Electronic Supplementary Information

Cascade CRISPR/Cas enables amplification-free microRNA sensing with fM-sensitivity and single-base-specificity

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S1. Experimental section

1.1. Reagents and instruments

All oligonucleotides, including miRNA, FAM and BHQ1-labeled poly-T reporter (FQTR) and hairpin probes, were purchased from Takara Biotechnology Co. Ltd (CN). The sequences are displayed in **Tables S1**. T7 RNA polymerase, LbCas12a, exonuclease I and NTP mix were purchased from New England Biolabs. RNase-free water, dNTP (deoxyribonucleotides) mix, RNase inhibitor, small RNA extraction kit, qPCR mix, and DNase I (RNase-free) were purchased from Takara. RNA purification kit and mini plasmid kit were bought from Tiangen Biotech Co., Ltd. (CN). A CFX Connect 96 real-time PCR instrument (Bio-Rad) was used for the real-time fluorescence monitoring.

1.2. Preparation of Cas/gRNA complexes.

The pET-Sumo-LbuCas13a plasmid was acquired as a generous gift from Prof. Yanli Wang (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China), and the LbuCas13a was expressed and purified as described in our previous work.[1] The pLBH531_MBP-Cas14a1 expression vectors were purchased from Addgene, and Cas14a was purified as previously description.[2]

The dsDNA template of crRNA for Cas13a and Cas12a was prepared by annealing two complementary ssDNA in 1×PCR buffer from 95ºC to 25ºC. The DNA template of Cas14a-sgRNA was obtained by annealing Cas14a-sgR-F with Cas14a-sgR-R and filling in the single-stranded regions with DNA polymerase. The *in vitro* transcription reactions were performed in 1×T7 RNA polymerase reaction buffer, 2 mM each NTP, 5 U μL^{-1} T7 RNA polymerase, 1 U μL^{-1} RNase inhibitor and 200 nM DNA template at 37°C overnight. The DNA templates were digested by DNase I. The transcription products were purified by RNA clean kit, quantified by Nanodrop 2000, and frozen at -80°C for later use.

1.3. Fluorescent assay of casCRISPR.

NUPACK and IDT oligoanalyzer 3.1 were used to analyze and design the hairpin structure. The hairpin denatured at 65°C for 5 min in 1×PCR buffer, and gradually cooled to room temperature (2°C min^{-1}). For casCRISPR v1 (Cas13a-Cas14a), 10 nM Cas13a, 2.5 nM 13a-crR, 40 nM ST-HP and target miRNA were mixed in 1×PCR buffer and incubated at 37°C for 40 min. Then, 10% (v/v) of the reaction products were mixed with 60 nM Cas14a, 30 nM 14a-sgRNA, 500 nM FQTR, and 1×T7 RNA polymerase reaction buffer with a total volume of 10 μL. For casCRISPR v2, the step 1 (the ST-HP was cleaved by Cas13a/crR) was the same with casCRISPR v1. In the step 2, 10% (v/v) of the reaction products were mixed with 60 nM Cas12a, 30 nM 12a-sgRNA, 500 nM FQTR, and 1×NEBuffer 3.1 with a total volume of 10 μL. The fluorescence signal of both casCRISPR v1 and v2 were monitored by a CFX Connect 96 real-time PCR instrument (Bio-Rad) at 37°C for 60 min with 1-minute intervals. According to the experimental results, the real-time fluorescence curve can reach a plateau in 60 minutes. Thus, the integral area of the fluorescence curve in 60-minute intervals was used to quantify the target miRNA concentration. The whole analytical time of the casCRISPR is about 100 minutes. According to the experimental results, the real-time fluorescence curve can reach a plateau in 60 minutes. Therefore, the integral area of the fluorescence in 60-minute intervals was used to quantify the miRNA concentration.

