Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

Clip-to-Release on Amplification (CRoA): a Novel DNA Amplification Enhancer on and off Microfluidics

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EXPERIMENTAL SECTION

CRoA enhancer and its analogs

Table S1 lists the sequences of the CRoA enhancers and some analogs. A series of CRoA named "AnQ" were synthesized (A means the loop is composed of oligo A, n means the stem is made of n base pairs, Q stands for a quencher attached to the 3' end). Besides, some CRoA analogs with similar structures were also tested. A4spacer had the same sequence as A4Q except that a C3 spacer instead of a quencher was on its 3' end to prevent mispriming, and it was used to test the necessity and importance of the quencher moiety.

To test the impact of the loop sequence on the enhancer's performance, we applied a molecular beacon probe (MB1) with its loop sequence complementary to the G269 symmetric PCR product. The stem sequence of the hairpin-structured oligonucleotides is marked in red.

To investigate whether the CRoA enhancer can maintain its hairpin structure during the PCR process, we also synthesized some CRoA analogs named "AnF&Q" (see the supporting information), by adding a CY5 fluorophore to the 5' end of "AnQ". Melting curves of "AnF&Q" should well represent the melting characteristics of the corresponding CRoA. To test, a melting profile from 25 °C to 95 °C with a 1 °C interval was performed.

Table S1. CRoA enhancer and its analogs.

Name	Sequence
A0Q	5'AAAAAAAAAA-BHQ2
A2Q	5'CCAAAAAAAAAAGG-BHQ2
A4Q	5'CCGCAAAAAAAAAAAAAAGCGG-BHQ2
A6Q	5'CCGCTGAAAAAAAAAAAAAACAGCGG-BHQ2
A8Q	5'CCGCTGCGAAAAAAAAAAAAAACGCAGCGG-BHQ2
A10Q	5'CCGCTGCGCGAAAAAAAAAAAAAACGCGCAGCGG-BHQ2
A15Q	5'CGTGCCGCTGGTCGCAAAAAAAAAAAAAAGCGACCAGCGGCACG-BHQ2
A20Q	5'CGTGCCGCTGGTTCGCTGGCAAAAAAAAAAAAAAGCCAGCGAACCAGCGGCACG-BHQ2
A4spacer	5'CCGCAAAAAAAAAAAAAAGCGG-C3 spacer
MB1	5'CY5-CCGGACTCTGCTTGTTATGCCGG-BHQ2

Templates and primers

For the proof-of-principle experiments, we performed symmetric PCR based on the G269 allelic of human *HEXA* gene, both in the benchtop Biorad CFX96 real-time PCR detection system (Biorad, USA), and on a digital microfluidic (DMF) platform. The G269 allelic of human *HEXA* gene is related to Tay-Sachs disease as previously described [1]. The template used was either plasmid inserted with a 311 bp long G269 sequence, or genomic DNA from human placenta (Sigma-Aldrich, USA). The target amplicon was 94 bp. To test CRoA's function on other microfluidic platforms besides DMF, we also performed asymmetric PCR on a polydimethylsiloxane (PDMS) based channel microfluidic chip. The template was the same as in the G269 symmetric PCR, but a different pair of primers used led to target amplicons including a 106 bp dsDNA and corresponding ssDNA.

Table S2. PCR templates, primers and probes

Name	Sequence
G269 template	5’CGAGGTCATTGAATACGCACGGCTCCGGGGTATCCGTGTGCTTGC AGAGTTTGACACTCCTGGCCACACTTTGTCCTGGGGACCAGGTAAG AATGATGTCTGGGACCAGAGGGACTCTGCTTGTTATGCTCAGAGTG AAGCTTCAGGGCACTGGCTCATGGAAGTGGCATATCCCAGCTTGGT CCTTAGAAGAATGTTTTCCATCGACTTCTTCCACCTGGGAATTTAGA TAGGAAGAACTCACTTTGGACAATGGAGGCTGCTTCTTACTATTAA AATATGTACTGTTAGACTATGTAAGGGCACAGCGC
G269 FW	5’GAATGATGTCTGGGACCAGA
G269 BW	5’GACCAAGCTGGGATATGCC
G269 XP	5’ATCGACTTCTTCCACCT
G269 LP	5’CTGTGCCCTTACATAGTCTAACAGT
G269 MB2	5’CY3-CGTGCTCCATTGTCCAAACACG-BHQ2
G269 FW target wt	5’AGTCCCTCTGGTCCCAGACATCATTCTTACCT
G269 FW target mt	5’AGTCCCCTAGGTCCCAGACATCATTCTTACCT
Kras FW	5’ACTTGTGGTAGTTGGAGCT
Kras BW	5’CCTCTATTGTTGGATCATATTCG
T. brucei FW	5’CTGTCCGGTGATGTGGAAC
T. brucei BW	5’CGTGCCTTCGTGAGAGTTTC
T. brucei FIP	5’GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA
T. brucei BIP	5’AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC
T. brucei LF	5’GCCTCCCACCCTGGACTC
T. brucei LB	5’AGACCGATAGCATCTCAG

