Supplementary Information

Integrating CRISPR-Cas12a with DNA Circuit as A Generic Sensing Platform for Amplified Detection of MicroRNA

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Experimental Section

Materials and Instruments

Hydrochloric acid (HCl), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), boric acid(H_3BO_3) and potassium chloride (KCl) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). dithiothreitol (DTT), glycerol, tris, tetrasodium salt (EDTA) and heparin sodium from Porcine Intestinal were purchased from Sangon (Shanghai, China). All chemical reagents were of analytical grade and used without further purification, and all solutions were prepared with DEPC (diethyl pyrocarbonate) treated water. The plasmid pET-LbCpf1-2NLS was a gift from Jennifer Doudna (Addgene plasmid # 102566)[1]. Cell culture medium (DMEM and RPMI-1640), trypsin and fetal bovine serum (FBS) were purchased from Invitrogen (Gibco, USA). The number of cells was measured with automated cell counter (Countstar, Shanghai, China). The quantitative real-time PCR (RT-qPCR) and fluorescence intensity testing was carried out on QuantStudio 7 Flex (Invitrogen Life Technologies, Carlsbad, CA, USA).

All the oligonucleotides were bought from Sangon (Shanghai, China). DNA and RNA strands were dissolved in DEPC (diethyl pyrocarbonate) treated water as stock solutions and quantified by UV−vis-absorption at 260 nm. DNA annealing process were performed on a S1000^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., USA).

Protein expression and purification

The plasmid pET-LbCpf1-2NLS was transformed into *E. coli* BL21 (DE3) by heat shock and induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C for 24 h. Cells were harvested by centrifugation and re-suspended in buffer containing 50 mM Tris (pH 8.0), 1.5 M NaCl, 1 mM DTT, and 5% glycerol and lysed by sonication on ice. Protein was purified by Ni-NTA agarose chromatography (ÄKTA, GE) by washing and eluting the resin with wash buffer (50 mM Tris, pH 8.0, 1.5 M NaCl, and 1 mM DTT and 30 mM imidazole) and elution buffer (50 mM Tris pH 8.0, 1.5 M NaCl, and 1 mM DTT and 600 mM imidazole). And then the buffer was exchanged into desalination buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT and 5% glycerol). Expression level and purity of the protein (MBP-LbCas12a) were investigated using SDS-PAGE. The purified protein was quantified by using the improved Bradford protein assay dye reagent kit with bovine serum albumin as the standard. The protein was stored at –80 °C until use.

CRISPR/Cas12a collateral cleavage assays

Generally, Cas12a-mediated cleavage assays were carried out in cleavage buffer consisting of 20 mM tris HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol and 1 mM DTT. The reaction was carried out in 20 μL reaction buffer composed by 50 nM CRISPR-Cas12a/gRNA, 50 nM dsDNA target,1 μM FQ reporter. The reactions were incubated in a fluorescence plate reader at 37 °C for 60 min with fluorescence measurements taken every 30 s.

Detection of miR-21 with CRISPR-CHA assay

The H1 and H2 strands were annealed to form hairpin structure using an automated PCR thermocycler. The annealing process was 95 °C for 5 min, Tm-5 °C for 30 min, 37 °C for 30 min, and 4 °C for 30 min. The buffer used in the annealing step and the CHA reaction was Tris-HCl buffer with 20 mM Tris-HCl (pH7.5), 140 mM NaCl, 5 mM KCl. A 20 μL CHA reaction sample contained 1 μM H1(H1, $H1_{s1}$ or $H1_{s2}$), 1 μM H2 and different concentrations of miRNA to conduct reaction at 37 °C for 1 h. Nextly, 1 μL of CHA reaction solution was added into the cleavage buffer containing 50 nM CRISPR-Cas12a/gRNA, 1 μM FQ reporter with a final volume of 20 μL. The reactions were incubated in a fluorescence plate reader at 37 °C for 60 min with fluorescence measurements taken every 30 s.