1.4. Detection of miRNA in cell extracts and human serum.

All cell lines used in this article were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human tumor cell lines (MDA-MB-231, HepG2) and human normal liver cell line (LO2) were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Life). The miRNA was extracted with Serum miRNA Extraction and Separation Kit (Tiangen) from 400 μL serum samples. The obtained products were stored at -80°C for further use. Human normal umbilical cord

serum was vortexed and incubated at room temperature for 15 min, and heated by following three processes: (1) 90°C for 2 min. (2) 55°C for 20 min. (3) 65°C for 5 min. And then the serum samples were diluted 20-times with RNase-free water.

The serum samples from volunteers used in this study were obtained from the First Affiliated Hospital of Guangzhou Medical University. The standard venous blood collection processes did not cause harm to the volunteers. The volunteers have known the research propose before taking blood. Conclusively, this research conforms to the national and international medical experiment ethics standards. The experiments were approved by the Research Ethics Committee of the Institute of Biophotonics, South China Normal University.

S2. General design experience of crRNA

The crRNA is composed of two functional domains, the Cas13a binding region and the targeting region. In principle, by changing the target region of crRNA, Cas13a/crRNA can be targeted to any RNA. Generally speaking, the design of crRNA needs to be considered from several aspects: (i) The sequence of the Cas13a binding region is related to the kinds of Cas protein. Cas13a from different genus matches different binding region of crRNA.8 (ii) The length of the targeting region should not be less than 20 nt to guarantee efficient *trans*-cleavage; (iii) Mismatches between crRNA and target RNA can affect Cas13a *trans*-cleavage activity in a number and position-dependent manner, especially within the seed region (9~14 nt); (iv) Introducing a mismatch into Cas13a-crR can improve the fidelity of Cas13a; (v) The complex high-level structure that may decrease the available of target region should be avoided.

S3. Purity and activity analysis of Cas14a

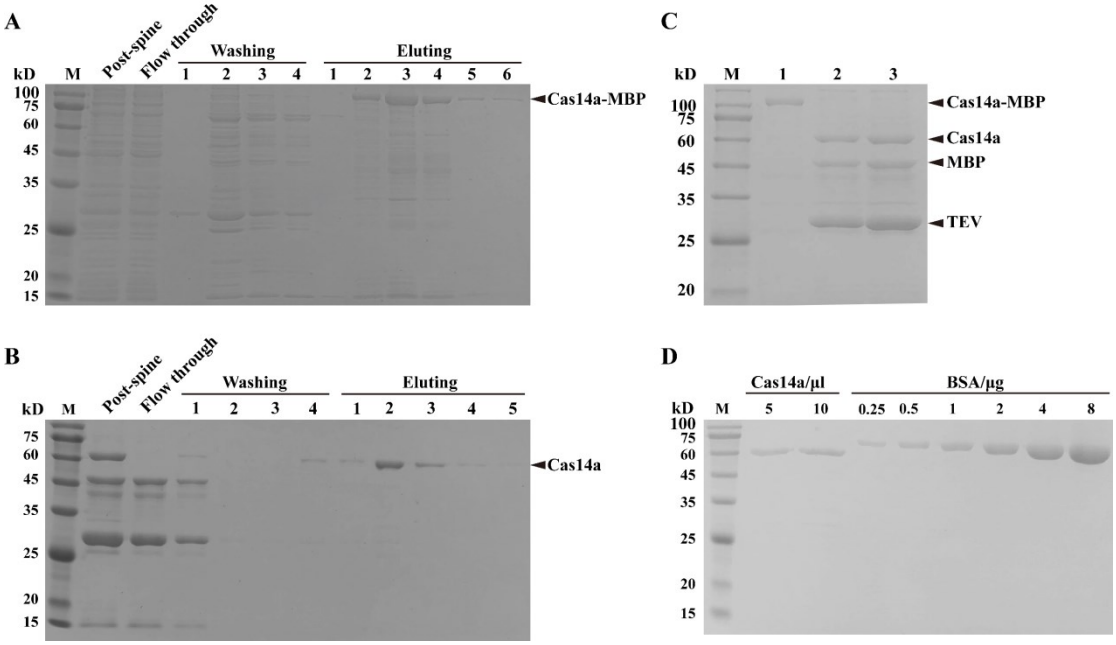
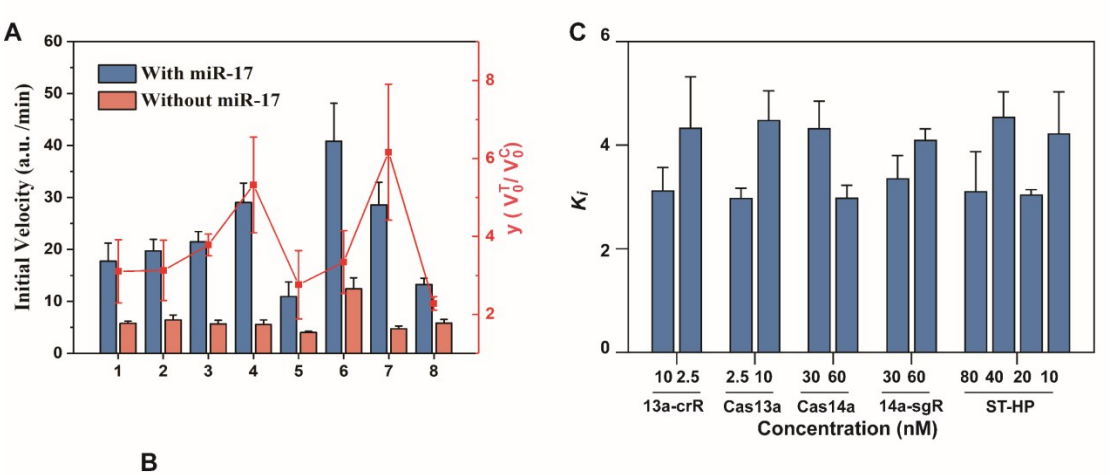


