Electronic Supporting Information

FnCas12a/crRNA Assisted Dumbbell-PCR Detection of IsomiRs with Terminal and Inner Sequence Variants

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Experimental section

Cell culture and small RNA isolation. Breast cancer cell line MCF-7 was purchased from the Typical Culture Preservation Commission Cell Bank, Chinese Academy of Sciences (Shanghai, China). Doxorubicin-resistant breast cancer cell line MCF-7R was purchased from Shanghai Aiyan Biological Technology Co. Ltd. (Shanghai, China). MCF-7 and MCF-7R cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), and 1% penicillin/streptomycin (Corning). Small RNA from all cell lines was extracted using EasyPure miRNA Kit (TransGen Biotech) according to the manufacturer's protocol, and was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher).

FnCas12a purification. 800 mL of Terrific Broth growth media with 100 mg/mL ampicillin were inoculated with 8 mL of Rosetta (DE3) pLyseScells (Millipore) containing the FnCas12a expression plasmid (a gift from Prof. Feng Zhang, Addgene plasmid #90094). Until OD600 reached 0.6~0.8, a final concentration of 500 mM IPTG was added, and cells were cooled to 18 °C for protein expression for 16 h. Cells were subsequently collected by centrifugation and ruptured by sonication in 40 mL of Lysis Buffer (50 mM Tris-HCl (pH 7.5-8.0), 500mM NaCl, 10 mM imidazole, 0.5mM PMSF, 1mM DTT, 5%(v/v) glycerol and 0.5 mg/ml lysozyme). Lysate was cleared by centrifugation. The supernatant was filtered through 0.22 micron filters, and mixed with 5 mL of Ni-NTA beads (ThermoFisher) and softly shaken for 1 h at 4 °C before being loaded onto a 30-mL column. The packing was then washed with wash buffer (lysis buffer supplemented with 25 mM imidazole) and eluted with elution buffer (lysis buffer supplemented with 250 mM imidazole). Afterward, the protein was purified with cation exchange chromatography. Finally, the protein was collected and diluted to a final concentration of 10 μM and mixed with an equal volume of 100% cold glycerol prior to being frozen at –80 °C. The purified protein was verified by Coomassie blue stained acrylamide gel (Fig S8).

crRNA preparation. The sequences of crRNAs are shown in Supplementary Table S2. The transcription templates were prepared through annealing of the synthesized oligonucleotides with T7-F (Table S2). crRNAs were synthesized using the HiScribe™T7 Quick High Yield RNA Synthesis Kit (NEB) and the reaction was performed at 37 °C overnight (about 16 h). RNA was purified using the RNA Clean & ConcentratorTM-5 (Zymo Research) and quantified with NanoDrop 2000 (ThermoFisher Scientific).

Cas-Db-PCR to quantify small RNA variants. The sequences of adaptors and primers are shown in Supplementary Table S1.

（1）Adaptors ligation

The sequences of adapters and primers used for Cas-Db-PCR are shown in Supplementary Table S2. To detect miRNAs by Cas-Db-PCR, two stem-loop adaptors with protruding end complementary to the terminal sequences of target miRNAs were designed. The adapters (1 μM) was hybridized to synthetic miRNAs or 1 ng of cellular small RNAs in a 5 μL reaction mixture containing 10 mM $MgCl_2$ (ThermoFisher) at 95 ℃ for 5 min, 37 ℃ for 30 min. To ligate the annealed adaptors to miRNA, 5 μL of the 1× reaction buffer containing 1 U of Rnl2 (New England Biolabs) was added to the mixture, and the entire mixture (10 μL) was incubated at 37 ℃ for 1 h. Afterward, 1 U Antarctic Thermolabile UDG (NEB), 10 U Endonuclease IV (NEB) and 1× NEBuffer 3 were added and incubation at 37 ℃ for 30 min.

（2）RT-PCR

For one-step RT-PCR, the ligated RNA (2 μL) was mixed with primers (0.5 μM each) and Luna® Universal Probe One-Step RT-qPCR Kit (NEB), the reaction mixture (20 μL) was incubated at 55 ℃ for 15 min, 95 ℃ for 1 min, followed by 45 cycles of 95 ℃ for 10 s, 60 ℃ for 30 s and 72 ℃ for 10 s.