Cell culture and miRNA extraction

Human non-small cell lung cancer cells (A549), human breast cancer cells (MCF-7) and human hepatocytes cells (LO2) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The A549 and LO2 cells were cultured in 1640 medium supplemented with 10% fetal calf serum. MCF-7 was cultured in a DMEM medium supplemented with 10% fetal calf serum,100 μg/mL of streptomycin, 100 units/mL of penicillin. All the cell lines were cultured at 37 ℃ with 5% CO_2. Total miRNA from the cells was extracted using miRNA Isolation Kit (OMEGA Bio-tek, American) following the product manual. Primers were designed and synthesized by Sangon (Shanghai, China) and the primer sequences were listed in Table S2. The cDNA was prepared by miRcute Plus miRNA First-Strand cDNA Synthesis Kit (TIANGEN Biotech Co., Ltd, Beijing, China). The 20 μL of reaction solution containing 10 μL of 2× miRNA RT Reaction Buffer, 2 μL of total RNA, 2 μL of miRNA RT Enzyme Mix and 6 μL of DEPC treated water. The reactions were incubated at 42 ℃ for 60 min, and followed by at 95 ℃ for 3 min. The cDNA was stored at –20 °C until use.

RT-qPCR analysis of miRNA expression

RT-qPCR analysis of miR-21 was performed with miRcute Plus miRNA qPCR Kit (SYBR Green) (TIANGEN Biotech Co., Ltd, Beijing, China). The 20 μL of reaction solution containing 10 μL of 2× miRcute Plus miRNA PreMix (SYBR&ROX), 0.4 μL of forward primer, 0.4 μL of reverse primer (provided by Kit), 2 μL of cDNA, and 7.2 μL DEPC treated water. The procedure was performed as follows: annealing at 95 ℃ for 15 min, then followed by 40 cycles at 94 ℃ for 20 s and 60 ℃ for 34 s. The relative expression of cell samples was evaluated by referring to the expression of U6 gene using the $2^{-\Delta\Delta Ct}$ method. Before the evaluation, amplification efficiency of miRNA-21 and reference U6 snRNA was verified according to the protocol from manufacturer.

Quantification of miR-21 in Serum Samples with CRISPR-CHA

The lung cancer serum samples were obtained from the Hunan Provincial Tumor Hospital (Hunan, Changsha). The healthy serum samples were obtained from the Maternal and Child Health Hospital (Hunan, Zhuzhou). Approval was obtained from the local agency review board and informed consent was obtained from all subjects for serum sampling. Eight serum samples were collected by standard surgical procedures. The serum samples from 4 lung cancer patients and 4 healthy donors were centrifuged at 10,000 × g for 10 min at 4 °C. Then, 50 μL of supernatant was mixed with 50 μL of DEPC treated water, and the solution was heated to 65 °C for 10 min to inactivate deoxyribonuclease. Finally, the serum of healthy donor was spiked with different concentrations of miR-21, and 1 μL of serum samples was added into CRISPR-CHA system. The reaction and fluorescent detection were performed with the above-mentioned procedures.

Tables

Table S1 Sequence of the oligonucleotides used in the LbCas12a assay.

Oligonucleotides	Sequence (5'-3')
[a]gRNA	UAAUUUCUACUAAGUGUAGAUGAUCGUUACGCUAACUAUGA
[b,c]TS	TAGCATTCCACAGACAGCCCTCATAGTTAGCGTAACGATCTAAAGTTTTGTCGTC
NTS	GACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGCTA
[d]NTS-1	GACGACAAAACTTTACATCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGCTA
NTS-2	GACGACAAAACTTTACTTCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGCTA
FQ reporter	FAM-TTATT-BHQ
FAM-ssDNA	FAM-TAGCTTATCAGACTGA

[a]The bases in purple are the sequence of spacer.

[b]The bases in blue are the sequence of TS and NTS.

[c]The bases in orange are the sequence of PAM.

[d]The bases in red are the substitutes.

Table S2 Sequence of the oligonucleotides used for miRNA assay in CRISPR-CHA.

