Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Ribonuclease -dependent cleavable beacon primer triggering DNA amplification for single nucleotide mutation detection with ultrahigh sensitivity and selectivity

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Experimental Section

Materials and Reagents

TEMED (*N, N, N', N'*-tetramethylethylenediamine), $(NH_4)_2S_2O_8$, 30% acrylamide/bis-acrylamide solution, and 10 × TBE Buffer were purchased from Bio-Rad Laboratories (Hercules, CA). EvaGreen dye (20 × in water) was purchased from Biotium (Fremont, CA). *Bacillus stearothermophilus* (Bst) 2.0 WarmStart™ DNA polymerase (8000 U/mL), *Taq* DNA polymerase (5000 U/mL), deoxynucleotide (dNTP) solution mix (10 mM of each), and Mg_2SO_4 (100 mM), 10 × Isothermal Amplification Buffer (200 mM Tris-HCl, 500 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1.0% Tween® 20 and pH 8.8 at 25°C) were all purchased from New England BioLabs (Ipswich, MA). DNeasy Blood & Tissue Kit for cell genomic DNA extraction was purchased from QIAGEN (Frederick, MD). LS 174T human colon cancer cell line with KRAS mutant (G12D) (No. CL-188) and HT-29 human colon cancer cell line with wild-type KRAS (No. HTB-38) were purchased from the American Type Culture Collection (ATCC). The ribonuclease RNase H2 (50 U at 2U/μL) and its Dilution Buffer, oligonucleotides (primers and probes), and the pUCIDT (Amp) plasmid containing 300-bp *KRAS* mutated or wild-type gene sequence were ordered from Integrated DNA Technologies (Coralville, IA).

Cell culture

Human colon tumor cell lines HT29 and LS174T from ATCC were cultured in Dulbecco's modified Eagle's medium (Gibco, Life Technologies), containing 10% fetal bovine serum (Gibco, Life Technologies),100 U/ml penicillin/streptomycin (Gibco, Life Technologies) at 37℃ in a humidified 5% CO_2 environment. Cells were collected after digestion by 0.25% Trypsin (Gibco, Life Technologies) in PBS buffer. LS174T and HT29 cells were mixed at the ratios of 0%，0.001%，0.01%，0.1%，1%，10%，50% and 100% (LS174T cells/all cells), and their DNA were extracted for analysis.

The principle for CBP design

To design a CBP with high efficiency, we can use the NUPACK software to check the free energy of secondary structure under the condition of 70 mM Na^+ and 6 mM Mg^{2+}. A well-designed CBP should be hairpin-structured at room temperature but linear at over 60 ºC. The ribonucleotide is inserted close to the 3'-end and the optimal location is the sixth base from 3'-end of the target-binding region in CBP. The 3'-end part complementary to the 5'-end part in CBP should be not over seven nucleotides. As required, the ribonucleotide is specific to single nucleotide mutation. For CBP-LAMP, CBP is designed using one of the loop primers. For CBP-PCR, CBP should have similar melting temperature (between 60 ºC and 72 ºC) as the reverse primer when annealing to the complementary region of the target. All the melting temperature should be calculated using the nearest neighbors formula.

Hybridization assays of CBP-Target

The target used for hybridization assays was a single-stranded DNA with a complementary sequence to the CBP (Table S1). The 10-μL reaction system consisted of 0.8 μM CBP, 20 mM Tris-

HCl, 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Tween® 20, and various concentrations of target (from 10pM to 1μM). In the assays with the presence of ribonuclease (RNase H2), 0.02 U RNase H2 was added. The real-time hybridization ran at 61 ºC for 60 min in the Bio-rad CFX96 Real-Time System. After incubation and cooling down to room temperature, the endpoint detection was conducted by using the Bio-Rad Gel Imaging System.

