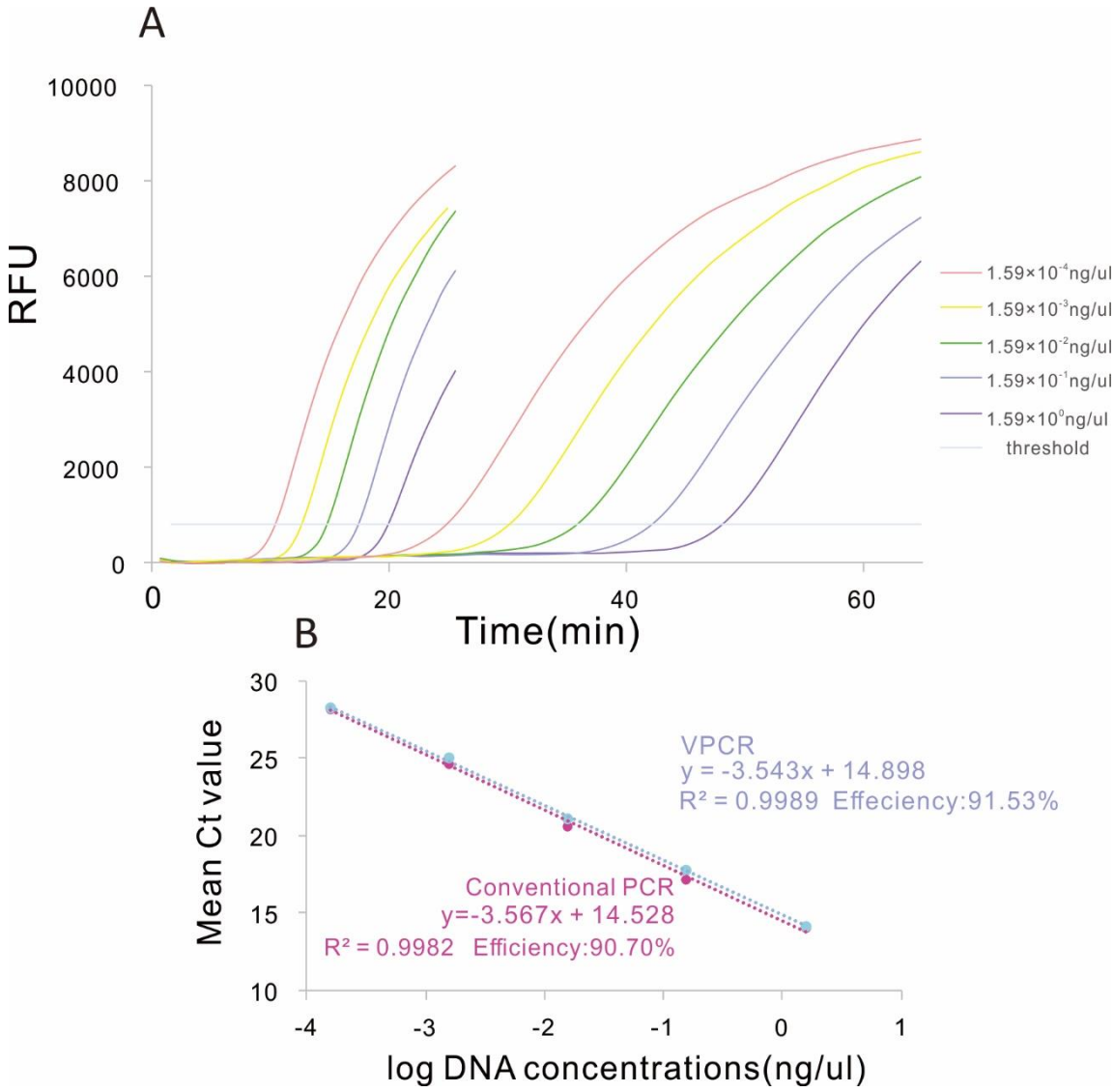

Figure S5. A Fluorescence amplification plot generated by known concentration of *Crocus sativus* genomic DNA. **B** Ct values versus log (DNA concentrations) plots for Real-time fluorescence assay. Reactions were performed in triplicate in a final volume of 10 µl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), additional 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.1U/µL of *EasyTaq* DNA polymerase, 0.3 µmol/L of each primer and 0.4µmol/L of probe. The template concentration ranges from 1.59×10^{-4} ng/µl to 1.59×10^{0} ng/µl. Conventional Real-time PCR cycles consist of 40 cycles of 94°C for 20 s, 60°C for 30s, while Real-time VPCR cycles consist of 40 cycles of 94°C for 0 s, 60°C for 0s.

2.6 Generality test: DNA templates from different sources.

Generality of VPCR in DNA templates from different sources is evaluated by amplifying DNA extracted from plasmid, virus, bacteria, plant or animal. It is shown in Figure S6 that DNA templates from different sources can be successfully amplified by the developed VPCR method.

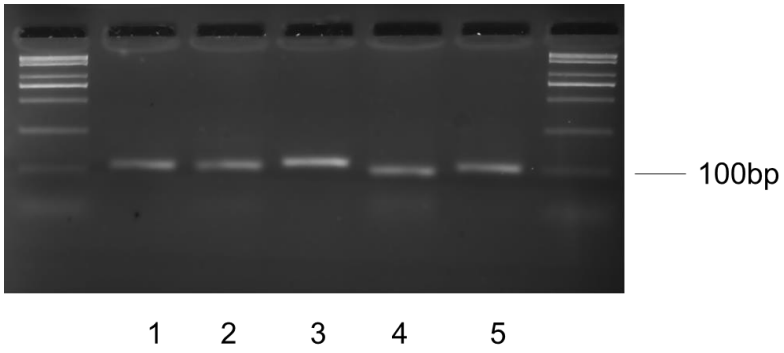

Figure S6. Amplification of DNA templates from different sources.Lane 1-5: plasmid pEGFP-N1, bacteriophage λ *c*|857, *Staphylococcus aureus*, *Carthamus tinctorius*, *Sus scrofa*. Reactions were carried out in a final volume of 10 μl mixture containing 1 μl of DNA extracts (final concentration: plasmid pEGFP-N1:75pg/μl, bacteriophage λ *c*|857 0.5pg/μl, *Staphylococcus aureus:0.53* ng/μl , *Carthamus tinctorius:0.59* ng/μl, *Sus scrofa:* 0.67ng/μl, 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase and a certain amount of primers (final concentration: VF/VR for plasmid pEGFP-N1: 0.5 μmol/L, LD/LRD for bacteriophage λ *c*|857: 0.4 μmol/L, Sta-F-1/ Sta-R-1 for *Staphylococcus aureus*:0.5μmol/L, F2/R2 for *Carthamus tinctorius* 0.8 μmol/L, PF/PR for *Sus scrofa:*1μmol/L). All corresponding VPCR amplifications were conducted under the following cycling conditions: 94 °C for 0 s,65°C (plasmid pEGFP-N1), 68°C (bacteriophage λ *c*|857), 61°C *(Staphylococcus aureus)*,65°C (*Carthamus tinctorius)*, 64°C *(Sus scrofa)* for 0 s,30 cycles.

2.7 Generality test: amplification of GC-rich templates.

A GC-rich template (GC content: 75%) was amplified either by conventional PCR or VPCR. Figure S7 shows that the templates in the two systems can both be amplified with similar efficiency, indicating that VPCR also works for the GC-rich templates.

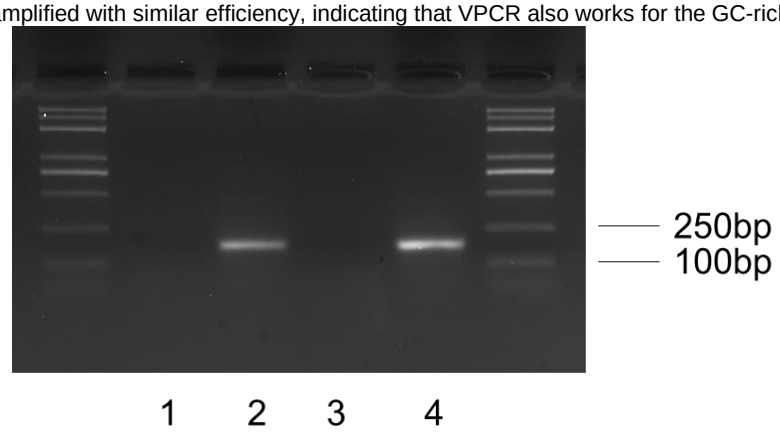

Figure S7. Agarose gel electrophoresis after amplifying GC rich templates using conventional PCR or VPCR. Lane 1-4: conventional PCR without template, conventional PCR with template, VPCR without template, VPCR with template. Reactions were carried out in a final volume of 10 μl mixture containing 1x Taq buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.5 μmol/L of each primer (AP1/ARP1), 0.48 ng/μl of genomic DNA extracted from *Pinellia ternate*. Conventional PCR amplifications were conducted under the following cycling conditions: 94 °C for 30 s, 60°C for 30 s, 72°C for 30 s, 35 cycles. VPCR amplifications were conducted as follows: 94 °C for 0 s, 60°C for 0 s,35 cycles.