Oligonucleotides	Sequence (5'-3')
[a]gRNA	UAAUUUCUACUAAGUGUAGAUGAUCGUUACGCUAACUAUGA
[b,c]H1	TCAACATCAGTCTGATAAGCTATTTAGATCGTTACGCTAACTATGATAGCTTATCAGACT
H2	TAAGCTATCATAGTTAGCGTAACGATCTAAATAGCTTATCAGACTTAGATCGTTACGCTAACTATG
[d]$H1_{s1}$	TCAACATCAGTCTGATAAGCTATTTACATCGTTACGCTAACTATGATAGCTTATCAGACT
[d]$H1_{s2}$	TCAACATCAGTCTGATAAGCTATTTACTTCGTTACGCTAACTATGATAGCTTATCAGACT
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-21 DNA	TAGCTTATCAGACTGATGTTGA
$H1_{miR-141}$	CCATCTTTACCAGACAGTGTTATTTAGATCGTTACGCTAACTATGATAACACTGTCTGGT
$H2_{miR-141}$	AGTGTTATCATAGTTAGCGTAACGATCTAAATAACACTGTCTGGTTAGATCGTTACGCTAACTATG
miR-141 DNA	TAACACTGTCTGGTAAAGATGG
$H1_{miR-155}$	ACCCCTATCACGATTAGCATTAATTTAGATCGTTACGCTAACTATGATTAATGCTAATCGTG
$H2_{miR-155}$	GCATTAATCATAGTTAGCGTAACGATCTAAATTAATGCTAATCGTGTAGATCGTTACGCTAACTATG
miR-155 DNA	TTAATGCTAATCGTGATAGGGGT
miR-21 inhibitor	TCAACATCAGTCTGATAAGCTA
R-21 forward primer	TAGCTTATCAGACTGATGTTGA
U6 forward primer	CTCGCTTCGGCAGCACA
FQ reporter	FAM-TTATT-BHQ

[a]The bases in purpleare the sequence of spacer.

[b]The bases in blue are the sequence of TS and NTS.

[c]The bases in orange are the sequence of PAM.

[d]The bases in red are the substitutes.

Table S3 Sequence of the oligonucleotides used for miR-21 assay in CHA.

Oligonucleotides	**Sequence (5'-3')**
H1-CHA	TCAACATCAGTCTGATAAGCTAGATGTTGAAACCTAGGTAGCTTATCAGACT
H2-CHA	ATAAGCTACCTAGGTTTCAACATCTAGCTTATCAGACTGATGTTGAAACCTAGGCC AAGTCATAGAGTAC
Reporter-FAM	FAM-CGAGTACTCTATGACTTGGCCTAGGTT
Reporter-BHQ	CCAAGTCATAGAGTACTCG-BHQ

Table S4 Average Ct values in RT-qPCR assay of miR-21.

Note	Ct_{U6}	Ct_{miR-21}	ΔCt	-ΔΔCt	Fold change
MCF-7	12.16	16.70	4.54	2.04	4.11
A549	12.98	16.79	3.81	2.77	6.82
LO2	10.22	16.80	6.58	-	1.00

Figures

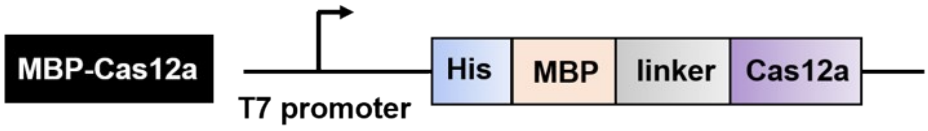

Fig. S1 Schematic for the expression vector of MBP-Lbcas12a protein.

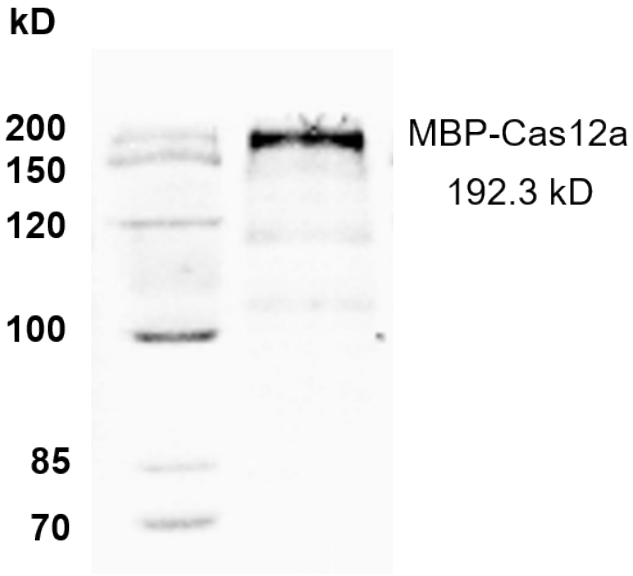

Fig. S2 SDS-PAGE (12%) analysis of the purified MBP-LbCas12a protein.