To further investigate CRoA’s broad usage as an amplification enhancer, we applied CRoA in two other nucleic acid amplification systems. For one, the human *KRAS* gene was amplified using PCR assay as previously described [2]. The *KRAS* gene is an oncogene in the human genome, and its mutations are found to have close relationship with several types of human cancers. The template used was genomic DNA from human placenta (Sigma-Aldrich, USA), and the target amplicon was 98 bp. For the other, the serum resistance-associated (*SRA*) gene of *T.brucei* was detected using a LAMP assay, as previously described [3]. *T.brucei* is a parasitic pathogen that can cause African sleeping sickness, and the *SRA* gene is specific to this pathogen. The template used was *T.brucei* genomic DNA isolated and purified from infected mice. Both assays were performed off-chip in the Biorad CFX96 real-time PCR detection system.

All oligonucleotides used throughout this work were synthesized by Sangon Biotech, China. The primers were desalt purified, and all the enhancers and probes were HPLC purified. Their sequences are listed in Table S2.

Off-chip nucleic acid amplification

For the G269 symmetric PCR assay, the off-chip reaction mixture was a 10 μL mixture consisting of 1 × PCR buffer (Invitrogen, USA), 3 mM $MgCl_2$ (Invitrogen, USA), 200 nM of each dNTP (Invitrogen, USA), 200 nM FW primer and 200 nM BW primer, 0.4 Unit Platinum™ *Taq* DNA polymerase (Invitrogen, USA).

1 × 10^6 copies of template plasmids were included, while in the no template control (NTC) samples deionized water was added. SGI (Invitrogen, USA) at various concentrations or other dsDNA-binding dyes such as EvaGreen (Biotium, USA) and Sytox Green (Invitrogen, USA) was added according to experimental design. The dyes were at the purity level for molecular analysis. The exact molar concentration of SGI was not disclosed by the manufacturer. Yet according to the references[4, 5], 1 × SGI was likely to be about 2 μM. As for EvaGreen, accordingly to manufacturer's instruction, 1 × EvaGreen had a molar concentration of 1.25 μM. The CRoA enhancer was used at a 500 nM concentration unless otherwise stated. The thermal profile included a step at 95 °C for 2 min, and 50 cycles of 95 °C 5 s, 58 °C 15 s, followed by melting analysis.

For the *KRAS* PCR assay, the reaction mixture was almost the same as the above G269 mixture, except that the primers were replaced with 500 nM *KRAS* FW and BW primers. 35 ng human genomic DNA was used as the template in each reaction. The extension temperature was 62 °C, and else were the same as above.

The *T.brucei* LAMP assay used a 10 μL reaction mixture containing 1 × reaction buffer (NEB, USA), 8 mM $MgSO_4$ (in total), 1.4 mM of each dNTP (Invitrogen, USA), 16 Unit *Bst* DNA polymerase (NEB, USA), 0.4 μM each F3 and B3, 1.6 μM each FIP and BIP, and 0.8 μM each LF and LB. SGI and the CRoA enhancer were also added according to experimental design. 0.4 ng *T.brucei* genomic DNA was used as the template. The off-chip LAMP reaction was run at 65 °C for 1 h, followed by melting analysis.

PCR on the DMF chip

The fabrication process of the DMF chip used for on-chip PCR in this work and the overall system setup (Figure S1) is illustrated below.