2.8 Generality test: different DNA polymerase.

Generality of VPCR in different DNA polymerase is investigated by amplifying λ-DNA using different polymerase from several mainstream suppliers. It turns out in Figure S8 that all the polymerase can work well in the rapid PCR system and give the expected amplification products.

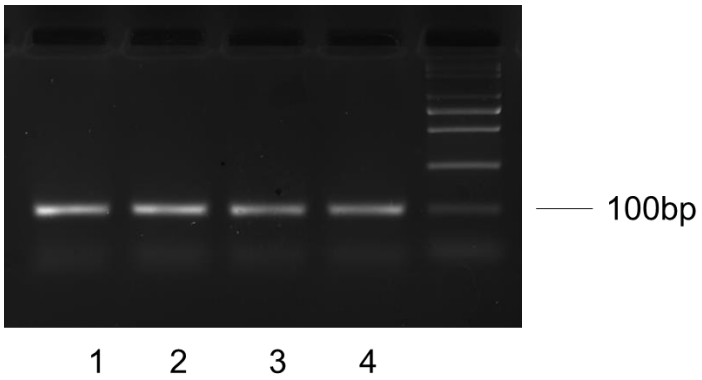

Figure S8. Agarose gel electrophoresis of VPCR products amplified with polymerase from different manufacturers. Lane 1-4: Easy *Taq* from TransGen Biotech, Ex *Taq* from Tarkara, Q5 High-Fidelity DNA Polymerase from New England Biolabs, *Taq* DNA polymerase from TIANGEN Biotech. Reactions were carried out in a final volume of 10 μl mixture containing 0.4 μmol/L of each primer(LD/LRD), 0.5pg/μl of template and recommended concentration of buffers and DNA polymerase. All VPCR amplifications were conducted under the following cycling conditions: 94 °C for 0 s, 68°C for 0 s,30 cycles.

2.9 Generality test: different reaction volumes

Generality of VPCR in different reaction volumes are studied by amplifying 5, 10, 25, 50ul samples using ordinary thermal cyclers. Figure S9 shows that samples of different volumes can be amplified with equivalent yield, indicating a wider application of the VPCR technology.

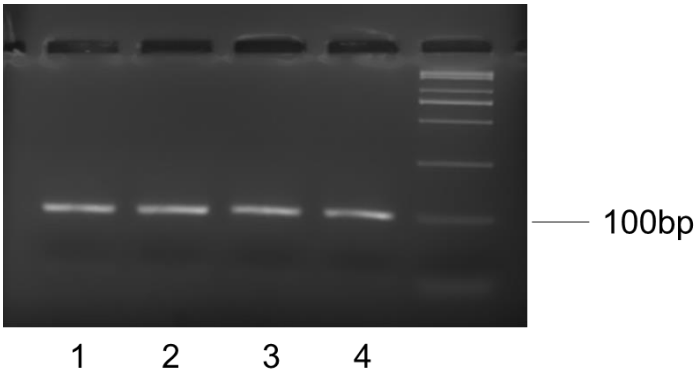

Figure S9. Amplification of 5, 10, 25, 50ul samples using VPCR protocol. Reactions were carried out in different volumes but containing same concentration of reagents: 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer (LD/LRD), 0.5pg/μl of λ-DNA. All amplifications were conducted under the following cycling conditions: 94 °C for 0 s, 68°C for 0 s,30 cycles.

2.10 Generality test: different instruments

Generality of VPCR on different instruments from different manufacturers are investigated by analyzing serially diluted λ-DNA with SYBR dyes. Good liner relationships and amplification efficiencies in Figure S10 prove that the developed amplification system is suitable for quantification. However, the total time for the 40 cycles of amplification are all reduced from nearly 1hour to about 20minutes, despite the instrument chosen. The amplification efficiency is determined by analyzing calibration curves using the equation: $E(100\%)=[10^{(-1/slope)}-1]\times100$

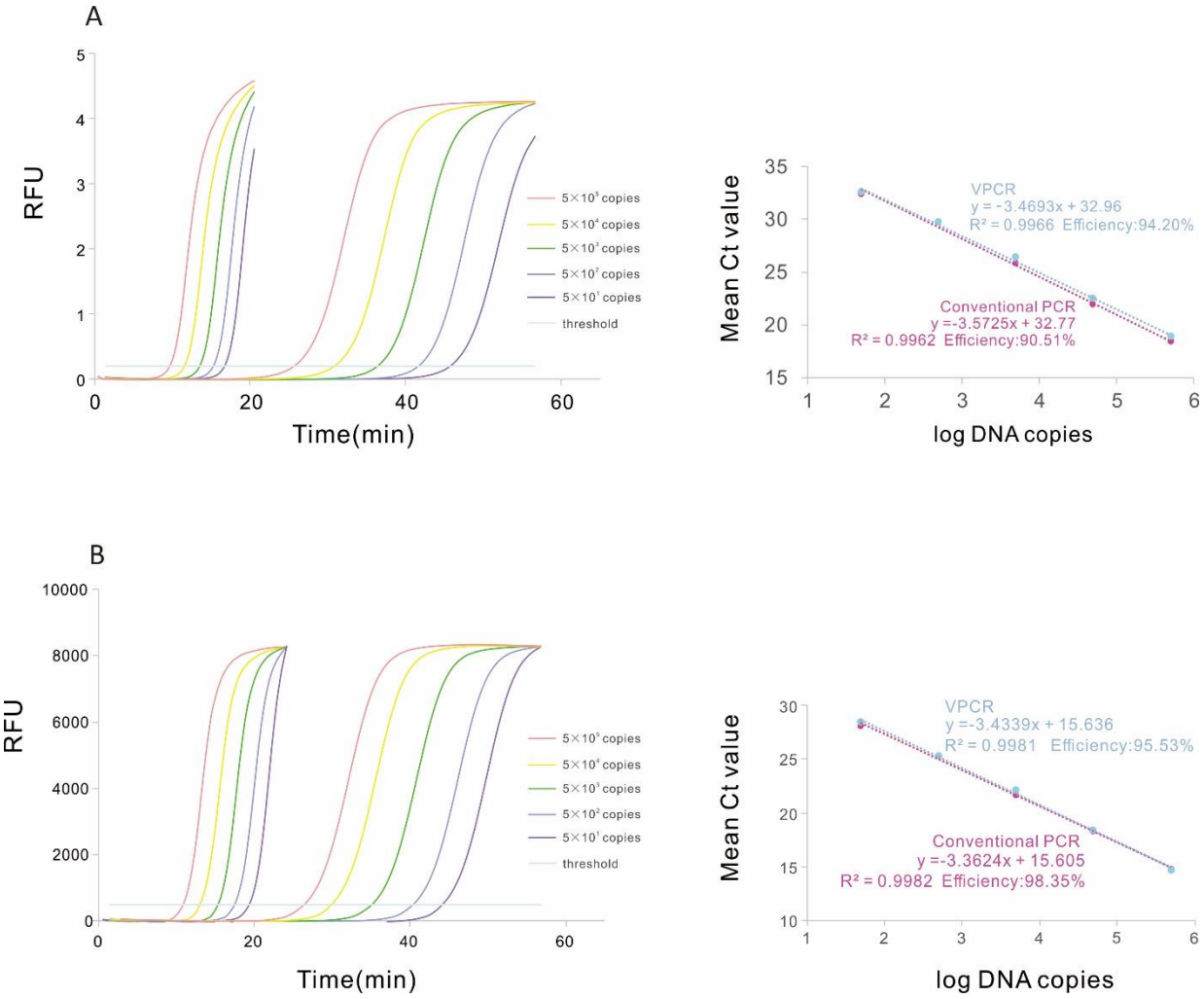

Figure S10. Fluorescence amplification plot and calibration curves generated by known concentration of λ DNA using **A**. Roche LightCycler96 **B**. Bio-Rad CFX connect Real Time PCR. Reactions were carried out in triplicate in a final volume of 10 μl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.4 μmol/L of each primer (LD/LRD), different concentration of λ-DNA, 0.4 x SYBR green dyes.