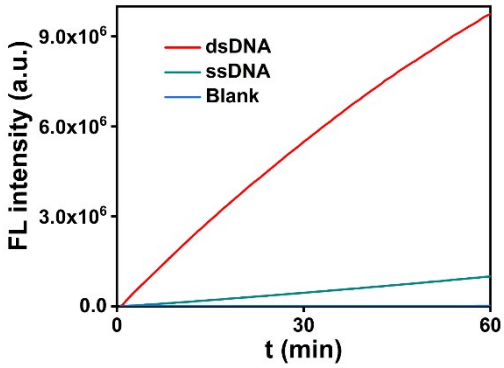

Fig. S3 Time-dependent fluorescent signals of the collateral cleavage activity of LbCas12a in the presence of dsDNA and ssDNA targets, respectively. Fluorescence emission at 520 nm of the system was monitored. Cas12a/gRNA, 100 nM; dsDNA, 50 nM; ssDNA, 50 nM and FQ reporter, 1.0 μM.

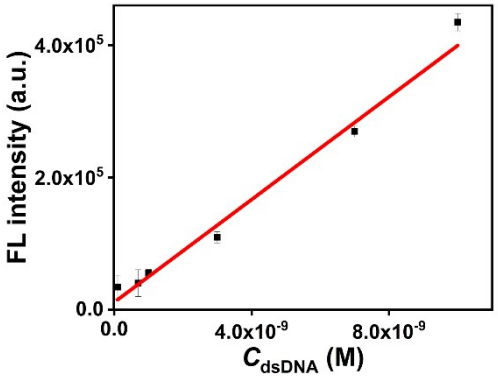

Fig. S4 Calibration curve of LbCas12a assay in response to different concentrations of dsDNA (0, 0.1, 0.7, 1, 3, 7 and 10 nM). Cas12a/gRNA, 100 nM; and FQ reporter, 1.0 μM. (The data are presented as mean ± s.d. of three replicate measurements.)

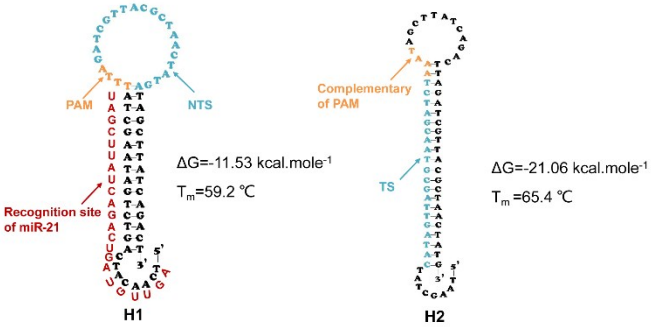

Fig. S5 Detailed sequences of H1 and H2 used for miR-21 detection in CRISPR-CHA. The annealing temperatures of H1 and H2 were calculated by Oligo Analyzer 3.1.[2]

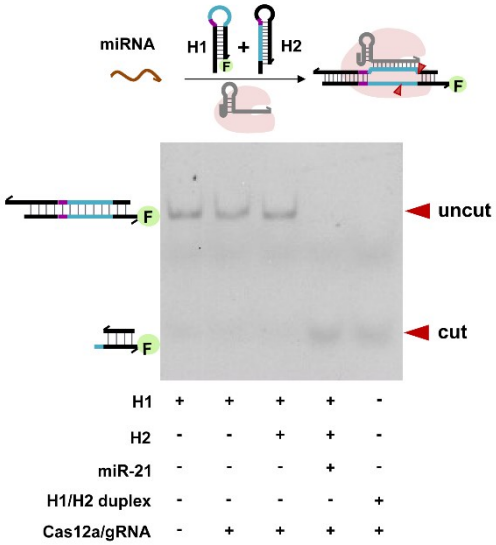

Fig. S6 Native PAGE gel (10%) analysis of the specific cleavage of H1/H2 duplex by LbCas12a/gRNA complex. Cas12a/gRNA, 200 nM; H1, 100 nM; H2, 100 nM; and H1/H2 duplex, 100 nM.

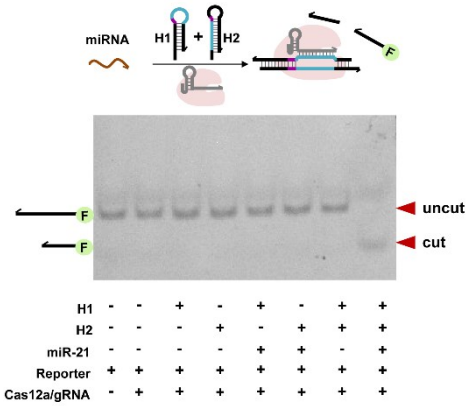

Fig. S7 Native PAGE gel (10%) analysis of the collateral activity of LbCas12a without and with addition of target miRNA using FAM-ssDNA as the reporter. Cas12a/gRNA, 200 nM; H1, 100 nM; H2, 100 nM; miR-21, 20 nM; and FAM-ssDNA, 100 nM.