（3）FnCas12a detection

FnCas12a detection was performed at 37 ℃ in Tris Buffer (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 9.0) for 3 h, employing 250 nM Cas12a, 500 nM synthesized crRNA, 10 μL of PCR reaction mixture, 250 nM collateral ssDNA (FAM-TTATT-BHQ1) in a 100 μL volume. Fluorescence emission was excited at 485 nm and detected at 530 nm using microplate reader (BioTek).

（4）Adaptors ligation optimization

For adaptors ligation optimization, 1 nM target let-7a was used for adaptors ligation and RT-PCR according to above described. Afterward, the PCR products were analyzed by native PAGE stained with GelRed (Biotium).

（5）Optimization of FnCas12a detection conditions

For FnCas12a reaction pH condition optimization, 1 nM target let-7a was used for adaptors ligation and RT-PCR according to above described. Afterward, Tris buffer (50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$) with different pH was used for optimizing the FnCas12a reaction pH condition.

For crRNA type optimization, 1 nM target miRNAs were used for adaptors ligation and RT-PCR according to above described. Afterward, crRNAs with different length or binding site were used for optimizing the crRNA type.

Real time RT-PCR to quantify miRNA variants. The specificity of the real-time PCR method with Evagreen (Biotium) was tested by Luna® Universal Probe One-Step RT-qPCR Kit (NEB) supplemented with 1× Evagreen and 2 μL ligation products (1 pM target let-7a for ligation) as the template. The assay was performed with the Real-Time PCR System (StepOne, ThermoFisher).