To fabricate the DMF chip used in this work, chromium electrodes with a size of 2 mm × 2 mm were patterned on the soda-lime glass substrate by Shaoguang Technology (Changsha, China). On top of the electrodes, a thin layer (10 μm) of SU-8 3010 photoresist (MicroChem Corp, USA) was coated as the dielectric. Another thick layer (50-60 μm) of SU-8 3050 photoresist (MicroChem Corp, USA) was coated with certain patterns to act as fences to keep droplets from drifting around during thermal cycling. Both the bottom plate and the top plate were treated with Teflon (Chemours, USA) to form a 100 nm hydrophobic

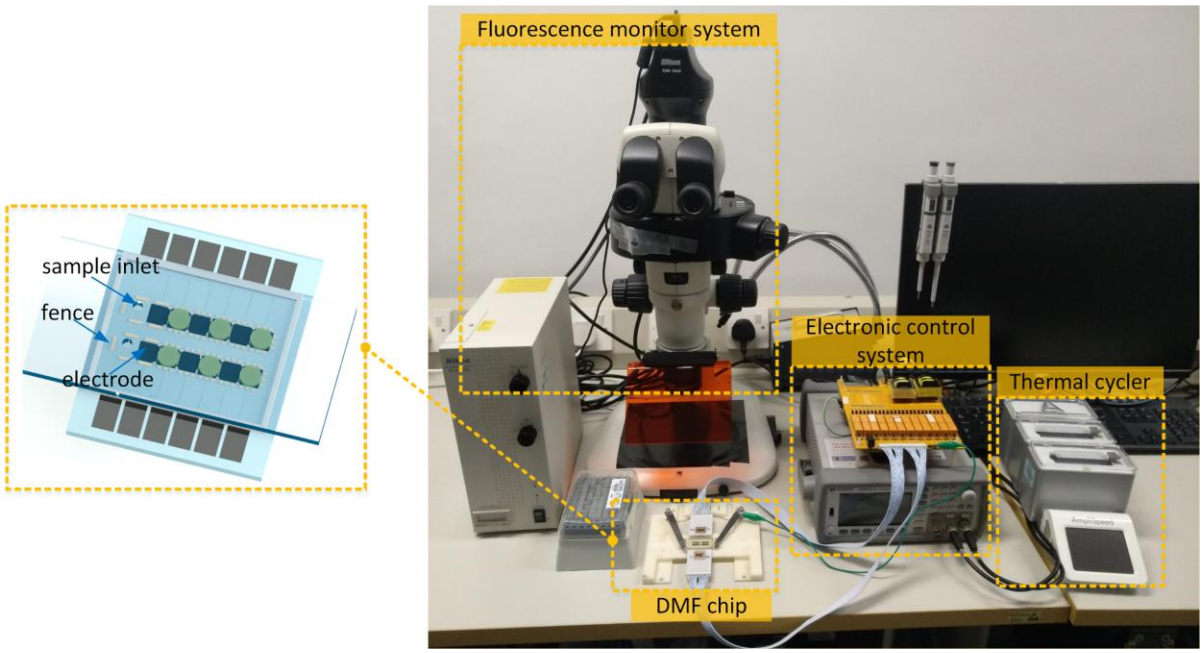

Figure S1. System setup for on-DMF PCR assays.

layer. Then the treated top plate and bottom plate were assembled by double-sided tape (3M, USA) to form a 300 μm height chamber. The ITO side of the top plate (Kaivo Optoelectronic Technology, China) faced downwards and acted as the ground.

The setup for the overall on-chip system is shown in Figure S1. The electronic control system included a signal generator for AC signal generation, and an in-house electronic control board for amplifying the AC signal, task execution, and electrical connection. For loading and moving the droplets, a sine wave (90-100 V_{rms}, 1 kHz) signal was used to actuate the electrodes. Thermal control of the PCR cycles was realized by placing the DMF chip onto a tiny flat-surface AmpliSpeed thermal cycler. Fluorescence data of the on-chip assays were monitored and collected by a fluorescence microscope and the corresponding imaging software. The fluorescence images of droplets on the chip were taken under room temperature, before and after amplification.

The G269 symmetric PCR assay performed on the DMF chip had the same reagent components and thermal profile as off-chip, except volume scaled down to 1.1 μL. The droplets were surrounded by 2cSt silicone oil (Zhongkeshangde Technology, China). After loading, the droplets were monitored by a fluorescence microscope (Nikon, Japan) under the GFP channel for the dsDNA-binding dyes' signal, with 1 s exposure and 64 × gain. The chip was then placed into the AmpliSpeed slide cycler (Advalytix, Germany) for thermal cycling. After on-chip amplification, the chip was cooled to room temperature before another microscopy image was taken. The on-DMF PCR assays were run in triplicate. For the initial few proof-of-principle trials, droplets were taken out of the chip after PCR for gel electrophoresis to identify amplification products. 3% agarose gel was prepared, with GelRed (Biotium, USA) as nucleic acid stain, and a low molecular weight DNA ladder (NEB, USA) as the marker.