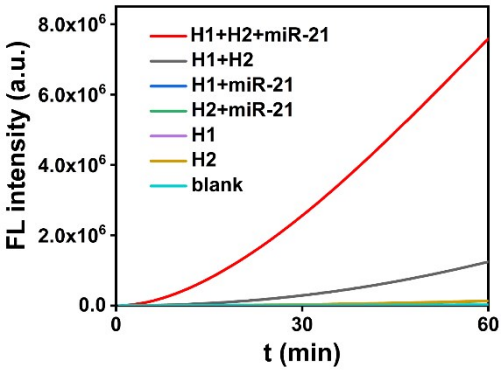

Fig. S8 Time-dependent fluorescent signals of the collateral cleavage activity of LbCas12a in the presence of different targets, including H1; H2; H1+miR-21; H2+miR-21; H1+H2 and H1+H2+miR-21. Cas12a/gRNA, 100 nM; H1, 50 nM; H2, 50 nM; miR-21, 10 nM; and FQ reporter, 1.0 μM.

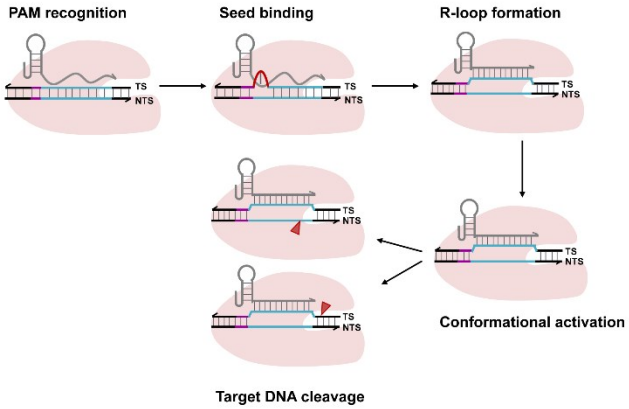

Fig. S9 Schematic model of gRNA-guided binding and cleavage of target DNA by Cas12a. This model was referred to a previously reported literature by Martin Jinek.[3]

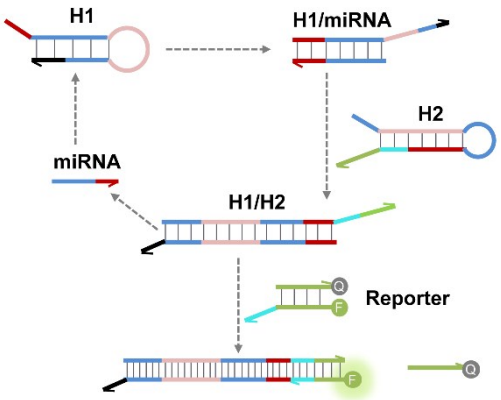

Fig. S10 Working principle of CHA method for the detection of miRNA. This principle was referred to a previously reported literature by Andrew D. Ellington.[4]

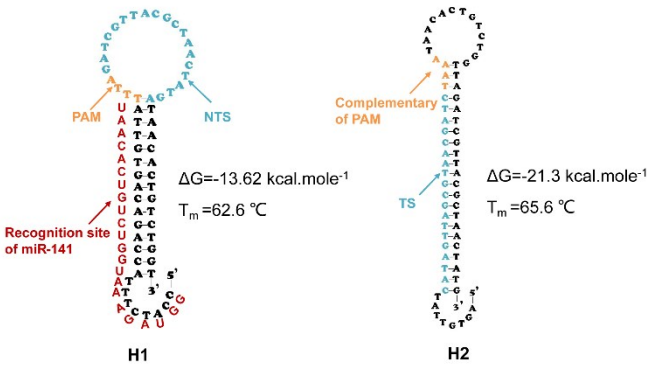

Fig. S11 Detailed sequences of H1 and H2 used in CRISPR-CHA for the detection of miR-141. The annealing temperatures of H1 and H2 were calculated by Oligo Analyzer 3.1.[2]