Figure S1. Purity and activity analysis of Cas14a. **(A)** SDS-PAGE analysis of Cas14a-MBP with a Ni-NTA column. MBP: maltose binding protein **(B)** SDS-PAGE analysis of Cas14a with a heparin column. **(C)** SDS-PAGE analysis of purified Cas14a. The MBP tag was digested by TEV enzyme overnight. TEV enzyme: Tobacco Etch Virus Protease. The molecular weight of Cas14a with MBP tag was 106.9 kD, and reduced to 66.6 kD after digesting by TEV enzyme. **(D)** 1.2 μM concentration of Cas14a was quantified by BSA-method.

S4. Optimization of the reaction conditions and detailed calculation of K_i



B

Sample	13a-crR (nM)	Cas13a (nM)	Cas14a (nM)	14a-sgR (nM)	ST-HP (nM)	Result $\bar{y}$ (V_T/ V_C)
1	10	2.5	30	30	80	3.11
2	2.5	10	60	60	80	3.13
3	10	2.5	60	60	40	3.78
4	2.5	10	30	30	40	5.32
5	2.5	2.5	60	30	20	2.76
6	10	10	30	60	20	3.34
7	2.5	10	30	60	10	6.16
8	10	2.5	60	30	10	2.28

Figure S2. Optimization of the reaction conditions of casCRISPR for miRNA detection. (A) The detection results of casCRISPR under different reaction conditions. V_0^T&V_0^C represent the initial velocity of fluorescence growth of the test samples and the control samples, respectively. The concentrations of miR-17 and FQTR for this experiment were 50 pM and 500 nM, respectively. (B) The scheme and the corresponding y value of the orthogonal experiment. (C) Analysis results of the influence of the concentration of the casCRISPR components for miR-17 detection.

The calculation of K_i

K_i is defined as the mean value of the evaluation indexes of each level in each factor,[3-4] and can be calculated by the following formula:

$$K_i = \frac{1}{N_i}\sum_{j=1}^{N_i} y_{i,j}$$

Wherein, N_i represents the number of samples with a certain level in factor i, $y_{i,j}$ is the

evaluation index. In this experiment, the effect of five variables (A: 13a-crR; B: Cas13a; C: 14a-sgR; D: Cas14a; E: ST-HP) on the detection performance were evaluated (**Fig. S3**).