Fluorescence images were analyzed by the NIS-Elements imaging software (Nikon, Japan). Each droplet's fluorescence intensity was measured with background subtraction (Figure S2) and recorded as F_{before} and F_{after}. Since the initial fluorescence intensity of samples before PCR might differ, the fluorescence increment was set to be $(F_{after}-F_{before}) / F_{before} \times 100\%$ to normalize the fluorescence change of samples, and better compare on-chip amplification results.

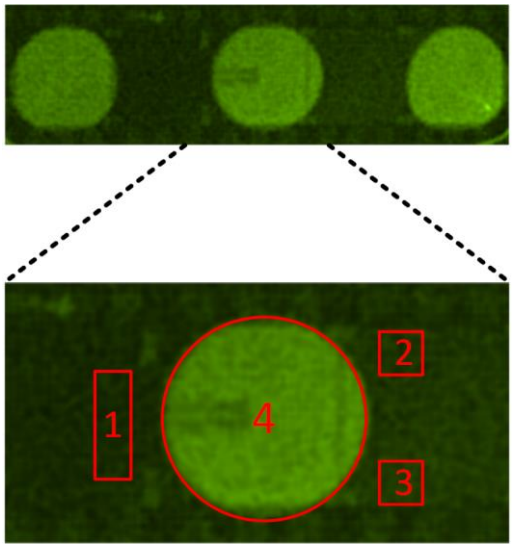

Figure S2. Measurement of on-chip fluorescence. The background intensity was measured by the fluorescence of three spots around the middle droplet (F1, F2, and F3). The mean fluorescence of the droplet itself was measured and marked as F4. Thus, the fluorescence intensity of the sample droplet was calculated by subtracting the background intensity from the mean intensity of the droplet itself, which equals to F4 - (F1 + F2 + F3)/3.

PCR on the PDMS chip

To testify CRoA's general enhancing performance for on-chip amplification, we performed the G269 asymmetric PCR assay in a PDMS-based channel microfluidic chip. Fabrication and system operation of the chip followed previously described procedures [6]. A brief description of the process is listed below.

To fabricate the PDMS-based channel microfluidic chip used in this work, a bilayer PDMS (RTV 615) was patterned using soft lithography, and bound onto the glass substrate. The upper layer was 5 mm thick with flow channels and the PCR chambers. The lower layer was 40 μm thick with reservoirs and control valves. The reservoirs underneath the PCR chambers were filled with deionized water to compensate for water evaporation during thermal cycling. For sample loading, 0.02'' Tygon tubing (Cole-Parmer, USA) was used to load the PCR reaction mixture through the inlet holes and 10 psi nitrogen air was applied. After sample loading, 25 psi nitrogen air was applied to close the valves. During the PCR process, a 10 psi pressure was applied to the reservoir channels filled with deionized water to keep compensating for the water loss during thermal cycling.

The G269 asymmetric PCR assay was performed on the PDMS chip, with each sample group in 8 replicates. The reaction mixture was consisted of the same concentration of PCR buffer, $MgCl_2$, and dNTP as the symmetric assay, as well as 100 nM limiting primer (LP) and 1000 nM excess primer (XP), 1 × additive reagent (0.2 mg/ml BSA, 150 mM Trehalose and 0.2% Tween-20), and 1 Unit Platinum™ *Taq* DNA polymerase (Invitrogen, USA). SGI was applied at 0.24 ×. Since the reaction chambers on this chip had a volume of 10 nL and were dead-ended, gel electrophoresis for PCR product confirmation was inapplicable for this system. We thus added 500 nM molecular beacon probe (MB2) targeting the ssDNA product to detect the specific amplification. Thermal control was conducted by the AmpliSpeed slide cycler, with a thermal profile including 5 min at 95 °C, and 50 cycles of 95 °C, 10 s and 58 °C, 40 s. After amplification, fluorescence images of the chip under the GFP channel (for SGI's signal) and the CY3 channel (for MB2's signal) were recorded respectively.