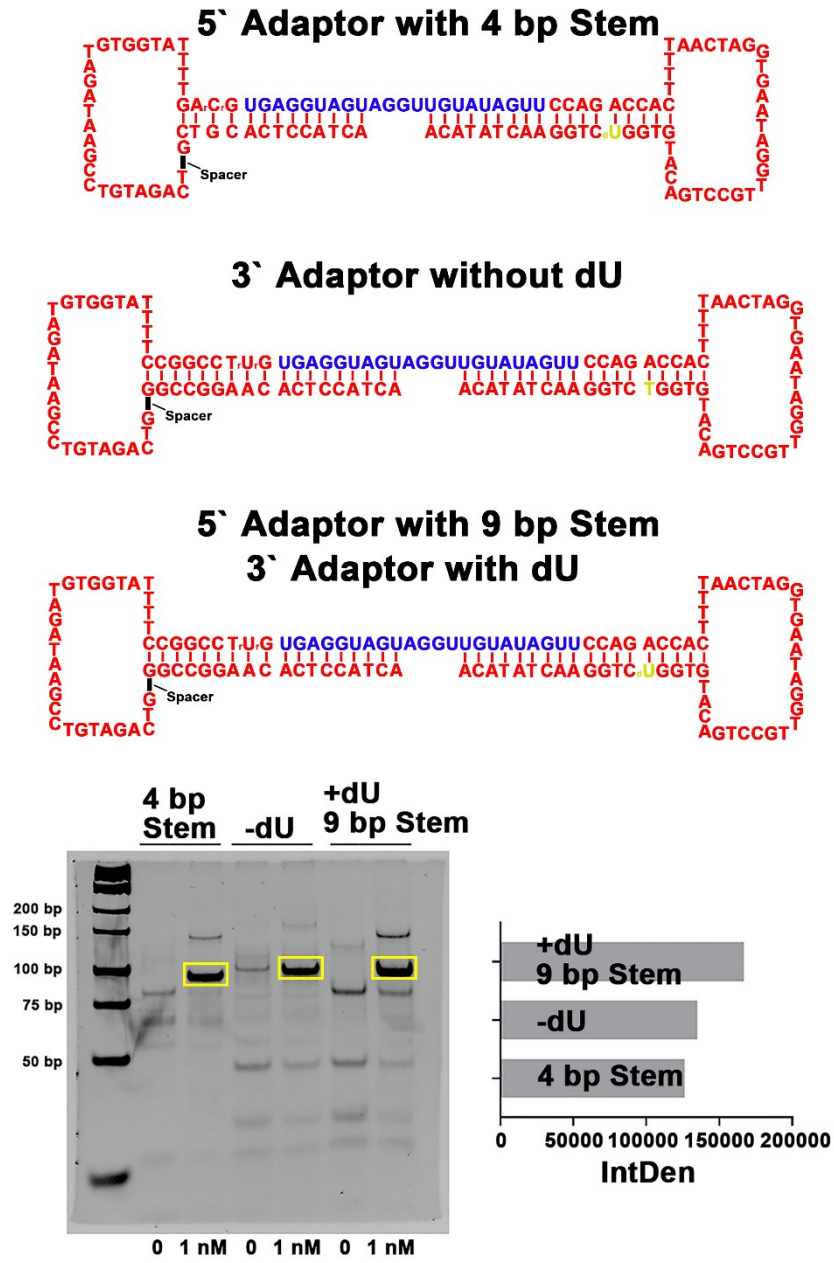

Figure S1. Design of different adaptors and native PAGE analysis of RT-PCR amplified products after different adaptors ligation. Target bands are marked by boxes. 0 or 1 nM Let-7a was used for ligation reaction. Lanes 4 bp Stem: 1 μM 5' Adaptor with 4 bp stem and 1 μM 3' Adaptor with base dU were used for ligation reaction; Lanes -dU: 1 μM 5' Adaptor with 9 bp stem and 1 μM 3' Adaptor without base dU were used for ligation reaction; Lanes +dU 9 bp Stem: 1 μM 5' Adaptor with 9 bp stem and 1 μM 3' Adaptor with base dU were used for ligation reaction. 5' Adaptor-4bp has 4 base pair; 5' Adaptor has 9 base pair; 3' Adaptor-dU has 9 base pair without dU base; 3' Adaptor has 9 base pair with dU base. With imageJ analysis, adaptors with 9 base pair and a dU base in 3' Adaptor produced highest gray scale value.

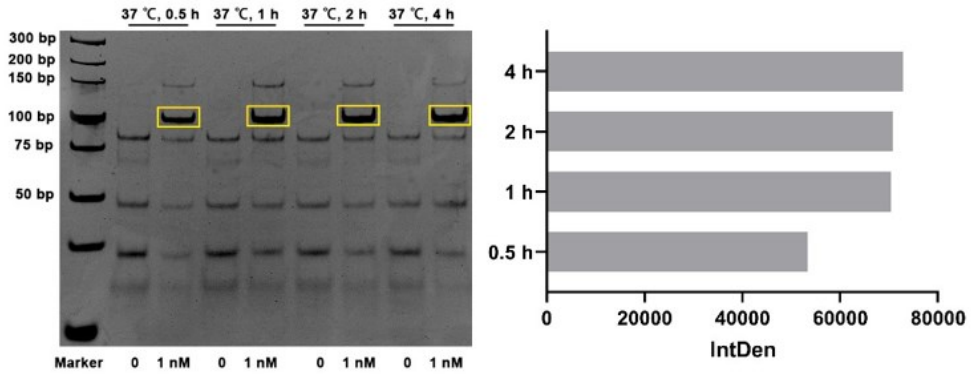

Figure S2. Adaptors ligation condition optimization. Adaptors ligation time were optimized using let-7a as target. Target bands are marked by boxes. 0 or 1 nM Let-7a was used for ligation reaction. With ImageJ analysis, ligation at 37℃ for 1 h were enough for analysis.

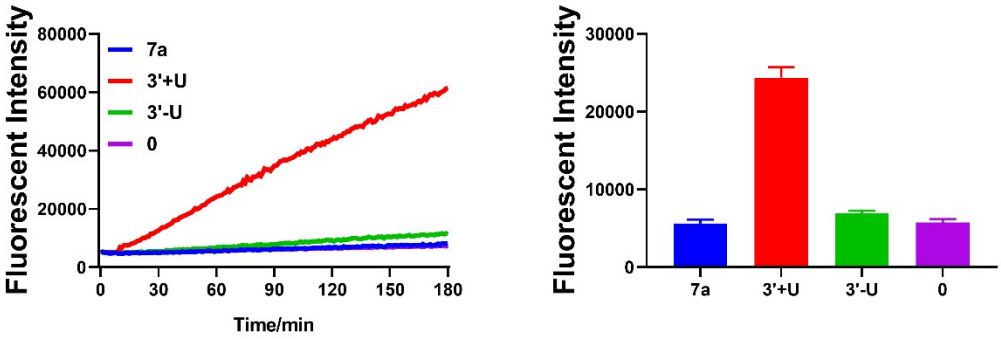

Figure S3. Cas-Db-PCR distinguished target let-7a 3' + U from similar variants. Each miRNA was 1 pM. Error bars were means and SDs from three independent repeats.