Taken the factor of concentration of 13a-crR as example, there are four samples with 10 nM 13a-crR, thus N_i is 4, $y_{i,j}$ is the value of V_0^T/V_0^C that obtained by analyzing the real-time fluorescence detection results (Fig. R3-A). The calculation of K_i of 13a-crR concentration is shown as follows:

$K_{A,1}$ = $K_{13a\text{-}crR,\ 10\ nM}$ = (Plus the y values of the four samples with 10 nM 13a-crR)/4 = (y1+y3+y6+y8)/4 = (3.11+3.78+3.34+2.28)/4 = 3.1275

$K_{A,2}$ = $K_{13a\text{-}crR,\ 2.5\ nM}$ = (y2+y4 +y5+y7)/4 = (3.15+5.32+2.76+6.16)/4 = 4.3425

Thus, $K_{13a\text{-}crR,\ 10\ nM} < K_{13a\text{-}crR,\ 2.5\ nM}$. Accordingly, 2.5 nM was considered as optimal concentration of 13a-crR.

S5. Michaelis-Menten analysis of *trans*-cleavage activity of Cas14a

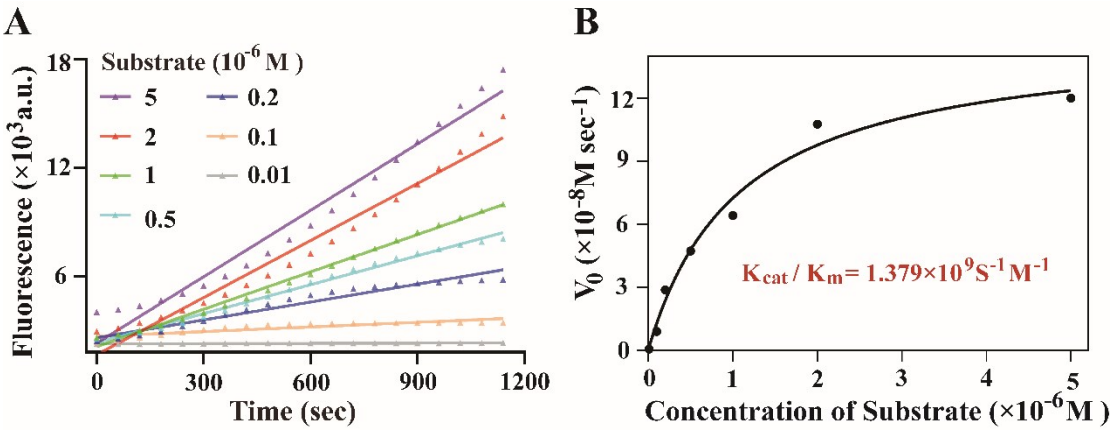


Fig. S3 Michaelis-Menten analysis of trans-cleavage activity of Cas14a. (A) Real-time fluorescence monitoring results of casCRISPR with different concentrations of FQTR reporter (0.01 nM~5 μM). 10 nM 14T-2 was used to activate the *trans*-cleavage of Cas14a/sgRNA. The concentration of the effective Cas14a/sgRNA complex was 30 nM. (B) Michaelis-Menten fit of *trans*-cleavage activity of Cas14a.