CBP-LAMP and EvaGreen-based LAMP assays

The LAMP primers for *KRAS* mutated gene sequence were all designed using the online PrimerExplorer (http://primerexplorer.jp/e/). The optimal CBP-LAMP (10 μL) was composed of 1 × Isothermal Amplification Buffer, 4 mM $MgSO_4$, 1.6 mM each of dNTPs, 0.002 U/μL RNase H2, 0.4 μM F3, 0.4 μM B3, 1.6 μM FIP, 1.6 μM BIP, 0.8 μM CBP, 0.8 μM LB, 0.32 U/μL Bst 2.0 WarmStart™ DNA polymerase, and 1 μL of template solutions (including the genomic DNA extracted from cancer cells and the plasmid templates with *KRAS* mutated or wild-type gene sequences). For real-time detection, CBP-LAMP was incubated at 61 ºC for 45 min in the Bio-Rad CFX96 Real-Time System. For endpoint detection, CBP-LAMP after 45-min reaction at 61 ºC was left to cool down at room temperature and subject to tube imaging in the Bio-Rad Gel Imaging System. The endpoint fluorescence intensity was determined using Image J software and the unpaired t-test was applied for the statistical analysis using Prism 8. The products of CBP-LAMP were analyzed using a denaturing 15% PAGE containing 8 M urea. The reaction system for optimal EvaGreen-based LAMP was the same as that of the CBP-LAMP, except for adding 1 × EvaGreen dye and replacing 0.8 μM CBP with 0.8 μM non-labeled CBP or 0.8 μM normal LF primer. The EvaGreen-based LAMP assay was incubated at 61 ºC for 60 min in the Bio-Rad CFX96 Real-Time System.

CBP-PCR assays

The PCR primers for *KRAS* mutated gene sequence were designed using the online PrimerQuest Tool (https://www.idtdna.com/PrimerQuest/Home/Index?Display=AdvancedParams). The optimal CBP-PCR (10 μL) reaction system contained 20 mM Tris-HCl, 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM each of dNTPs, 0.01% Trition X100, 0.2 μM CBP, 0.2 μM reverse primer, 0.5 U *Taq* DNA polymerase, 0.005 U RNase H2, and 1 μL of template solutions (including the genomic DNA extracted from cancer cells and the plasmid templates with *KRAS* mutated or wild-type gene sequences). For real-time fluorescence detection, CBP-PCR was incubated at 98 ºC for 2.5 min, and then 35 or 40 cycles of 98 ºC for 15 s and 61 ºC for 30 s in the Bio-Rad CFX96 Real-Time System. For endpoint fluorescence detection, CBP-PCR after the thermal cycling was left to cool down at room temperature and subject to fluorescence imaging in the Bio-Rad Gel Imaging System. The products of CBP-PCR were analyzed using the denaturing 15% PAGE (8 M urea). The endpoint fluorescence intensity was determined using Image J software and the unpaired t-test was applied for the statistical analysis using Prism 8.

Supporting Table

Table S1. List of sequence information of the primers and targets used in this study.

Name	Sequence (5′-3′) [a]	Description
KRAS mutated gene sequence inserted into the plasmid	AAGGTACTGGTGGAGTATTTGATAGTGTATTAACCTTATGTGTGAC ATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAGGCCTGCT GAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTG**A**TGGCGT AGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGT GGACGAATATGATCCAACAATAGAGGTAAATCTTGTTTTAATATGC ATATTACTGGTGCAGGACCATTCTTTGATACAGATAAAGGTTTCTC TGACCATTTTCATGAGTACTTATT	The 300-bp target sequence inserted into the pUCIDT (Amp) plasmid
KRAS wild-type gene sequence inserted into the plasmid	AAGGTACTGGTGGAGTATTTGATAGTGTATTAACCTTATGTGTGAC ATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAGGCCTGCT GAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTG**G**TGGCGT AGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGT GGACGAATATGATCCAACAATAGAGGTAAATCTTGTTTTAATATGC ATATTACTGGTGCAGGACCATTCTTTGATACAGATAAAGGTTTCTC TGACCATTTTCATGAGTACTTATT	The 300-bp target sequence inserted into the pUCIDT (Amp) plasmid
Target for CBP hybridization assay	GCGGCCTCAGATCCTGATGGCGTAGGCAAGAGTGCCTCGGTTGTA TAGTTTTT	Used for assays in Figure 1d, 1e, and S1b
F3 targeting *KRAS* mutated gene sequence	TTTTATTATAAGGCCTGCTGAA	LAMP forward outer primer
B3 targeting *KRAS* mutated gene sequence	AAATGGTCAGAGAAACCTTTAT	LAMP backward outer primer
FIP targeting *KRAS* mutated gene sequence	TGATTCTGAATTAGCTGTATCGTCATGACTGAATATAAACTTGTGG T	LAMP forward inner primer
BIP targeting *KRAS* mutated gene sequence	TGTGGACGAATATGATCCAACAATTGTATCAAAGAATGGTCCTG	LAMP backward inner primer
LF targeting *KRAS* mutated gene sequence	CACTCTTGCCTACGCCA**T**	LAMP forward loop primer
LB targeting *KRAS* mutated gene sequence	ATCTTGTTTTAATATGCATATTACTGGTG	LAMP backward loop primer
CBP targeting *KRAS* mutated gene sequence	**/56-FAM/**CACTCTTGCCTACGCCA**U**CAGCTAGAGTG**/3Dabcyl/**	Labelled CBP for LAMP and CBP-Target hybridization
CBP targeting *KRAS* mutated gene sequence	**/56-FAM/**ACTTGTGGTAGTTGGAGCTG**A**TGGCGCACAAGT**/3Dabcyl/**	Labelled CBP for PCR as forward primer
RP targeting *KRAS* mutated gene sequence	AGAATGGTCCTGCACCAG	Reverse primer for PCR