2.11 Generality test: applications in clinical-relevant assays.

A TaqMan based Hepatitis B Virus (HBV) quant assay and sequencing assisted Single Nucleotide Polymorphism (SNP) genotyping of a multi-drug resistance gene (ABCB1) were performed to test the applicability of the developed VPCR in the clinically-relevant assay.

The HBV quant assay was conducted using a commercially available kit. An unknown clinical serum sample was analyzed by the VPCR and the conventional PCR in parallel. Figure S11 shows the corresponding fluorescence amplification curves and calibration curves generated by VPCR and conventional PCR. The amplification efficiency is 92.96% in VPCR compared to the 92.46% in conventional PCR (amplification efficiency: $E(100\%)=[10^{(-1/slope)}-1]\times100$). The liner relationships are both pretty good. R^2 value of 0.9999 compared to 0.9963 of conventional PCR successfully demonstrates that VPCR is suitable for DNA quantification. The final concentration of the unknown sample determined by these two methods is both 4×10^5 IU/ml. However, 40cycles of VPCR only takes 24min22s, while 40cycles of conventional PCR requires 73min27s.

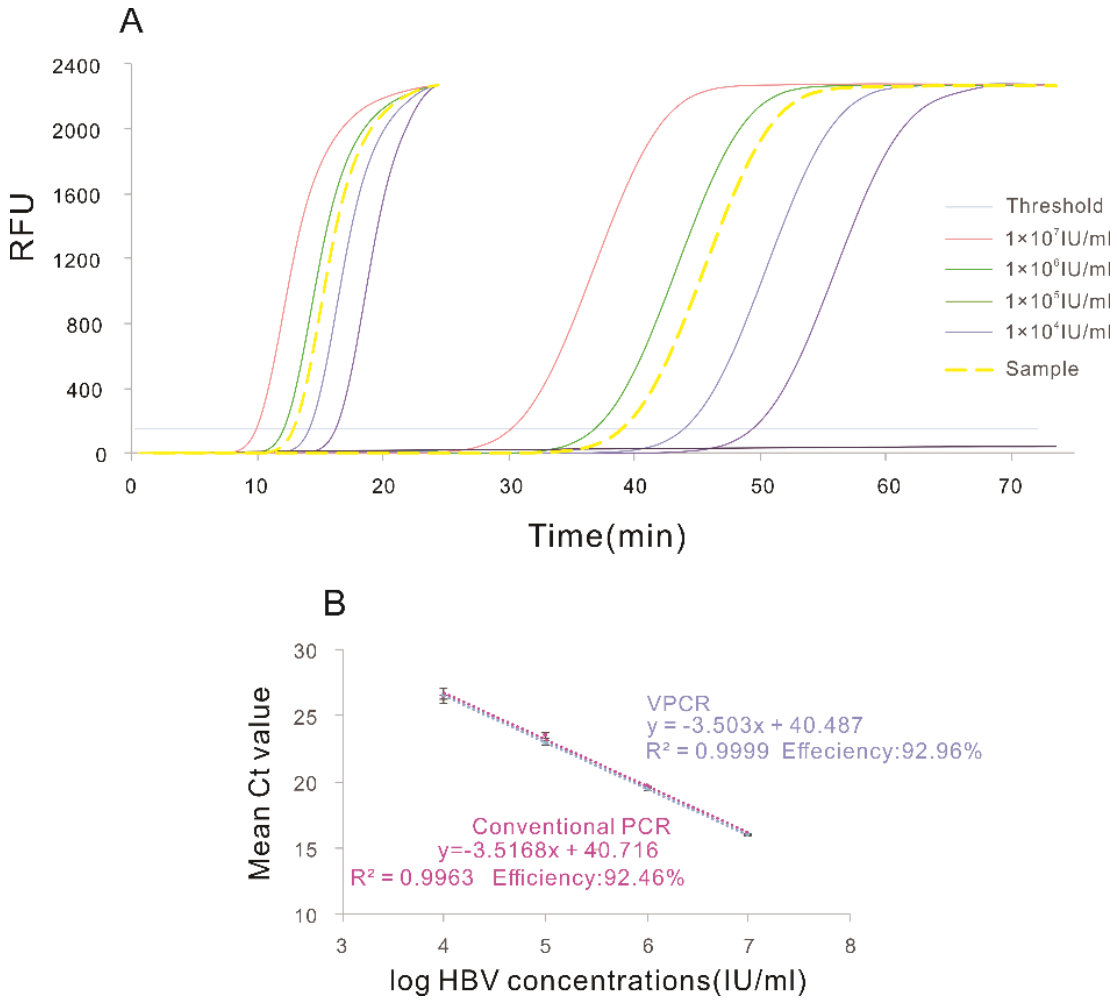

Figure S11 A. Fluorescence amplification plot generated by genomic DNA extracted from reference standards and an unknown clinical serum sample. B. Ct values versus log (HBV concentrations) plots for Real-time fluorescence assay. Reactions were performed in triplicate in a final volume of 10 μl mixture containing 9 μLof reaction buffer, 0.9 μL of DNA extracts and 0.1 μLof DNA polymerase. Conventional Real-time PCR cycles consist of 40 cycles of 94°C for 15 s, 60°C for 60s(set as the manufacturer's indications), while Real-time VPCR cycles consist of 40 cycles of 94°C for 0 s, 60°C for 0s.

The SNP genotyping of the ABCB1 gene was conducted by VPCR or conventional PCR coupled with sequencing. For all the three different genotypes, Figure S12 and FigureS13 show that the amplification results and sequencing results of VPCR products are the same as those of the conventional PCR products, but the amplification time was reduced from 77min to 20min.

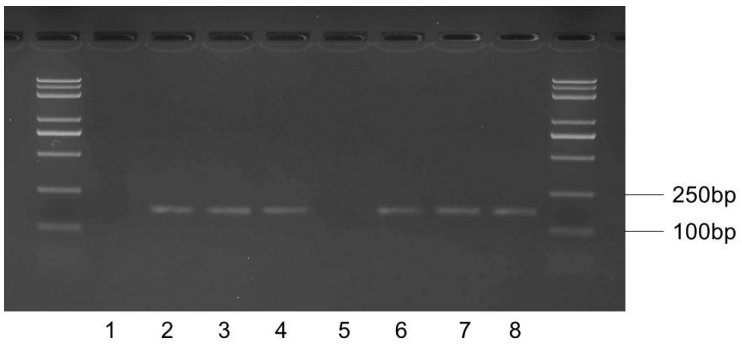

Figure S12. Agarose gel electrophoresis after amplifying 3 different samples using conventional PCR or VPCR. Conventional PCR: Lane 1-4 (1.no template control, 2.CC genotype, 3. CT genotype, 4.TT genotype); VPCR: Lane 5-8 (5.no template control, 6.CC genotype, 7. CT genotype, 8.TT genotype). Reactions were carried out in a final volume of 10 μl mixture containing 1x Taq buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/μL of *EasyTaq* DNA polymerase, 0.5 μmol/L of each primer (M1-F1/M1-R1), 1μl of genomic DNA extracted from saliva samples. Conventional PCR amplifications were conducted under the following cycling conditions: 94 °C for 30 s, 60°C for 30 s, 72°C for 30 s, 35 cycles. VPCR amplifications were conducted as follows: 94 °C for 0 s, 60°C for 0 s,35 cycles.