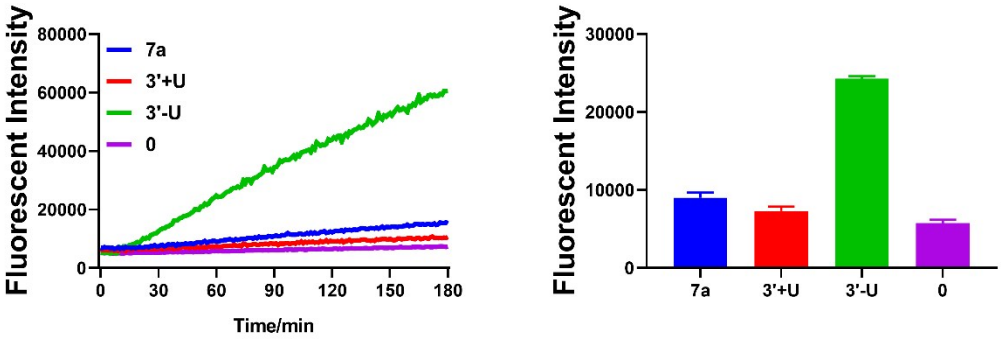

Figure S4. Cas-Db-PCR distinguished target let-7a 3' - U from similar variants. Each miRNA was 1 pM. Error bars were means and SDs from three independent repeats.

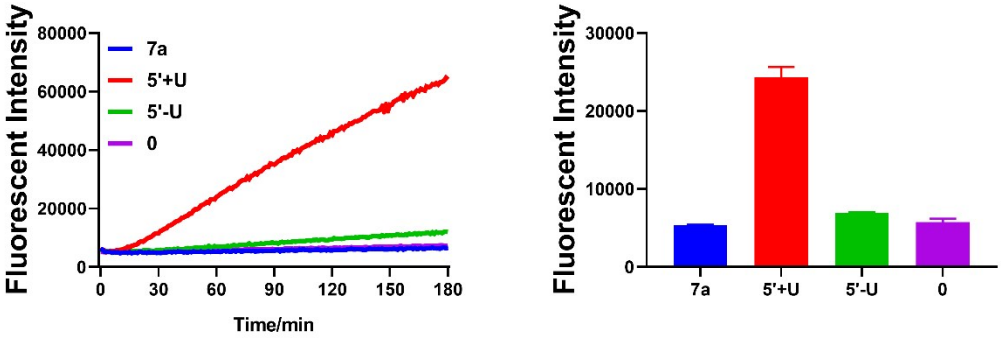

Figure S5. Cas-Db-PCR distinguished target let-7a 5' + U from similar variants. Each miRNA was 1 pM. Error bars were means and SDs from three independent repeats.

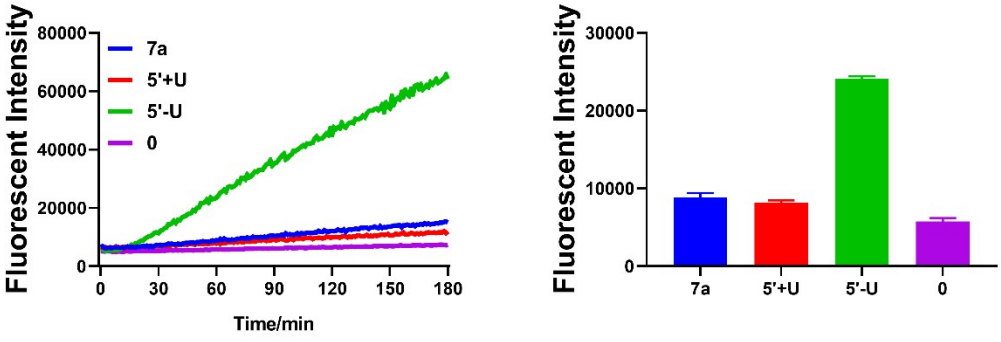

Figure S6. Cas-Db-PCR distinguished target let-7a 5' - U from similar variants. Each miRNA was 1 pM. Error bars were means and SDs from three independent repeats.