SUPPLEMENTARY DATA

Sanger Sequencing of some amplification products

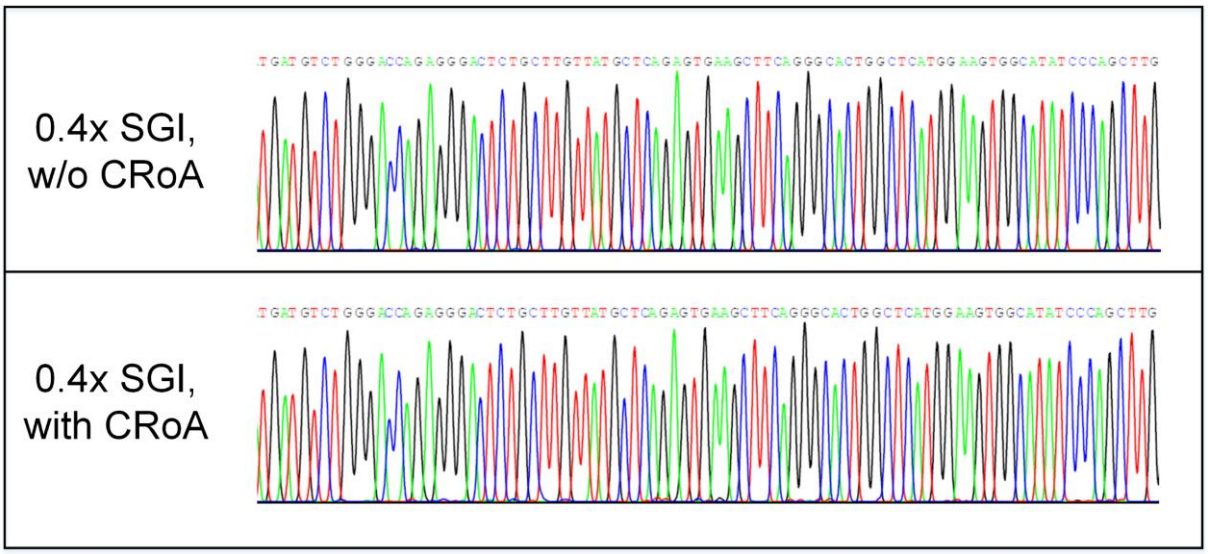

Figure S3. Sanger sequencing results for the G269 symmetric PCR product. Amplification products of the G269 symmetric PCR assay at 0.4 × SGI, both without and with CRoA, were sent to Sangon Biotech (China) for Sanger sequencing. Since the amplicon was only 94 bp, for better results the PCR products were cloned into pESI-T vector and sequenced with M13F or M13R sequencing primers. Both samples, without and with CRoA, were 100% identical to the target sequence.