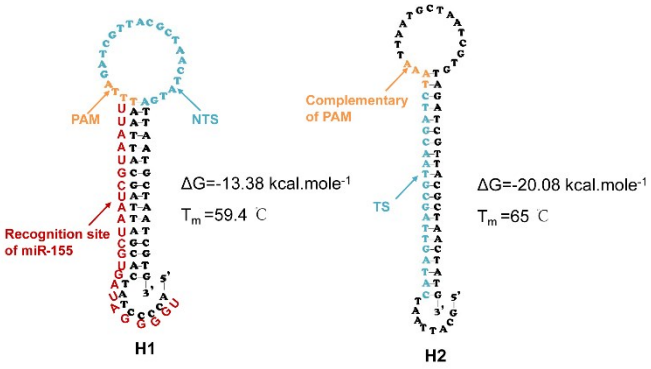

Fig. S12 Detailed sequences of H1 and H2 used in CRISPR-CHA for the detection of miR-155. The annealing temperature of H1 and H2 were calculated by Oligo Analyzer 3.1.[2]

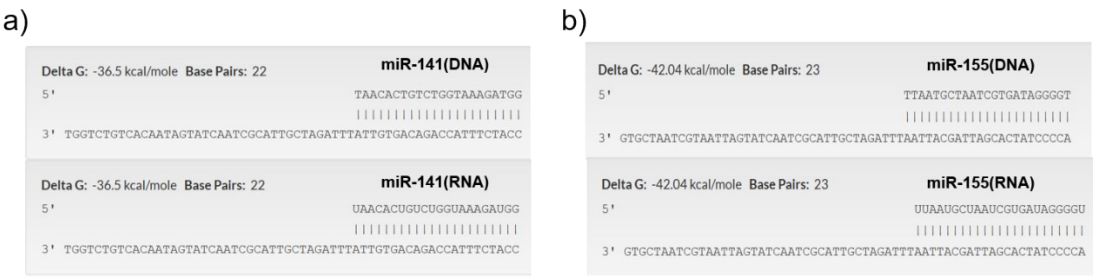

Fig. S13 (a) Analysis of the hybridization reaction between miR-141 (and its DNA mimic) and $H1_{miR\text{-}141}$ by Oligo Analyzer 3.1. (b) Analysis of the hybridization reaction between miR-155 (and its DNA mimic) and $H1_{miR\text{-}155}$.

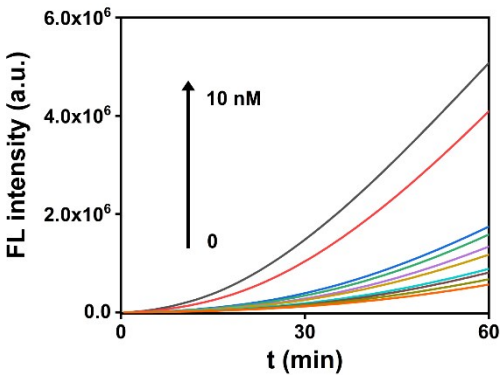

Fig. S14 Time-dependent fluorescent signals of CRISPR-CHA in response to different concentrations of miR-21 (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1.0 and 10 nM). Cas12a/gRNA, 100 nM; H1, 50 nM; H2, 50 nM and FQ reporter, 1.0 μM.

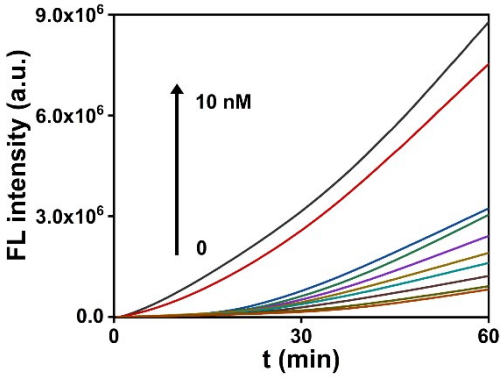

Fig. S15 Time-dependent fluorescent signals of CRISPR-CHA in response to different concentrations of miR-141 (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1.0 and 10 nM). Cas12a/gRNA, 100 nM; H1, 50 nM; H2, 50 nM; and FQ reporter, 1.0 μM.