[a] Red base in the sequence denotes the single nucleotide mutation from G to A. Underlined base represents the ribonucleotide specific to the single nucleotide mutation.

Supporting Figures

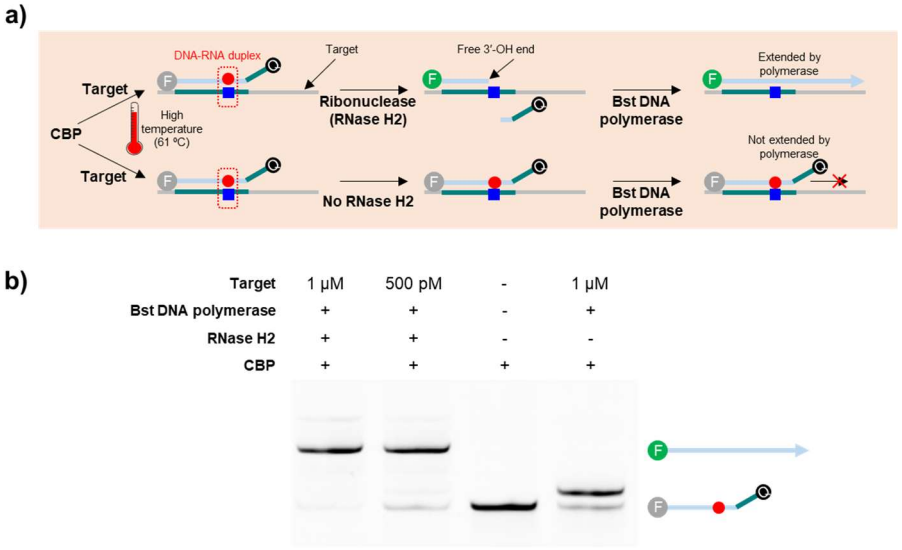

Figure S1. a) Schematic illustrating the cleaved CBP can be extended by *Bst* DNA polymerase in LAMP assay. b) Product analysis by using a denaturing PAGE.

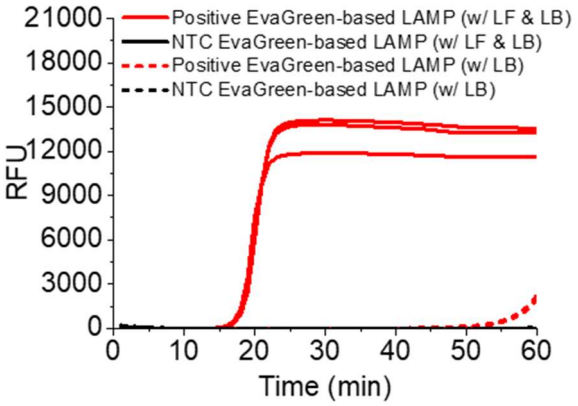

Figure S2. Real-time fluorescence curves of EvaGeen-based CBP-LAMP using two loop primers (LF&LB) or only one loop primer (LB) at 61 ºC. Positive, the reaction with 1.2×10^5 copies of plasmid templates containing *KRAS* mutated gene sequence. NTC, non-template control.