Melting characteristics of CRoA

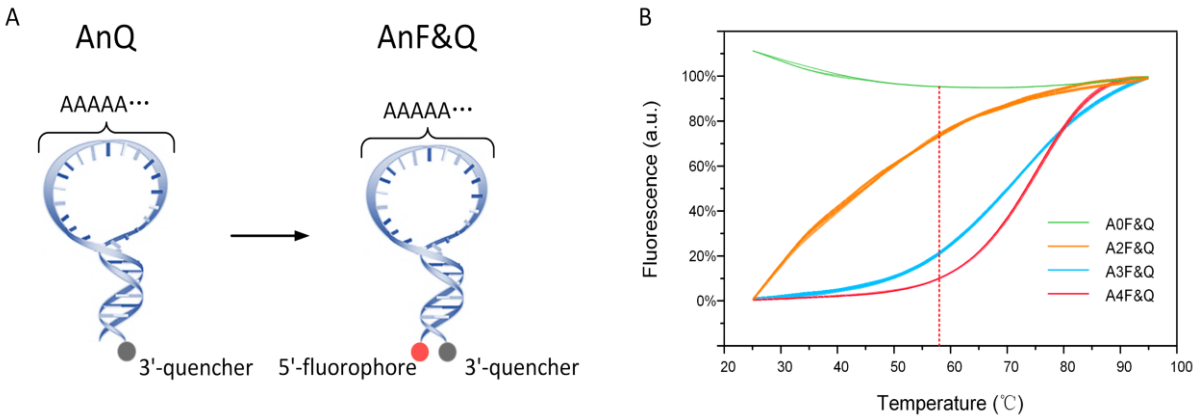

Figure S4. Simulational test of CRoA's melting characteristics through its analog "AnF&Q" (n=0, 2, 3, 4). (A) "AnF&Q" was synthesized by adding a fluorophore to the 5' end of "AnQ". Melting characteristics of "AnF&Q" could be a good simulation of the corresponding CRoA enhancer. (B) Normalized fluorescence contours of melting for "AnF&Q". The normalized fluorescence reversely indicates the portion of CRoA maintaining hairpin structure. At the extension temperature of 58 °C in G269 symmetric PCR, even with the shortest 2 bp stem, a small portion of the enhancers kept with the hairpin structure. As the stem length increased, a larger portion of CRoA maintained the hairpin structure.

CRoA's impact on primer Tm

To explore the possible reason for CRoA's ability to improve amplification specificity, we tested CRoA's impact on primer's melting temperature at 0.4 × SGI and 2.0 × SGI, respectively. The forward primer for the G269 symmetric PCR system was used. The primer's corresponding complementary sequence (G269 FW target wt), and a mutant sequence (G269 FW target mt) that has three point mutations in locations corresponding to the primer's 3' binding site, were synthesized and used. As shown in Figure S5, adding CRoA decreased primer's Tm with both wild type target and mutant target, at both SGI concentrations. However, primer's Tm with the mutant target was affected to a larger extent than the completely complementary target. In this way, with the annealing temperature of PCR being constant, the presence of CRoA might lower the Tm of the mispriming complex to a level that block nonspecific amplification, thus increase specificity.

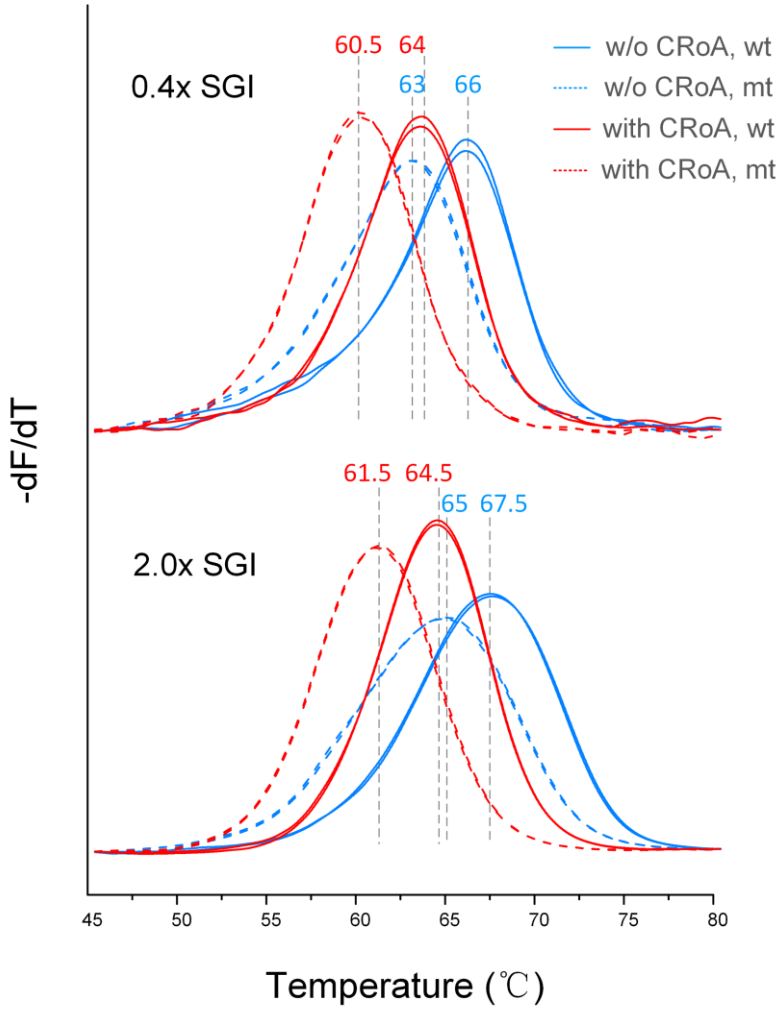

Figure S5. Primer's Tm with a completely complementary target (wt) and a mutant target (mt) under various conditions. At both 0.4 × SGI and 2.0 × SGI, primer's Tm was tested in a PCR buffer system containing the primer and its corresponding target sequence, and determined by SGI's fluorescence change during melting analysis. Samples with CRoA contained 1 μM A8Q, while samples without CRoA were tested as control groups.