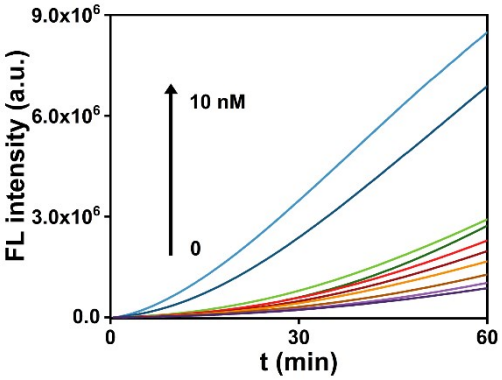

Fig. S16 Time-dependent fluorescent signals of CRISPR-CHA in response to different concentrations of miR-155 (0, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1.0 and 10 nM). Cas12a/gRNA, 100 nM; H1, 50 nM; H2, 50 nM; and FQ reporter, 1.0 μM.

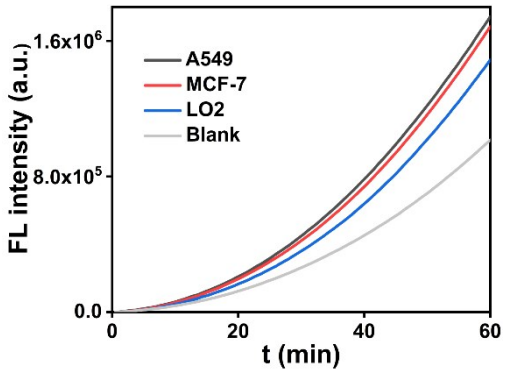

Fig. S17 Time-dependent fluorescent signals of CRISPR-CHA in response to microRNA extracted from different cell lines. Cas12a/gRNA, 100 nM; H1, 50 nM; H2, 50 nM; microRNA extraction solution, 1.0 μL; and FQ reporter, 1.0 μM.

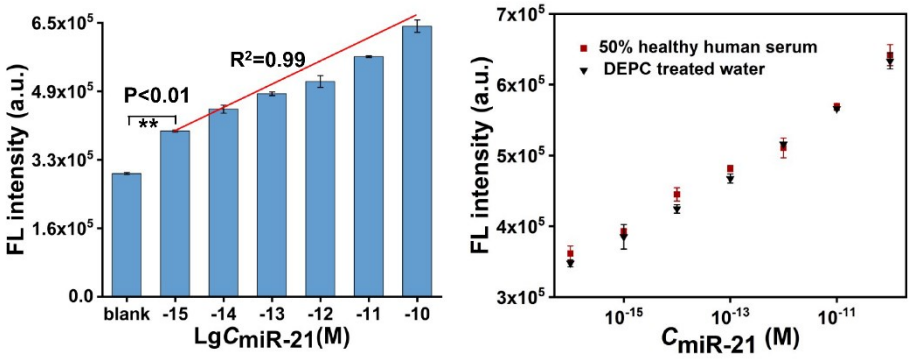

Fig. S18 (a) Fluorescence signals of CRISPR-CHA in response to miR-21 spiked in 50% healthy human serum at different concentrations. Cas12a/gRNA, 100 nM; H1, 50 nM; H2, 50 nM and FQ reporter, 1.0 μM. **(b)** Fluorescence signals of CRISPR-CHA in response to miR-21 spiked in 50% healthy human serum and DEPC treated water at different concentrations. Cas12a/gRNA, 100 nM; H1, 50 nM; H2, 50 nM; and FQ reporter, 1.0 μM. The data are presented as mean ± s.d. of three replicate measurements (**$P < 0.01$, Student's t-test).

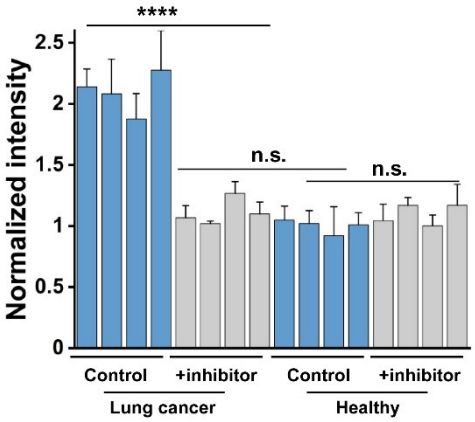

Fig. S19 Normalized intensity of CRISPR-CHA in response to microRNA extracted from healthy serum and lung cancer serum samples. The fluorescence signals are normalized to that of the average intensity of healthy group. The data are presented as mean ± s.d. of three replicate measurements (****$P < 0.0001$, n.s. not significant, Student's *t*-test).

Reference

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