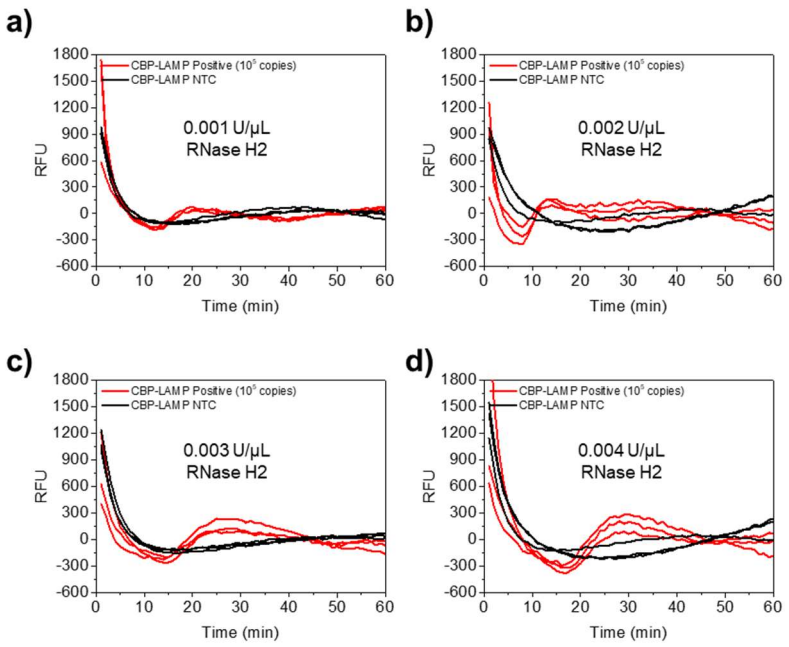

Figure S3. Effect of various concentrations of RNase H2 on the CBP-LAMP. a-d) Real-time fluorescence curves of the CBP-LAMP using 0.001 U/μL, 0.002 U/μL, 0.003 U/μL, and 0.004 U/μL RNase H2. Three replicates ran for each test. Positive, the reaction with 10^5 copies of targets (the plasmids containing the *KRAS* mutated gene sequence). NTC, non-template control. The high background signals of the real time CBP-LAMP curves were attributed to the unique structure and mechanism of the CBP. As shown in Figure 1b, the CBP opens the loop of its hairpin structure when the temperature reaches to 61 ºC during CBP-LAMP amplification.

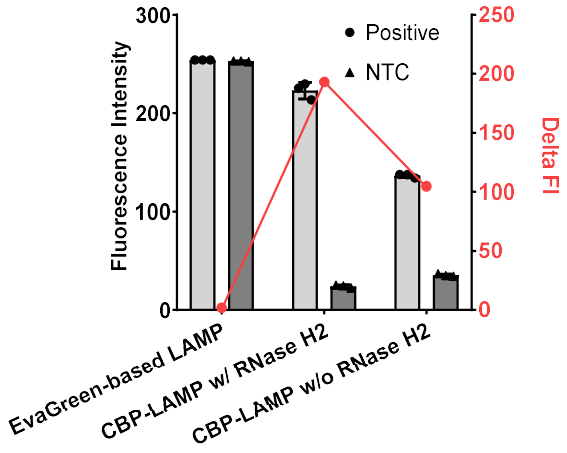

Figure S4. Endpoint fluorescence intensity of the EvaGreen-based LAMP, CBP-LAMP with RNase H2, CBP-LAMP without RNase H2, and their fluorescence difference (Delta FI) between positive and NTC. Positive, the reaction with 1.2×10^5 copies of plasmid templates containing *KRAS* mutated gene sequence. Three replicates ran for each reaction.