CRoA's impact on amplification sensitivity and specificity with EvaGreen

CRoA's ability to increase amplification sensitivity and specificity also worked for PCR systems using EvaGreen as the amplification indicator. As shown in Figure S6A, in the absence of CRoA, the detection limit of the amplification system was 10 cp, however, with nonspecific products as indicated by melting peaks that were not at 84 °C. By adding 1 uM A8Q in the reaction, target gene from single copy of the genomic DNA template could be amplified and detected, without junks at all dilution levels, as Figure S6B reveals. Therefore the addition of CRoA changed the detection limit of this assay from 10 cp to 1cp, and improved the amplification specificity.

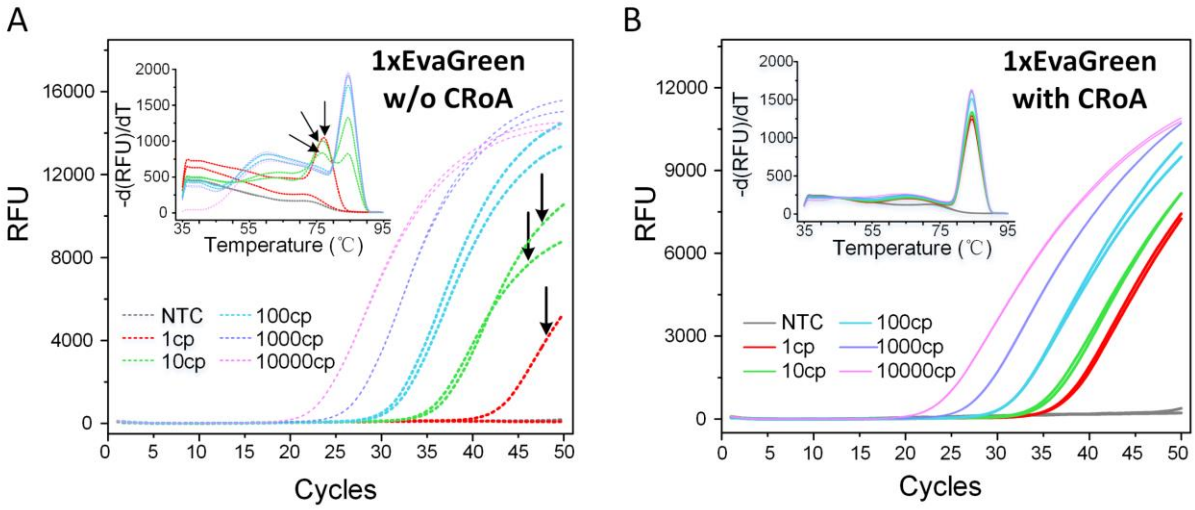

Figure S6. (A) Off-chip amplification of human genomic DNA, from 1 cp to 1 × 10^4 cp, for samples w/o CRoA at 1 × EvaGreen. The arrows stand for nonspecific products. (B) Off-chip amplification of human genomic DNA, from 1 cp to 1 × 10^4 cp, for samples with 1 uM A8Q, at 1 × EvaGreen.

REFERENCES

1. J. E. Rice, J. A. Sanchez, K. E. Pierce, A. H. Reis Jr, A. Osborne and L. J. Wangh, *Nature Protocols*, 2007, **2**, 2429.
2. J. Li, L. Wang, H. Mamon, M. H. Kulke, R. Berbeco and G. M. Makrigiorgos, *Nat Med*, 2008, **14**, 579-584.
3. L. Wan, J. Gao, T. Chen, C. Dong, H. Li, Y. Z. Wen, Z. R. Lun, Y. Jia, P. I. Mak and R. P. Martins, *Biomedical microdevices*, 2019, **21**, 9.
4. H. Zipper, H. Brunner, J. Bernhagen and F. Vitzthum, *Nucleic acids research*, 2004, **32**, e103-e103.
5. H. Gudnason, M. Dufva, D. D. Bang and A. Wolff, *Nucleic acids research*, 2007, **35**, e127-e127.
6. Y. Jia, P. I. Mak, C. Massey, R. P. Martins and L. J. Wangh, *Lab on a chip*, 2013, **13**, 4635-4641.