S6. The admixture experiment with perfectly matched and mismatched miRNAs

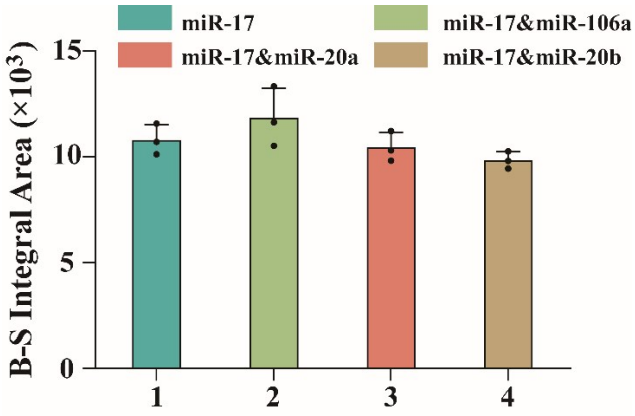
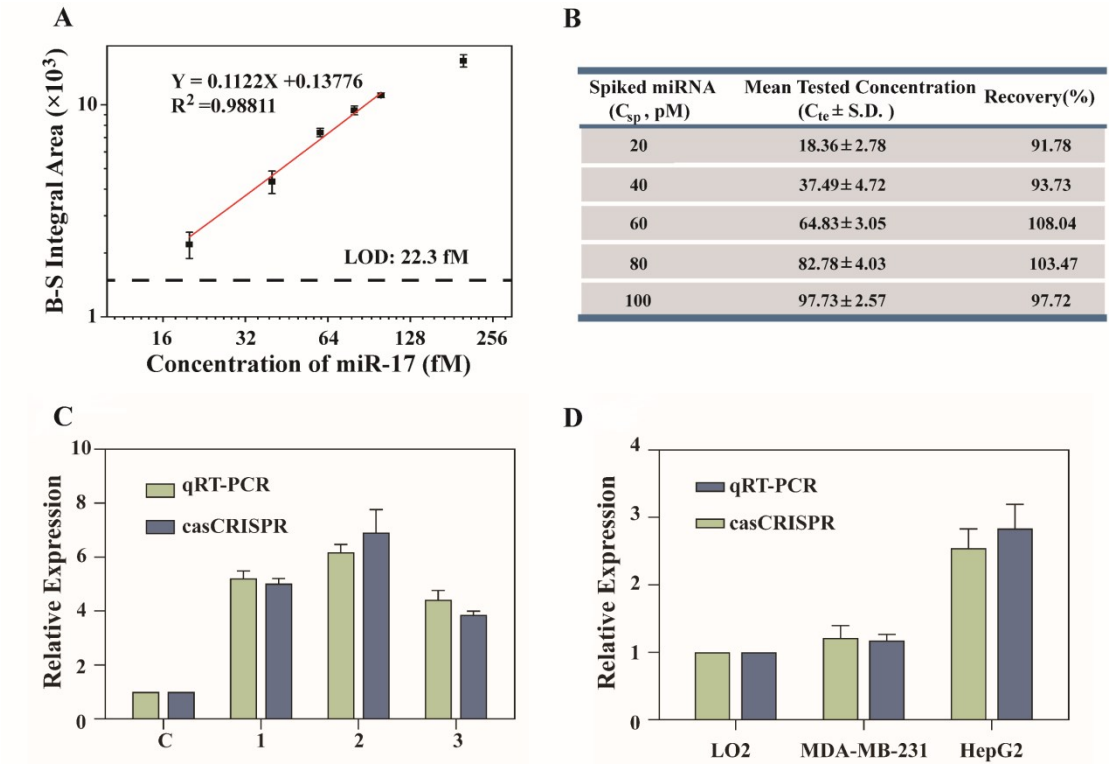


Figure S4. Analyzing the performance of casCRISPR with 13a-crR-M for detecting miR-17 in the admixture samples containing different homologous miRNA (miR-106a, miR-20a and miR20b). The concentration of all the miRNA was 100 fM.