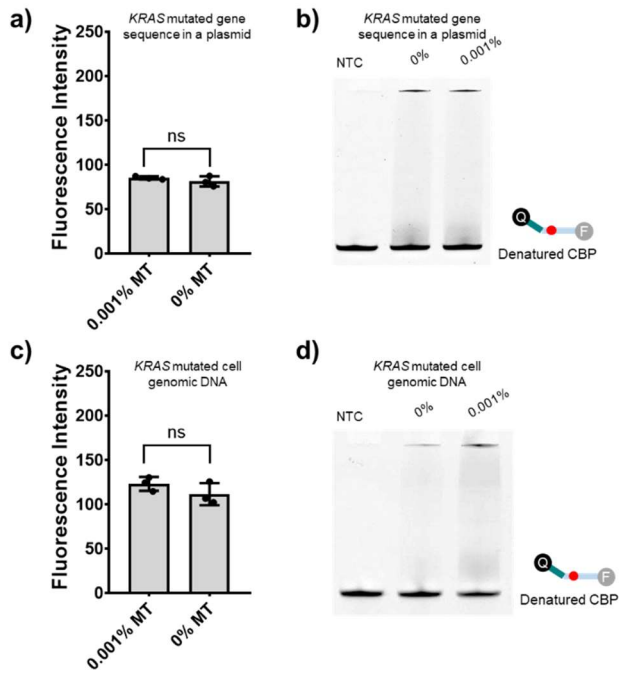

Figure S5. a) Endpoint fluorescence intensity comparison of the CBP-LAMP products to test 0.001% and 0% *KRAS* mutated gene sequence. b) Product analysis using denaturing PAGE for the CBP-LAMP to test 0.001% and 0% *KRAS* mutated gene sequence. c) Endpoint fluorescence intensity comparison of the CBP-LAMP products to test 0.001% and 0% *KRAS* mutated cell genomic DNA. d) Product analysis using denaturing PAGE for the CBP-LAMP to test 0.001% and 0% *KRAS* mutated cell genomic DNA. Three replicates ran for each reaction or test. Error bars represent the standard deviations at three replicates (n = 3).

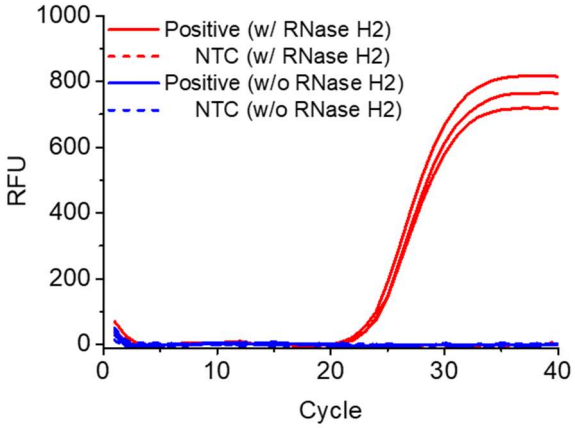

Figure S6. Real-time fluorescence curves of real-time CBP-PCR with and without RNase H2. Three replicates ran for each test.

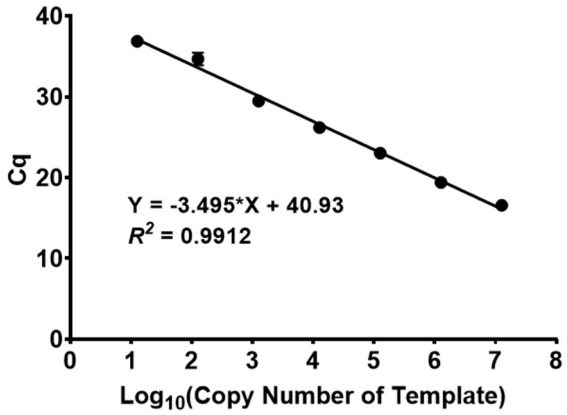

Figure S7. The linear relationship of quantitation cycle (Cq) and $\log_{10}$ of template copy number for real-time CBP-PCR. Three replicates ran for each test. Error bars represent the standard deviations at three replicates (n = 3).