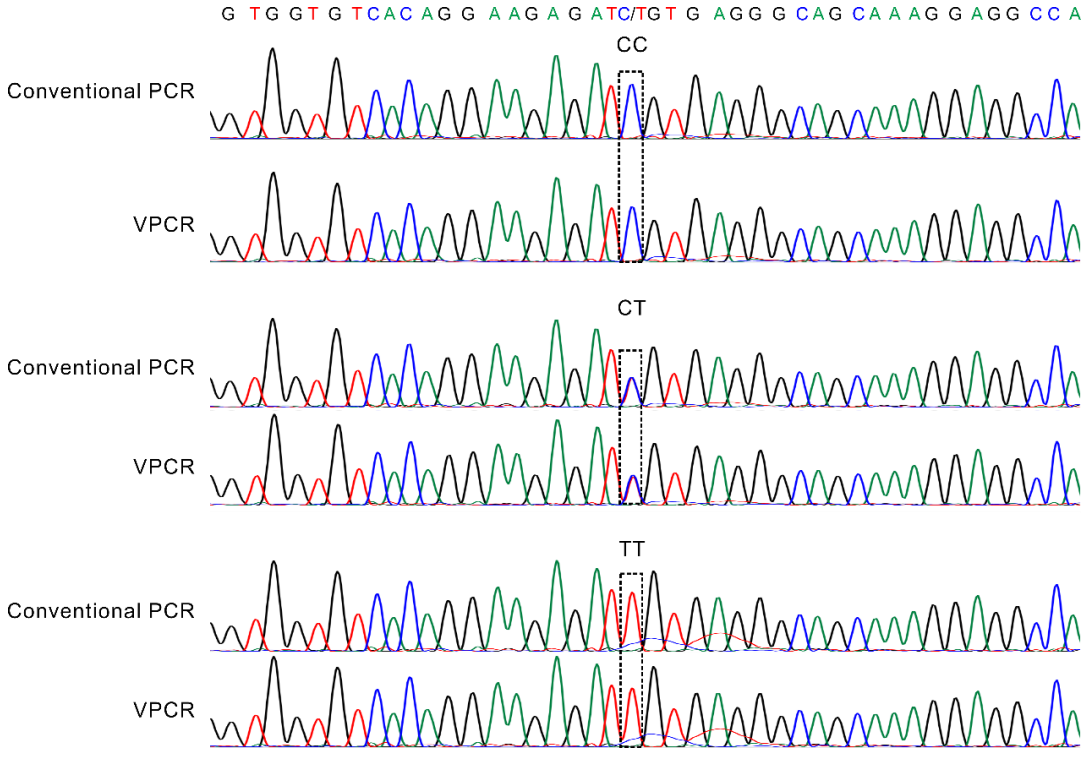

Figure S13. Genotyping of the ABCB1^{C3435T} Polymorphism: Sequencing results of corresponding VPCR products and conventional PCR products.

2.12 Optimal thermal ramp rates for templates with different length using *Taq* DNA polymerase

The effect of thermal ramp rates on the amplification time of templates with different length is examined by changing the cooling and heating speeds successively. It is shown in Figure S14 A and C that the optimal thermal ramp rate for 98bp and 200bp template are not available because the limit of the current instrument.

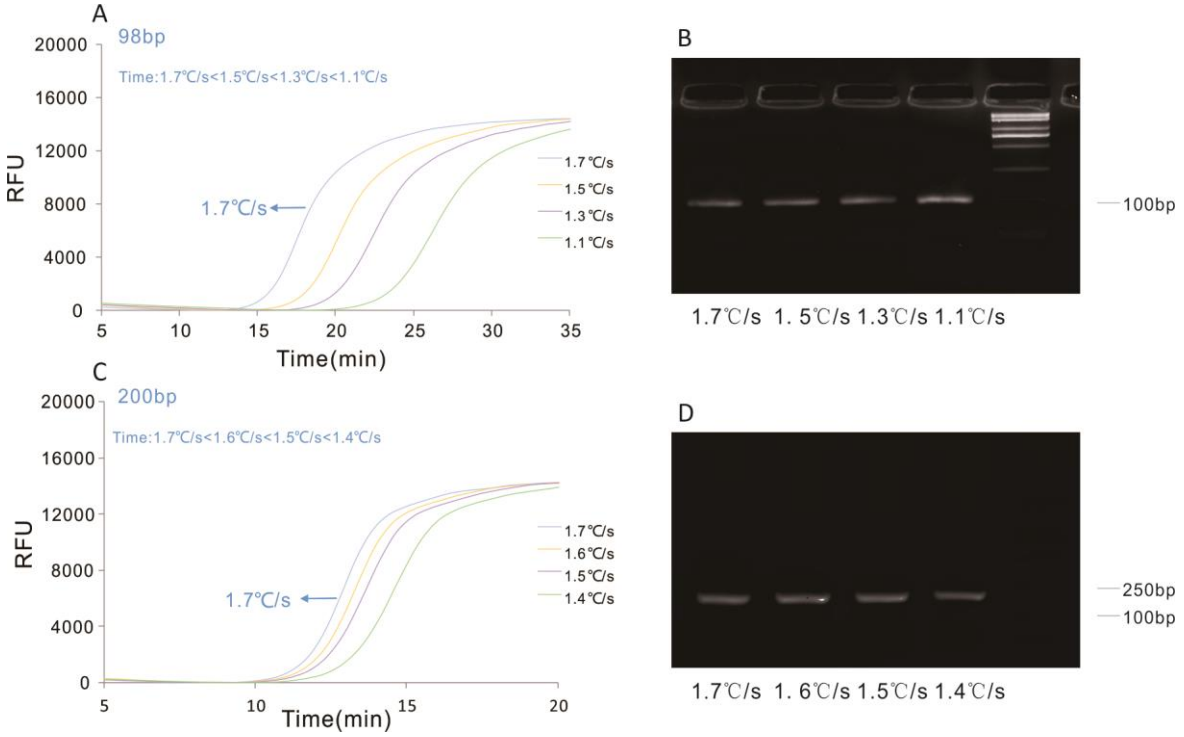

Figure S14. A-D Optimal thermal ramp rates for 98 and 200bp fragment using *Taq* DNA polymerase. **A** and **C** present the fluorescence amplification plot while **B** and **D** are corresponding agarose gel electrophoresis of the amplification products. Reactions were carried out in triplicate in a final volume of 10 µl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/µL of *EasyTaq* DNA polymerase, 0.4 x SYBR green dyes and a certain amount of primers (final concentration: LA/LRA, LD/LR200 for λ-DNA: 0.4 µmol/L) and templates (λ DNA 0.5pg/µl,). VPCR amplifications were conducted under the following cycling conditions: 94 °C for 0 s,60°C (LA/LRA)for 0 s or 68°C (LD/LR200)for 0 s,40 cycles, thermal ramp rates are set as shown in the figures.

Then we further extended the sequences, it is shown in Figure S14 E-L that the optimal thermal ramp rate is inversely related to the product length. The best ramp rates decrease from 1.6°C /s for 250bp to 0.7°C /s for 500bp fragment.