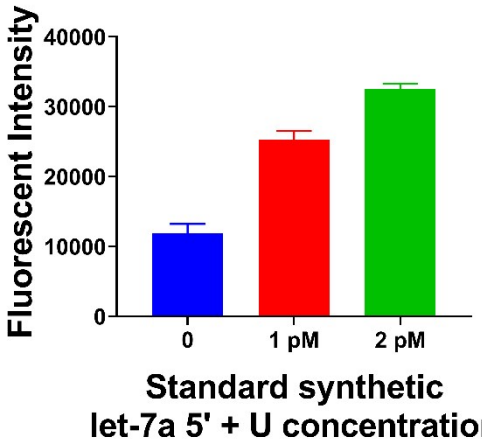

Figure S7. Cas-Db-PCR detected let-7a 5' + U in MCF-7 small RNA (1 ng) with 70 (1 pM) and 140 fg (2 pM) standard synthetic additions. Error bars were means and SDs from three independent repeats.

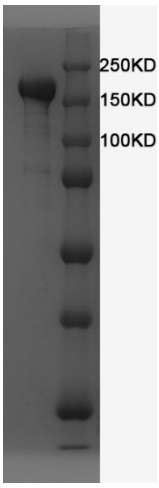

Figure S8. Coomassie blue stained acrylamide gel of purified FnCas12a.

Table S1. Sequences of let-7a and its variants.

Name	Sequence
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
Let-7c	UGAGGUAGUAGGUUGUAUGGUU
Let-7f	UGAGGUAGUAGAUUGUAUAGUU
Let-7a 5' + U	UUGAGGUAGUAGGUUGUAUAGUU
Let-7a 5' - U	GAGGUAGUAGGUUGUAUAGUU
Let-7a 3' + U	UGAGGUAGUAGGUUGUAUAGUUU
Let-7a 3' - U	UGAGGUAGUAGGUUGUAUAGU
Let-7a 5' + U / 3' - U	UUGAGGUAGUAGGUUGUAUAGU
Let-7a 5' - U / 3' +	GAGGUAGUAGGUUGUAUAGUUU

U	

Table S2. Sequences used for crRNAs transcription.

Name	Sequence
T7-F	TAATACGACTCACTATAGG
7a-5' 16	CAACCTACTACCTCACatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
7a-5' 17	ACAACCTACTACCTCACatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
7a-5' 18	TACAACCTACTACCTCACatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
7a-3' 16	GTAGGTTGTATAGTTCatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
7a-3' 17	AGTAGGTTGTATAGTTCatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
7a-3' 18	TAGTAGGTTGTATAGTTCatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
7f-3' 17	AGTAGATTGTATAGTTCatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
7f-5' 17	ACAATCTACTACCTCACatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
(3' + U)- 3' 17	GTAGGTTGTATAGTTTCatctacaacagtagaaattattCCTATAGTGAGTCGTATTA
(3' - U)- 3' 17	TAGTAGGTTGTATAGTCatctacaacagtagaaattattCCTATAGTGAGTCGTATTA

Table S3. Sequences of adaptors and primers.

5' Adaptor	actacctcacaaggccgg/dSpacer/gtcagatgtccgaatagatgtggtattttccggcct/rUrG/
5' + U Adaptor	actacctcaacaaggccgg/dSpacer/gtcagatgtccgaatagatgtggtattttccggcct/rUrG/
5' - U Adaptor	actacctccaaggccgg/dSpacer/gtcagatgtccgaatagatgtggtattttccggcct/rUrG/
5' Adaptor-4bp	actacctcaCGtcg/dSpacer/tcagatgtccgaatagatgtggtattttga/rC//rG/
3' Adaptor	PO_4-ccagaccacttttaactaggtgaataggttgcc/dU/gacatgtggtctggaactataca
3' + U Adaptor	PO_4-ccagaccacttttaactaggtgaataggttgcc/dU/gacatgtggtctggaaactataca
3' - U Adaptor	PO_4-ccagaccacttttaactaggtgaataggttgcc/dU/gacatgtggtctggactataca
3' Adaptor-dU	PO_4-ccagaccacttttaactaggtgaataggttgcctgacatgtggtctggaactataca
Reverse primer	tgtcaggcaacctattcacctagtt
Forward primer	gtcagatgtccgaatagatgtggta