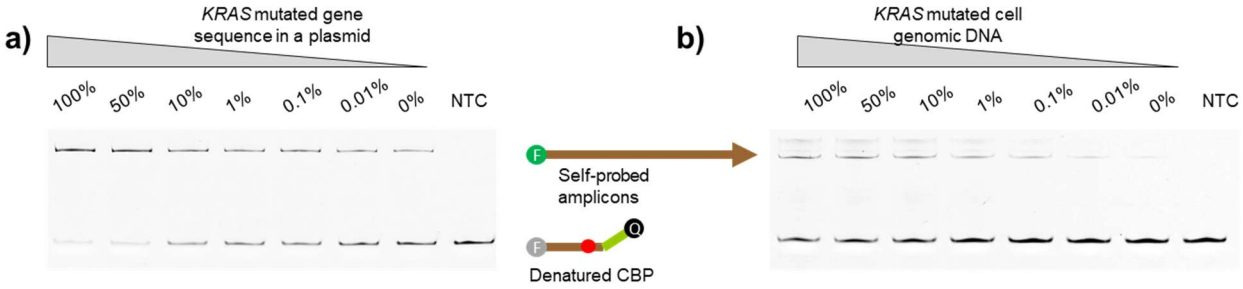

Figure S8. Product analysis using the denaturing PAGE for the CBP-PCR. a) Products of the CBP-PCR to test various percentages of *KRAS* mutated gene sequence inserted into a plasmid. b) Products of the CBP-PCR to test various percentages of *KRAS* mutated genomic DNA extracted from cancer cells. NTC, non-template control.

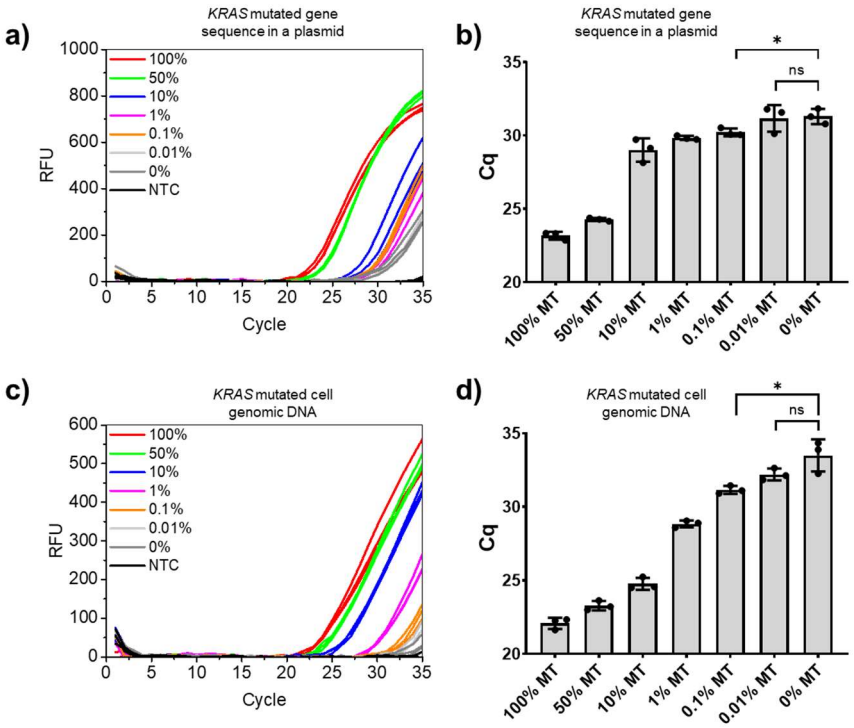

Figure S9. Real-time CBP-PCR assays for the SNM detection. a) and b) The real-time fluorescence curves and Cq comparison for the CBP-PCR to test various percentages of *KRAS* mutated gene sequence inserted into a plasmid. c) and d) The real-time fluorescence curves and Cq comparison for the CBP-PCR to test various percentages of *KRAS* mutated genomic DNA extracted from cancer cells. Three replicates ran for each reaction or test. Error bars represent the standard deviations at three replicates (n = 3). **P* < 0.05 using unpaired t test. NTC, non-template control.