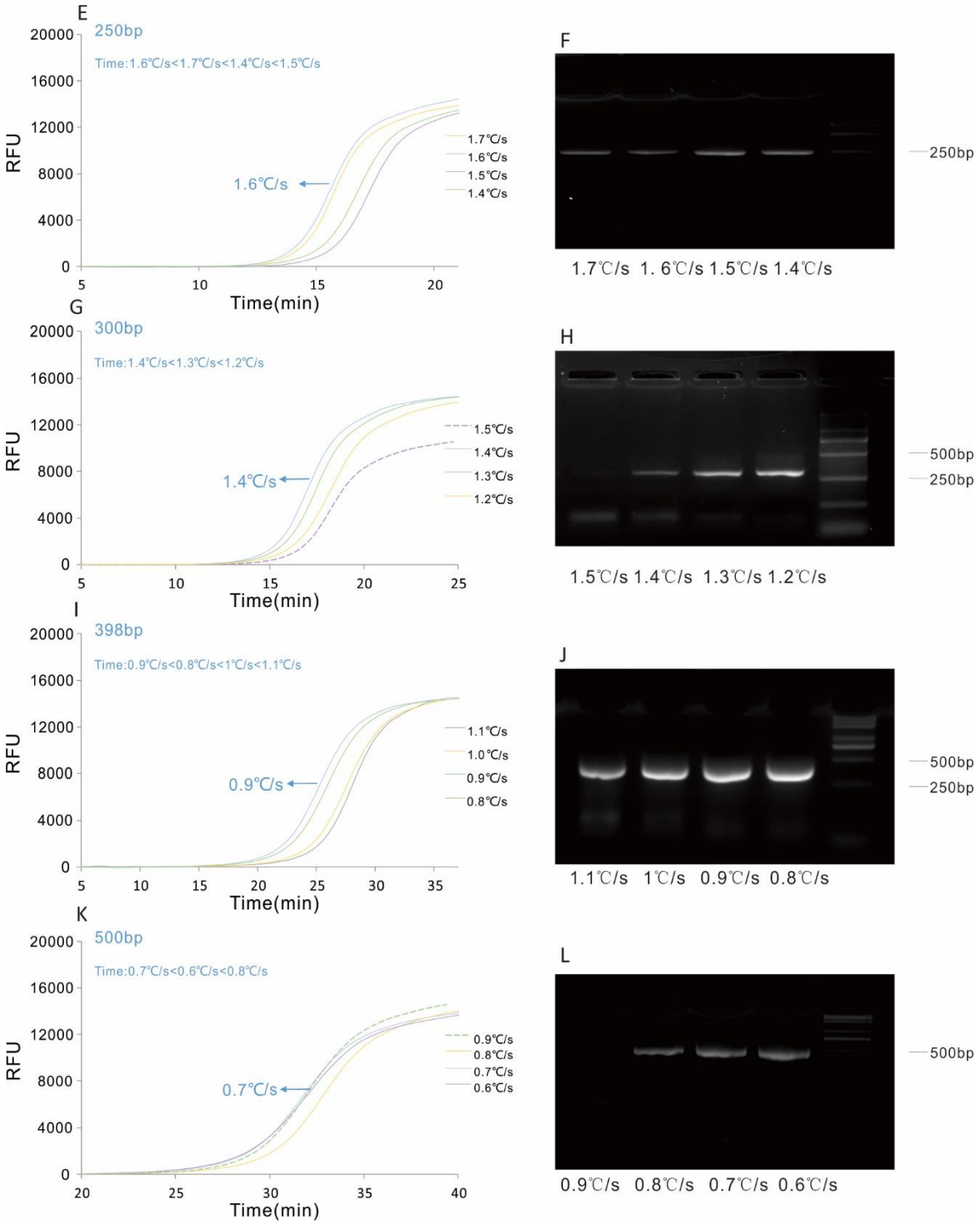

Figure S14. E-L Optimal thermal ramp rates for 250-500bp fragment using *Taq* DNA polymerase. **E, G, I** and **K** present the fluorescence amplification plot while **F, H, J** and **L** are corresponding agarose gel electrophoresis of the amplification products. Dotted lines in fluorescence figures are generated by non-specific amplification as no obvious amplification bands on agarose gel were detected. Reactions were carried out in triplicate in a final volume of 10 µl mixture containing 1x *Taq* buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), 0.2 mM dNTPs, 0.1U/µL of *EasyTaq* DNA polymerase, 0.4 x SYBR green dyes and a certain amount of primers (final concentration: LD/LR250, LD/LR300，LD/LR500 for λ-DNA: 0.4 µmol/L, Pig-P/Pig-RP for *Sus scrofa*: 0.4 µmol/L) and templates(λ DNA 0.5pg/µl, *Sus scrofa:* 0.67ng/µl,). All corresponding VPCR amplifications were conducted under the following cycling conditions: 94 °C for 0 s,68°C for 0 s,40 cycles, thermal ramp rated are set as shown in the figures.

2.13 Optimal thermal ramp rates for templates with different length using KAPA2G Robust DNA Polymerase

Optimal thermal ramp rates for templates with different length using KAPA2G Robust DNA Polymerase is investigated by similar way with *Taq* DNA polymerase. With the ability to extend 155 nucleotides per second, optimal temperature ramp rates for the same templates is much higher than that of *Taq* DNA polymerase. But it also shows a negative correlation with the product length. The best ramp rates decrease from 1.5°C /s for 898bp to 0.7°C /s for 2101bp fragment.

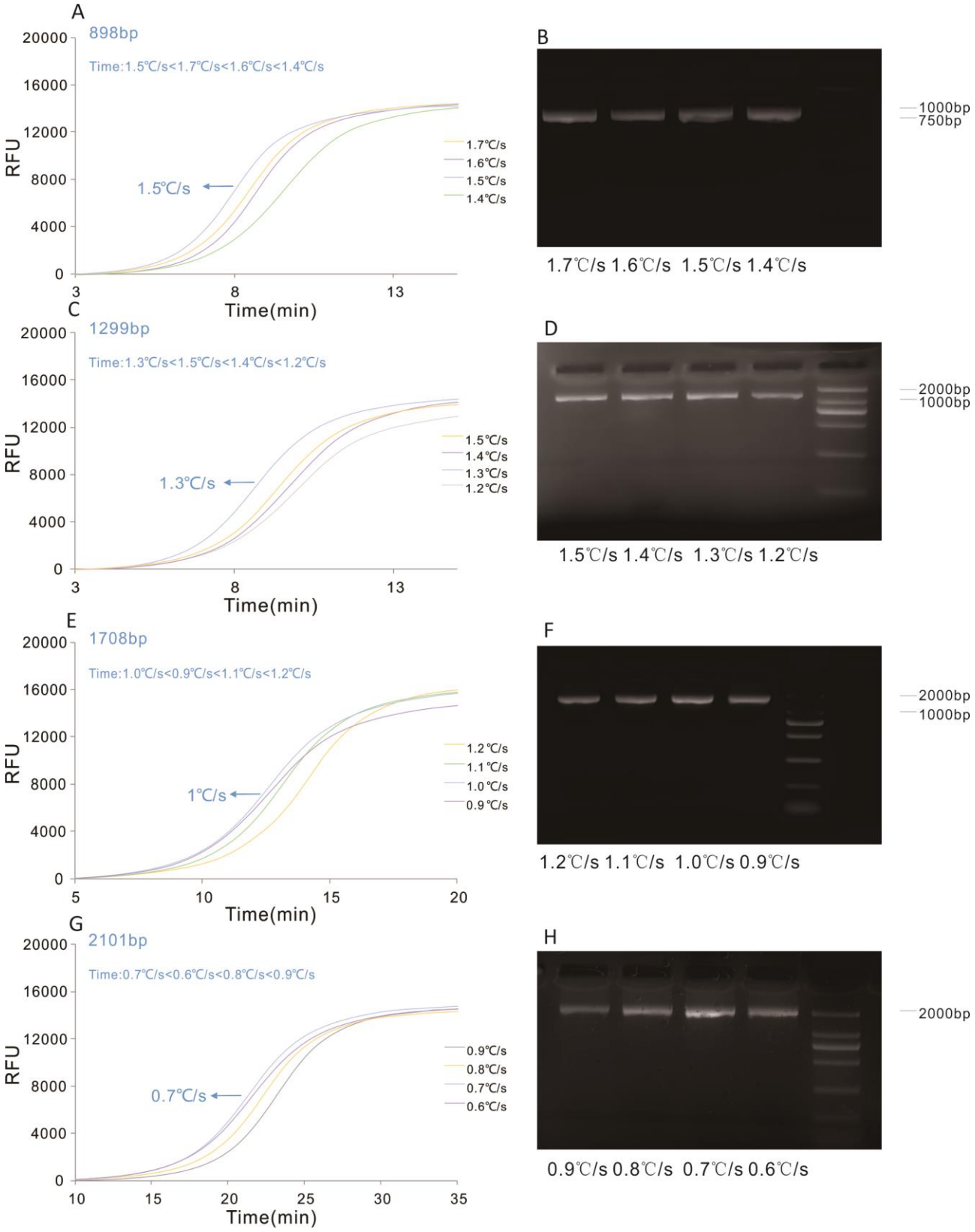

Figure S15.A-H Optimal thermal ramp rates for 898-2101bp fragment using KAPA2G Robust DNA DNA polymerase. **A, C, E** and **G** present the fluorescence amplification plot while **B, D, F** and **H** are corresponding agarose gel electrophoresis of the amplification products. Reactions were carried out in triplicate in a final

volume of 10 μl mixture containing 1x KAPA2G Buffer A, additional 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of KAPA2G Robust DNA Polymerase, 0.4 x SYBR green dyes and a certain amount of primers (final concentration: GF898/GR898, GF1299/GR1299, GF1708/GR1708, GF2101/GR2101:0.375 μmol/L) and templates (plasmid pEGFP-N1:75pg/μl). All corresponding VPCR amplifications were conducted under the following cycling conditions: 94 °C for 0 s,68°C for 0 s,40 cycles, thermal ramp rated are set as shown in the figures.

2.14 Optimization of the fastest VPCR

In this section, the KAPA2G Robust DNA Polymerase with a faster extension rate which is commonly used in rapid PCR cycling is applied to save more amplification time. All the amplification parameters for amplifying the previously mentioned 98bp target were fully optimized based on the KAPA2G Robust DNA Polymerase system.