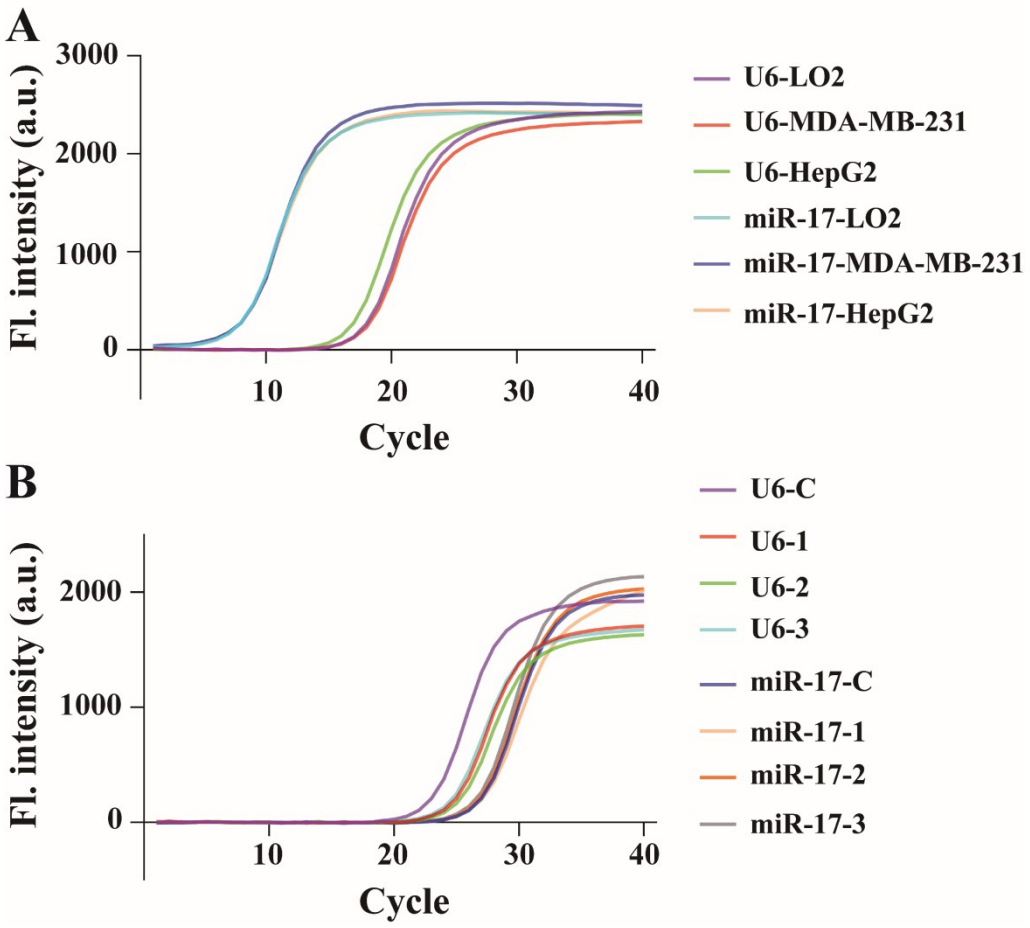
S7. Evaluation the application ability of casCRISPR for the detection of miR-17 in cell extracts and human serum samples



Spiked miRNA (C_{sp}, pM)	Mean Tested Concentration (C_{te} ± S.D.)	Recovery(%)
20	18.36 ± 2.78	91.78
40	37.49 ± 4.72	93.73
60	64.83 ± 3.05	108.04
80	82.78 ± 4.03	103.47
100	97.73 ± 2.57	97.72

Figure. S5 Evaluation the application ability of casCRISPR for the detection of miR-17 in cell extracts and human serum samples. (A) The linear analysis results of casCRSIPR for various concentrations of target miR-17 in diluted serum. (B) The recovery of casCRISPR for detecting miR-17 in serum. C_{te} ± S.D. represents the average of the net fluorescence signal (background-subtracted signal) from three batches of experiment. (C) Comparison of the detection results of casCRISPR and qRT-PCR for the relative expression of miR-17 in serum extract. The serum sample marked C is from healthy person and other 3 tissue samples marked 1-3 is from 3 different breast adenocarcinoma patients. (D) Comparison of casCRISPR and qRT-PCR for miR-17 quantification in cell extracts (300 ng).