Firstly, the optimal primer pair in the *Taq* DNA polymerase system LD/LRD is used to initiate the optimization process. The T_H is investigated from 86 °C to 94 °C while the T_L is set at 68 °C (which is the optimal temperature optimized by the previous study). As is shown in Figure S16A, there is no significant difference in the time spent to reach the threshold when the T_H is set at 88 °C, 90 °C or 92 °C. Therefore, 88 °C is chosen as the T_H in the following experiment.

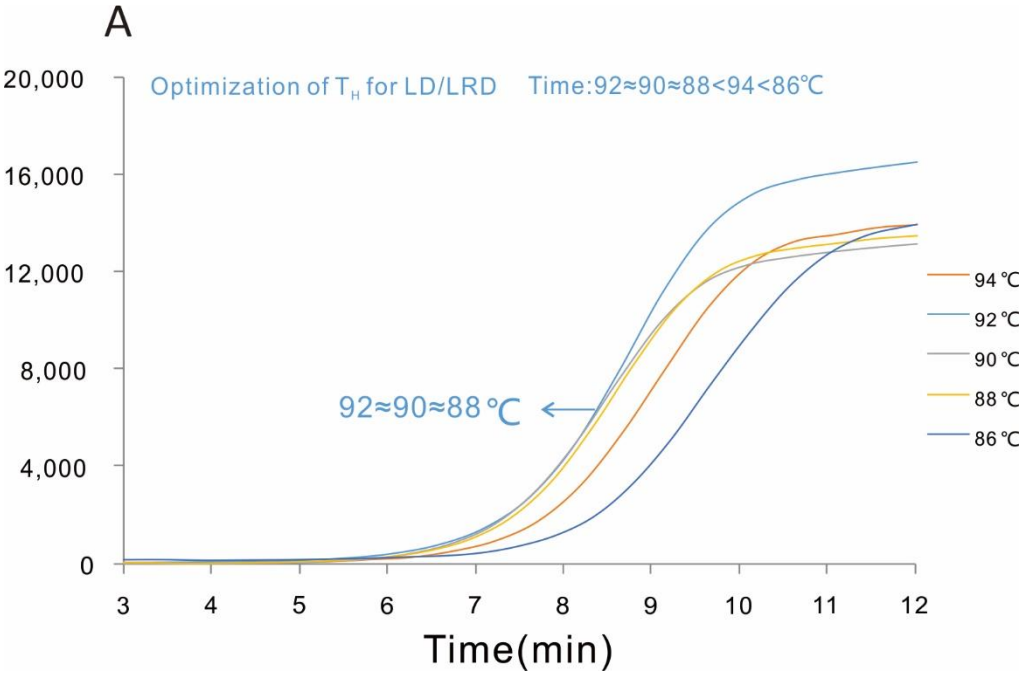

Figure S16.A Optimal T_H of LD/LRD for amplifying the 98bp fragment from λ-DNA using KAPA2G Robust DNA Polymerase is investigated by changing the temperature from 86°C to 94°C while the T_L is set at 68 °C. Reactions were performed in a final volume of 10 μl mixture containing 1x *taq* Buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), additional 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of KAPA2G Robust DNA Polymerase, 0.4 μmol/L of each primer (LD/LRD), 0.4 x SYBR green dyes, 0.1ng/μl of λDNA. VPCR were conducted under the following cycling conditions: 86-94°C for 0 s(set as shown in the Figure S16.A), 68°C for 0 s,40 cycles.

Then the T_L is studied by changing the temperature from 68ºC to 76ºC while the T_H is set at 88ºC. As shown in Figure S16B, the best result of Real-time VPCR in terms of time is obtained when the T_L is set at 72ºC or 74 ºC.

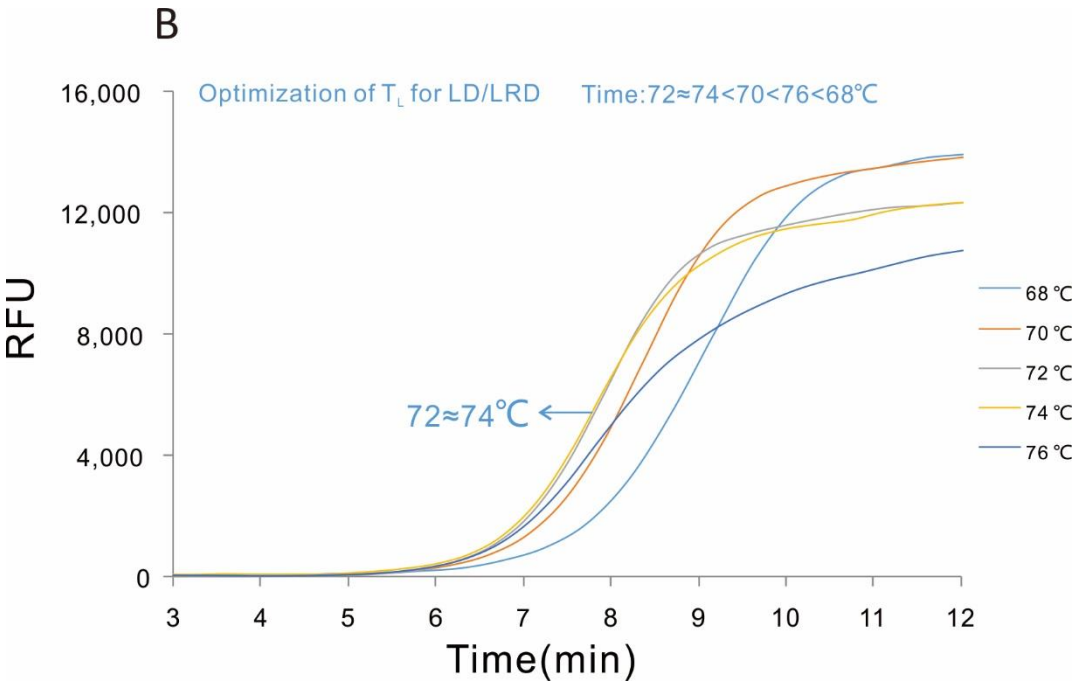

Figure S16.B Optimal T_L of LD/LRD for amplifying the 98bp fragment from λ-DNA using KAPA2G Robust DNA Polymerase is investigated by changing the temperature from 68°C to 76°C while the T_H is set at 88 ºC. Reactions were performed in a final volume of 10 µl mixture containing 1x *taq* Buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), additional 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of KAPA2G Robust DNA Polymerase, 0.4 µmol/L of each primer (LD/LRD), 0.4 x SYBR green dyes ,0.1ng/µl of λDNA. VPCR were conducted under the following cycling conditions: 88°C for 0 s, 68-76°C (set as shown in the Figure S16. B) for 0 s,40 cycles.

The T_L of LE/LRE, LF/LRF, LG/LG were optimized by the same strategies when the T_H is set at 88 ºC (data not shown). Then the shortest amplification time of each primer is compared. As shown in Figure S16C, the biggest time-saver in this system is primer pair LG/LRG and the corresponding T_L and T_H is 78 ºC and 88 ºC, respectively.