S8. Quantitative real-time PCR experiment



图表 1

Fig. S6. Quantitative real-time PCR experiment. The real-time reverse transcription PCR for miRNA17 and U6 small nuclear RNA detection in various cell lines. U6 was employed as the universal endogenous control and the relative expression was calculated by the equation: Fold change=$2^{-\Delta\Delta Ct}$. (A) qRT-PCR experiment of different cell lines. (B) qRT-PCR experiment of different serum extract samples.

S9. Table S1. Sequences used in this study §

Name	Sequence (5'-3')
miR-17	CAAAGUGCUUACAGUGCAGGUAG
miR-10b	UACCCUGUAGAACCGAAUUUGUG
miR-21	UAGCUUAUCAGACUGAUGUUGA

miR-155	UUAAUGCUAAUCGUGAUAGGGGU
miR-106a	AAAAGUGCUUACAGUGCAGGUAG
miR-20a	UAAAGUGCUUAUAGUGCAGGUAG
miR-20b	CAAAGUGCUCAUAGUGCAGGUAG
LbuCas13a-crRNA-17	GACCACCCCAAAAAUGAAGGGGACUAAAACCCUGCACUGUAAGCAC UUUG
LbuCas13a-crRNA-17-M	GACCACCCCAAAAAUGAAGGGGACUAAAACCCUGCACUGUAAGCAG UUUG
LbuCas13a-crR-17-F	***GAAATTAATACGACTCACTATAGGG***ACCACCCCAAAAATGAAGGGGAC TAAAACCTACCTGCACTGTAAGCACTTTG
LbuCas13a-crR-17-R	CAAAGTGCTTACAGTGCAGGTAGGTTTTAGTCCCCTTCATTTTTGGGGT GGTCCCTATAGTGAGTCGTATTAATTTC
LbuCas13a-crR-17-M-F	***GAAATTAATACGACTCACTATAGGG***ACCACCCCAAAAATGAAGGGGAC TAAAACCTACCTGCACTGTAAGCAGTTTG
LbuCas13a-crR-17-M-R	CAAACTGCTTACAGTGCAGGTAGGTTTTAGTCCCCTTCATTTTTGGGGT GGTCCCTATAGTGAGTCGTATTAATTTC
Cas14a-sgRNA-S	CUUCACUGAUAAAGUGGAGAACCGCUUCACCAAAAGCUGUCCCUUA GGGGAUUAGAACUUGAGUGAAGGUGGGCUGCUUGCAUCAGCCUAAU GUCGAGAAGUGCUUUCUUCGGAAAGUAACCCUCGAAACAAAUUCAU UUGGAAUGCAACUACCUUACACCGCUUGCGAA
Cas14a-sgR-S-F	***GAAATTAATACGACTCACTATAGGG***TTCACTGATAAAGTGGAGAACCG CTTCACCAAAAGCTGTCCCTTAGGGGATTAGAACTTGAGTGAAGGTGG GCTGCTTGCATCAGCCTAA
Cas14a-sgR-S-R	TTCGCAAGCGGTGTAAGGTAGTTGCATTCCTTCATTCTTTCAAATGAAT TTGTTTCGAGGGTTACTTTCCGAAGAAAGCACTTCTCGACATTAGGCT GATGCAAGCAGCCCACCT
14T-1	TTTATATGTTTCTCCTGGAGATAACGCAATCGTGACAACTTTCGCAAG CGGTGTAAGGTAGCAGGCTTCCGAATTCCGCGTTTTTACGGC
Cas14a-sgR	CUUCACUGAUAAAGUGGAGAACCGCUUCACCAAAAGCUGUCCCUUA GGGGAUUAGAACUUGAGUGAAGGUGGGCUGCUUGCAUCAGCCUAAU GUCGAGAAGUGCUUUCUUCGGAAAGUAACCCUCGAAACAAAUUCAU UUGGAAUGCAACUCAACUGGGAGAAUGUAACU
Cas14a-sgR-F	***GAAATTAATACGACTCACTATAGGG***TTCACTGATAAAGTGGAGAACCG CTTCACCAAAAGCTGTCCCTTAGGGGATTAGAACTTGAGTGAAGGTGG GCTGCTTGCATCAGCCTAA
Cas14a-sgR-R	AGTTACATTCTCCCAGTTGAGTTGCATTCCTTCATTCTTTCAAATGAAT TTGTTTCGAGGGTTACTTTCCGAAGAAAGCACTTCTCGACATTAGGCT GATGCAAGCAGCCCACCT
14T-2	TTTTTCATAGTTACATTCTCCCAGTTGATCTGCTTTTTT
N-14T-1	CATGCGACTGGAACAGACTCTTTGTTTCCTATACACCTACTACCTGAT
FQTR	FAM-TTTTTTTTTTT-BHQ1
FQ5U	FAM-UUUUU-BHQ1
14-S	CCGGCAAGCTGCCCGTGCCCAAAAGCACCGACTCGGTGCCACTTTTTC AAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAA

	AAC
LbCas12a-crRNA	UAAUUUCUACUACUAAGUGUAGAUUCAACUGGGAGAAUGUAACU
LbCas12a-crR-F	***GAAATTAATACGACTCACTATAGGG***TAATTTCTACTAAGTGTAGATTCA ACT GGG AGA ATG TAA CT
LbCas12a-crR-R	AGTTACATTCTCCCAGTTGAATCTACACTTAGTAGAAATTACCCTATA GTGAGTCGTATTAATTTC
HP-SB	CGTTCAACTGUUGGAGUUAATGTCCAGTTACATTCTCCCAGTTGAACG
HP1	CGTAGTTACATTCTCCCAGTTGAUUCTGGGAGAATGTAACTACG
HP2	GGCGTAGTTACATTCTCCCAGTTGAUUCTGGGAGAATGTAACTACGCC
HP3	CGTGGCGTAGTTACATTCTCCCAGTTGAUUCTGGGAGAATGTAACTAC GCCACG
HP4	CAACGTGGCGTAGTTACATTCTCCCAGTTGAUUCTGGGAGAATGTAAC TACGCCACGTTG
HP5	CGCAACGTGGCGTAGTTACATTCTCCCAGTTGAUUCTGGGAGAATGTA ACTACGCCACGTTGCG
U6-F	TTGGAACGATACAGAGAAGATT
U6-R	GGAACGCTTCACGAATTTG
miR-17-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAA CA
miR-17-F	GCCCGCTAGCTTATCAGACTGATG
miR-17-R	GTGCAGGGTCCGAGGT

[§]The targeting regions of the Cas13a-crR/Cas14a-sgR are marked in blue and green, respectively; The mismatch in Cas13a-crR is marked in purple; The T7 promoter is marked in red bold italics; The complementary regions of the hairpin probe are underlined.

S10. Table S2. The C_t value of stem-loop RT-PCR for different cell lines

Note	Ct_{miR-17}	Ct_{U6}	-ΔΔCt	Fold change
LO2	9.10	19.05	-	1
MDA-MB-231	9.13	19.25	0.18	1.13
HepG2	9.17	17.98	1.32	2.49

Table S3. The C_t value of stem-loop RT-PCR for different serum extracts

Note	Ct_{miR-17}	Ct_{U6}	-ΔΔCt	Fold change
C	28.14	24.06	-	1
Sample 1	27.81	26.15	2.42	5.35
Sample 2	28.02	26.54	2.61	6.10
Sample 3	27.77	25.88	2.19	4.56

S11. Table S4. Comparisons of casCRISPR and qRT-PCR for miRNA detection.

	casCRISPR	qRT-PCR
Reverse transcription	No	Yes
Amplification	No	PCR
Enzyme	Cas13a & Cas14a	Reverse Transcriptase, DNA polymerase
Temperature	Isothermal (37°C)	thermal cycle(95°C→65°C→72°C)
Specificity	High	Medium
Limit of detection	1.33 fM	~ aM
Time	~1.5 h	~3.5 h
Cost	>1 $ /per sample	~0.3 $ /per sample

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