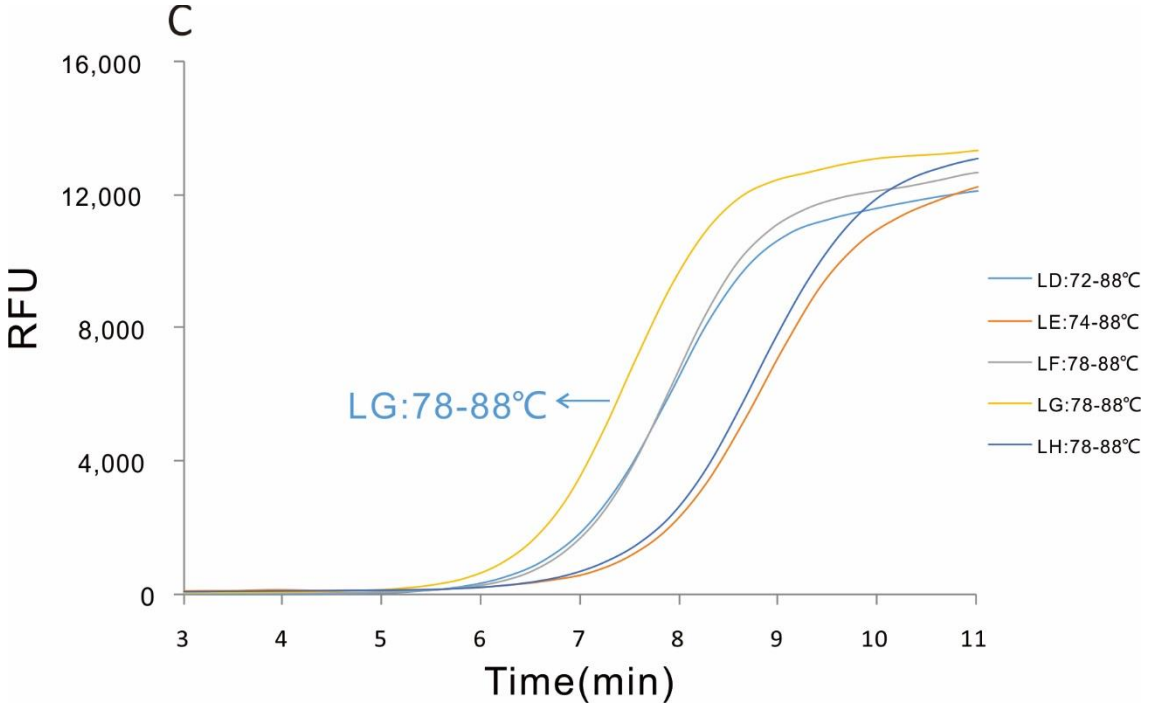

Figure S16.C Comparison of the amplification time comsumed by different primers at their own optimal condition. Reactions were performed in a final volume of 10 µl mixture containing 1x *taq* Buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), additional 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 units of KAPA2G Robust DNA Polymerase, 0.4 µmol/L of each primer, 0.4 x SYBR green dyes, 0.1ng/µl of λDNA.All corresponding VPCR amplifications were conducted under the following cycling conditions: 88 °C for 0 s, 72-78°C (set as shown in the Figure S16 C) for 0 s,40 cycles.

As Mg^{2+} is required for the activity of DNA polymerase, increased concentration of Mg^{2+} can improve the amplification efficiency and reduce the DNA amplification time. To achieve a shorter amplification time, the concentration of Mg^{2+} was optimized in this section.

Original concentration of Mg^{2+} in Taq buffer is 2mM, additional 1-4 mM of Mg^{2+} is added to the reactions. The effect of Mg^{2+} concentration on the fastest VPCR is shown in the figure below: little product is observed with original buffer (lane 2) or additional 1 mM $MgCl_2$ (lane 3), but the amount of product increases continuously through additional 2-3 mM $MgCl_2$ (lane 4-6). As there is no significant difference between the lane 5 and lane 6, additional 3 mM $MgCl_2$ was added in the final reaction system.

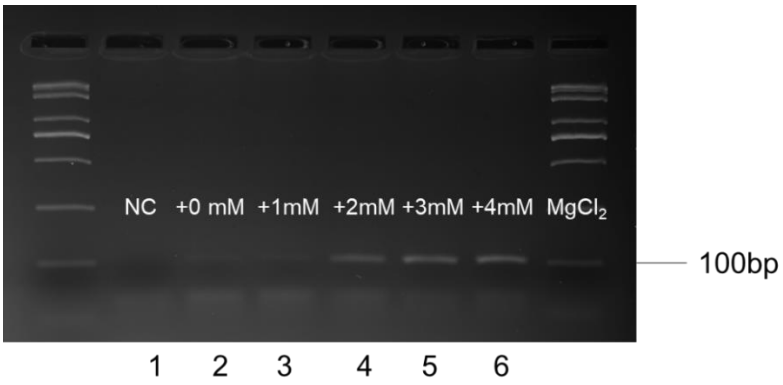

Figure S16.D The effect of Mg^{2+} concentration on the fastest VPCR. Lane 1-6：no template control, 2-6mM $MgCl_2$. Reactions were performed in a final volume of 5 μl mixture containing 1x *taq* Buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM $(NH_4)_2SO_4$ and 2 mM $MgSO_4$), additional 0-4 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 units of KAPA2G Robust DNA Polymerase, 0.5 μmol/L of each primer (LG/LRG), 0.1ng/μl of λDNA. The amplification was conducted under the following cycling conditions: 89 ºC for 0 s (temperature decreases 0.1 ºC per cycle), 77 ºC for 0 s (temperature increases 0.1 ºC per cycle), 25 cycles.

3.DNA sequences

3.1 Primers with different length and the sequence of 98bp template

A 98bp fragment was amplified from λ-DNA using primers with different length to generate the amplicon CATCGTCTGCCTGTCATG GGCTGTTAATCATTACCGTGATAACGCCATTACCTACAAAGCCCAGCGCGACAAAAATGCCAGAGAACTGAAGCTGGCGA

Table S1. Primers with different length which can generate the 98bp amplicon

Primer	Sequence	Length(nt)	Tm(°C)
LA	CATCGTCTGCCTGTCAT	17	61
LRA	TCGCCAGCTTCAGTT	15	60
LB	CATCGTCTGCCTGTCATG	18	63
LRB	TCGCCAGCTTCAGTTCT	17	63
LC	CATCGTCTGCCTGTCATGG	19	65
LRC	TCGCCAGCTTCAGTTCTCT	19	66
LD	CATCGTCTGCCTGTCATGGG	20	68
LRD	TCGCCAGCTTCAGTTCTCTGG	21	69
L-E	CATCGTCTGCCTGTCATGGGC	21	71
LRE	TCGCCAGCTTCAGTTCTCTGGC	22	72
LF	CATCGTCTGCCTGTCATGGGCTGTT	25	74
LRF	TCGCCAGCTTCAGTTCTCTGGCATTTTTG	29	74
LG	CATCGTCTGCCTGTCATGGGCTGTTAATCATTACCG	36	77
LRG	TCGCCAGCTTCAGTTCTCTGGCATTTTTGTCG	32	76
LH	CATCGTCTGCCTGTCATGGGCTGTTAATCATTACCGTGATAACGCC	46	80
LRH	TCGCCAGCTTCAGTTCTCTGGCATTTTTGTCGCG	34	79

3.2 Primers, probes and sequences used in TaqMan Real-time VPCR

In the *Taq*Man Real-time VPCR system: A 112bp fragment was amplified from *Crocus sativus* genomic DNA, using primers *F1: TCG TTGTTTGTGCCTACTCTC* and *R1: AGGACGGTTCCTTCTTCTTATTC* and to generate the amplicon *TCGTTGTTTGTGCCTACTCT*

CCGTGTCCTTGCCCTCAACAATGCGACATGTCGTCGTCGGACCCCTCACCATGGACCCTTCCGGCTTCCGAATAAGAAGAAGGA ACCGTCCT. The *Taq*Man probe used for fluorescence assay is *5'FAM-CCTTGCCCTCAACAATGCGACATG-3'BHQ1.*

3.3 Primers and sequences used in amplification of genomic DNA from different sources (including plasmid DNA, genomic DNA of virus, bacteria, plant and animal)

A 100bp fragment was amplified from plasmid pEGFP-N1 using primers VF: TACCTCGCTCTGCTAATCCT and VRF: GCGCCTTAT CCGGTAACTATC to generate the amplicon TACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTG TCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGC

A 98bp fragment was amplified from bacteriophage λ c|857 Sam7 genomic DNA(λ-DNA) using primers LD: CATCGTCTGCCTGTCA TGGG and LRD: TCGCCAGCTTCAGTTCTCTGG to generate the amplicon CATCGTCTGCCTGTCATGGGCTGTTAATCATTACCG TGATAACGCCATTACCTACAAAGCCCAGCGCGACAAAAATGCCAGAGAACTGAAGCTGGCGA

A 110bp fragment was amplified from *Staphylococcus aureus* genomic DNA, using primers Sta-F-1: CTGCGACATTAATTAAAGCGA T and Sta-R-1: AGGATGCTTTGTTTCAGGTG to generate the amplicon CTGCGACATTAATTAAAGCGATTGATGGTGATACGGTTA AATTAATGTACAAAGGTCAACCAATGACATTCAGACTATTATTAGTTGATACACCTGAAACAAAGCATCCT

An 87bp fragment was amplified from *Carthamus tinctorius* genomic DNA, using primers F2: TAGTGGTGGTTGTAAAGGACTTC and R2: CGTCGTAAGACGACACGTTAG to generate the amplicon TAGTGGTGGTTGTAAAGGACTTCGTAACGAGCCGTGTTGATGCT AGGGAATTGCTCTCTAAAGACCCTAACGTGTCGTCTTACGACG

A 100bp fragment was amplified from *Sus scrofa* mitochondrial DNA using primers PF: CACACATCTGTCGAGACGTAAA and ARP1: TCGTCCACCACTCGCCG to generate the amplicon CACACATCTGTCGAGACGTAAATTACGGATGAGTTATTCGCTACCTACATG CAAACGGAGCATCCATGTTCTTTATTTGCCTATTCATCCACGTAGGCCG

3.4 Primers and sequences used in amplification of GC Rich template

A 169bp fragment was amplified from *Pinellia ternate* genomic DNA using primers AP1: CCTGCCTGGGCGTCAC and ARP1: CGGC CTACGTGGATGAATA to generate the amplicon CCTGCCTGGGCGTCACGCCCCACGTCGCTCCCCAGCCCCCCCCACGCACTGC GGCACCCGTGCGCGCGTGGAGGGACGAGGGATGCGGAGATTGGCCCACCGTGCACTCGCGCGGCGGGCTCAAGAGCTCGGC CCTCCCGCCGGGCGAGCAAACGGCGAGTGGTGGACGA

3.5 Primers and sequences used in SNP genotyping of ABCB1 gene

A 162bp fragment was amplified from ABCB1 gene using primers M1-F1: CACACCTGGGCATCGT and M1-R1: TACATTAGGCAGT GACTCGATG to generate the amplicon CACACCTGGGCATCGTGTCCCAGGAGCCCATCCTGTTTGACTGCAGCATTGCTGAGAA CATTGCCTATGGAGACAACAGCCGGGTGGTGTCACAGGAAGAGATC/TGTGAGGGCAGCAAAGGAGGCCAACATACATGCCTTC ATCGAGTCACTGCCTAATGTA

3.6 Primers and sequences used for amplifying fragments with different length

A 200bp fragment was amplified from λ-DNA using primers LD: CATCGTCTGCCTGTCATGGG and LR200: GAGCATCATTTTCAGC TTTAGCATCAG to generate the amplicon CATCGTCTGCCTGTCATGGGCTGTTAATCATTACCGTGATAACGCCATTACCTACAAA GCCCAGCGCGACAAAAATGCCAGAGAACTGAAGCTGGCGAACGCGGCAATTACTGACATGCAGATGCGTCAGCGTGATGTTGC TGCGCTCGATGCAAAATACACGAAGGAGTTAGCTGATGCTAAAGCTGAAAATGATGCTC

A 250bp fragment was amplified from λ-DNA using primers LD: CATCGTCTGCCTGTCATGGG and LR250: GACTGCTTTGATGTGC AACCGA to generate the amplicon CATCGTCTGCCTGTCATGGGCTGTTAATCATTACCGTGATAACGCCATTACCTACAAAGCCC AGCGCGACAAAAATGCCAGAGAACTGAAGCTGGCGAACGCGGCAATTACTGACATGCAGATGCGTCAGCGTGATGTTGCTGCG CTCGATGCAAAATACACGAAGGAGTTAGCTGATGCTAAAGCTGAAAATGATGCTCTGCGTGATGATGTTGCCGCTGGTCGTCGT CGGTTGCACATCAAAGCAGTC

A 300bp fragment was amplified from λ-DNA using primers LD: CATCGTCTGCCTGTCATGGG and LR300: GCTGCATTATCCACGC CGG to generate the amplicon CATCGTCTGCCTGTCATGGGCTGTTAATCATTACCGTGATAACGCCATTACCTACAAAGCCCAGC GCGACAAAAATGCCAGAGAACTGAAGCTGGCGAACGCGGCAATTACTGACATGCAGATGCGTCAGCGTGATGTTGCTGCGCTC GATGCAAAATACACGAAGGAGTTAGCTGATGCTAAAGCTGAAAATGATGCTCTGCGTGATGATGTTGCCGCTGGTCGTCGTCGG TTGCACATCAAAGCAGTCTGTCAGTCAGTGCGTGAAGCCACCACCGCCTCCGGCGTGGATAATGCAGC

A 398bp fragment was amplified from *Sus scrofa* mitochondrial DNA using primers Pig-P: GACCTCCCAGCCCCCTCAAACATCTCA TCATGATGAAA and Pig-RP: GCTGATAGTAGATTTGTGATGACCGTA to generate the amplicon GACCTCCCAGCCCCCTCAAACA TCTCATCATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAACAGGCCTGTTCTTAGCAATACATTAC ACATCAGACACAACAACAGCTTTCTCATCAGTTACACACATCTGTCGAGACGTAAATTACGGATGAGTTATTCGCTACCTACATGC

AAACGGAGCATCCATGTTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGCCTATACTACGGATCCTATATATTCCTAGAAACAT
GAAACATTGGAGTAGTCCTACTATTTACCGTTATAGCAACAGCCTTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTGA
GGAGCTACGGTCATCACAAATCTACTATCAGC

A 500bp fragment was amplified from λ-DNA using primers LD: CATCGTCTGCCTGTCATGGG and LR500: ACTTTAGGCATTTATA
CTCCGCTGG to generate the amplicon CATCGTCTGCCTGTCATGGGCTGTTAATCATTACCGTGATAACGCCATTACCTACAAAG
CCCAGCGCGACAAAAATGCCAGAGAACTGAAGCTGGCGAACGCGGCAATTACTGACATGCAGATGCGTCAGCGTGATGTTGCT
GCGCTCGATGCAAAATACACGAAGGAGTTAGCTGATGCTAAAGCTGAAAATGATGCTCTGCGTGATGATGTTGCCGCTGGTCGT
CGTCGGTTGCACATCAAAGCAGTCTGTCAGTCAGTGCGTGAAGCCACCACCGCCTCCGGCGTGGATAATGCAGCCTCCCCCCG
ACTGGCAGACACCGCTGAACGGGATTATTTCACCCTCAGAGAGAGGCTGATCACTATGCAAAAACAACTGGAAGGAACCCAGAA
GTATATTAATGAGCAGTGCAGATAGAGTTGCCCATATCGATGGGCAACTCATGCAATTATTGTGAGCAATACACACGCGCTTCCA
GCGGAGTATAAATGCCTAAAGT

A 898bp fragment was amplified from pEGFP-N1 using primers GF898:GCCACAACGTCTATATCATGGC and GR898:GCTCCTTTC
GCTTTCTTCCC to generate the amplicon GCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCA
AGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGT
GCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGC
TGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCGACTCTAGATCATAATCA
GCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATT
GTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCAC
TGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGGCGTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTA
AATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGG
GTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAG
GGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAA
GGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGC

A 1299bp fragment was amplified from pEGFP-N1 using primers GF1299: AAATGTGCGCGGAACCC and GR1299: CAGAAGAACT
CGTCAAGAAGGC to generate the amplicon AAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCC
GCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTCCTGAGGCGGAAAGAACCAGCTGTGGAATG
TGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCA
GGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCC
TAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGA
GGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGATCGATC
AAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTAT
TCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTT
TTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAAGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGT
TCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCC
TGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCT
GCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTG
GACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGAGCATGCCCGACGGCGAGGATCTCGTCG
TGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTG
TGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTC
GTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTG

A 1708bp fragment was amplified from pEGFP-N1 using primers GF1708: ATTCGACCACCAAGCGAAAC and GR1708: GCATGGC
GGTAATACGGTTATC to generate the amplicon ATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAA
GCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGA
GCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCT
GGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCT
TGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCT
TGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCC
ACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCAT
GCTGGAGTTCTTCGCCCACCCTAGGGGGAGGCTAACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGG
CAATAAAAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCG
ATACCCCACCGAGACCCCATTGGGGCCAATACGCCCGCGTTTCTTCCTTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCC
CAGGGCTCGCAGCCAACGTCGGGGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTTCA
TTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGC
GTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCAC
CGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATAC
CAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAAT
CCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCA
GCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGT
GAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGC
GCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTT
TGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTT
TTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCATGC

A 2101bp fragment was amplified from pEGFP-N1 using primers GF2101:CGATCAAGAGACAGGATGAGGA and GRF2101:GGGATAACGCAGGAAAGAACAT to generate the amplicon CGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAAGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGAGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCTAGGGGGAGGCTAACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGGCAATAAAAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCACCGAGACCCCATTGGGGCCAATACGCCCGCGTTTCTTCCTTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGGGGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCC