Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Extraction-Free, One-Pot CRISPR/Cas12a Detection of MicroRNAs Directly from Extracellular Vesicles

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EXPERIMENTAL METHODS

Materials. RNA and DNA oligos were purchased from Integrated DNA Technologies (IDT), SplintR buffer, dNTP, BSA, Phi29 polymerase, SplintR ligase, T4 DNA ligase, and Cas12a were purchased from NEB (New England Biolabs Inc). SeraMir exosome RNA column purification kit (RA808A-1) was purchased from SBI (System Biosciences). miRCURY LNA™ RT kit, miRCURY LNA™ SYBR Green PCR kit, and miRNA specific primers were purchased from Qiagen.

Procedure of EXTRA-CRISPR assay. To prepare the RNP, a mixture of 2 μL of water, 0.5 μL of 2.1 buffer (NEB), 1 μL of Cas12a (1 μM), and 1.5 μL of crRNA (1 μM) was incubated at 37°C for 30 min. In a 20-μL reaction, padlock (2 μL) and miRNA (2 μL) in buffer (0.4 μL) were first heated to 80°C for 5 min, then cooled to room temperature in about 1 minute. This mixture was then added to the reaction system containing other components (15.6 μL in total), including 0.8 μL of dNTP, 0.2 μL of BSA, phi29 polymerase, SplintR ligase, RNP, reporter DNA, 1.6 μL of 10× SplintR buffer, and water. After thorough mixing, the assay was conducted in a qPCR device (Bio-Rad, CFX connect real-time system) at 37°C for a specific duration, with fluorescence intensity monitored every 1 minute.

RT-qPCR detection of miRNAs. The reverse transcription (RT) reaction, with a total volume of 10 μL, comprised of 2 μL of 5× miRCURY RT reaction buffer, 1 μL of 10× miRCURY RT enzyme mix, 1 μL of total RNA including miRNA, and 6 μL of water. The RT system was incubated at 42°C for 60 min, followed by inactivation at 95°C for 5 min. Subsequently, the cDNA solution was diluted 60-fold before being introduced into the qPCR system, which consisted of 5 μL of 2× miRCURY SYBR green master mix, 1 μL of the PCR primer, 3 μL of cDNA template, and 1 μL of water. The temperature program involved an initial step of 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 56°C for 60 seconds.

Cell culture. The cell culture procedures and collection of EV-containing conditioned SFM (serum-free medium) were performed following previously described methods.[1, 2] Briefly, immortalized PDAC cell lines (MIA-PaCa-2 and PANC-1), primary patient xenograft-isolated PDAC cell lines (PC1), and primary normal human pancreatic fibroblast cell lines (HPPF) were cultured in DMEM-F12 supplemented with 10% FBS and 1% antibiotic antimycotic solution. When the cells reached approximately 85% confluence, the culture medium was replaced with DMEM-SFM. After 48 hours, the conditioned SFM was collected and stored at -80°C until further analysis.

EV isolation from cell culture medium. All centrifugation steps were carried out at 4°C with the following protocol: 300 g for 10 min to remove cells, 2,000 g for 20 min to remove dead cells and cell debris, 10,000 g for 30 min to remove larger EV particles such as microvesicles, and 100,000 g for 2 h to collect the sEV pellet. The sEVs were then washed and resuspended in 100 μL of PBS for subsequent NTA analysis, thermolysis, and RNA extraction.

NTA analysis. Particle number and size distribution of EVs were determined by nanoparticle tracking analysis (NTA) using a ZetaView system (Particle Metrix Inc.) Samples were diluted in PBS to an acceptable concentration, according to the manufacturer's recommendations.

Detection of sEV miRNAs by thermolysis. sEVs isolated by UC were heated using a PCR device. After lysis, 2 μL of lysate was mixed with the padlock for hybridization, followed by the EXTRA-CRISPR assay.

Supplementary figures and tables

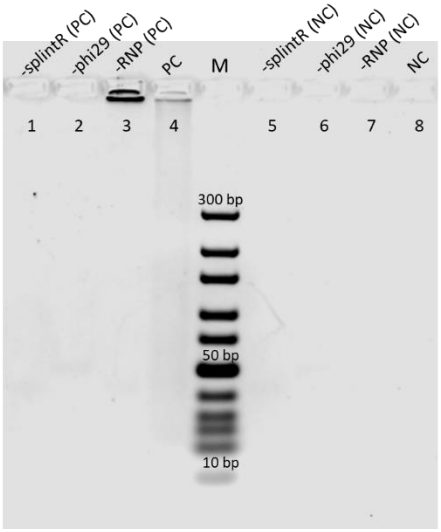

Figure S1. Gel electrophoresis of EXTRA-CRISPR assay. miR-21 target concentration in this experiment was 1 nM. Negative reactions (no miR-21 added, lane 5-8) and reactions lacking the addition of splintR ligase (lane 1) or phi29 polymerase (lane 2) exhibited no detectable RCA products. However, a distinct and clear band near the sample inlet was observed in the reaction without RNP (lane 3), signifying the successful occurrence of the RCA reaction. In the presence of RNP (lane 4), long DNA amplicons were transformed into a smeared band, serving as evidence for the trans- and cis-cleavage of the RCA products by Cas12a RNP.

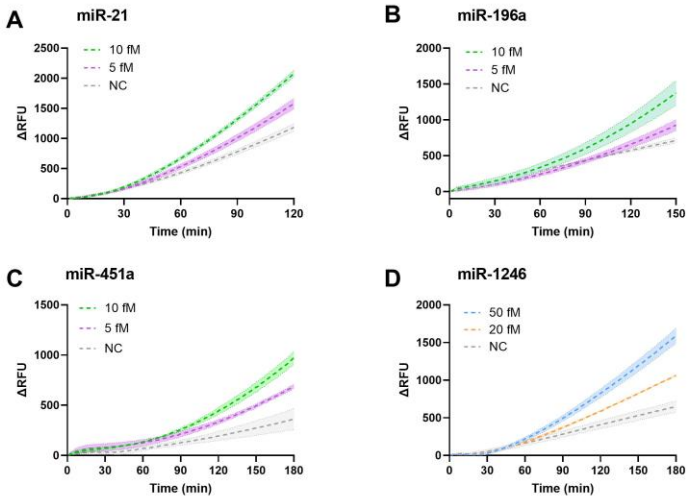

Figure S2. Real-time curves of EXTRA-CRISPR assay at low concentrations. Real-time curves of EXTRA-CRISPR for miR-21 **(A)**, miR-196a **(B)**, miR-451a **(C)**, and miR-1246 **(D)** at low concentrations with shaded bands indicating one S.D. (n = 5 for miR-21, n = 3 for miR-196a, miR-451a, and miR-1246). RFU is the relative fluorescence units, and ΔRFU is the signal subtracted by that of 0 min.

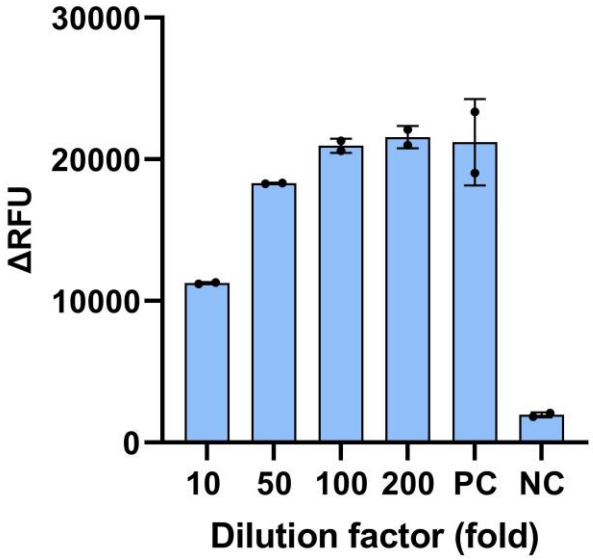

Figure S3. Dilution factor of sEVs from human plasma. MiR-21 (100 fM, final concentration in the EXTRA-CRISPR assay) was spiked into the diluted sEV suspension. PC is the positive control of 100 fM miR-21, and NC is the negative control. Error bar: one S.D. (n = 2).

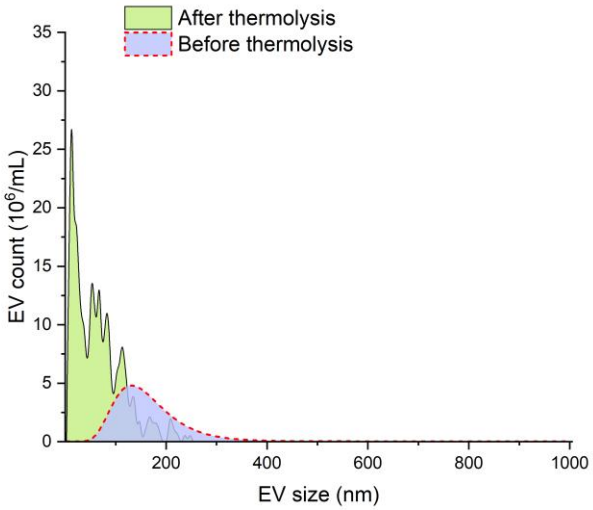

Figure S4. NTA characterization of EVs before and after thermolysis. Original EVs which have a size peak around 120 nm appear to be largely disrupted into smaller particles (highest size peak < 20 nm) by thermolysis.

Table S1. Nucleic acid sequences

DNA Sequences	
Padlock-miR-21	5'p-CTGATAAGCTAAGATACCCTAACCATCGATCGTCGCCGTCCAGCTCGACCTCAACATCAGT
Padlock-miR-196a	5'p-GAAACTACCTAAGATACCCTAACCATCGATCGTCGCCGTCCAGCTCGACCCCCAACAACAT
Padlock-miR-451a	5'p-GGTAACGGTTTAGATACCCTAACCATCGATCGTCGCCGTCCAGCTCGACCAACTCAGTAAT
Padlock-miR-1246	5'p-AAATCCATTAGATACCCTAACCATCGATCGTCGCCGTCCAGCTCGACCCCTGCTCCAA
Reporter	5-FAM-TTATT-IABkFQ-3'
RNA sequences	
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-196a	UAGGUAGUUUCAUGUUGUUGGG
miR-451a	AAACCGUUACCAUUACUGAGUU
miR-1246	AAUGGAUUUUUGGAGCAGG
crRNA	UAAUUUCUACUAAGUGUAGAUCGUCGCCGUCCAGCUCGACC

Table S2. Summary of the recipes for EXTRA-CRISPR detection of four miRNAs.

Components (μL)	**miR-21**	**miR-196**	**miR451a**	**miR-1246**
Buffer (10×)	2 μL	2 μL	2 μL	2 μL
dNTP	400 nM	400 nM	400 nM	400 nM
BSA	0.2 mg/mL	0.2 mg/mL	0.2 mg/mL	0.2 mg/mL
Padlock	100 nM	100 nM	10 nM	10 nM
miRNA	2 μL	2 μL	2 μL	2 μL
Phi29	0.1 U/μL	0.1 U/μL	0.05 U/μL	0.05 U/μL
SplintR	0.625 U/μL	1.25 U/μL	1.25 U/μL	1.25 U/μL
RNP	1 nM	1 nM	1 nM	1 nM
Reporter	1 μM	2 μM	4 μM	1 μM
Water	Adjust water volume accordingly (total reaction volume: 20 μL).			

1. S. Han, D. Delitto, D. Zhang, H. L. Sorenson, G. A. Sarosi, R. M. Thomas, K. E. Behrns, S. M. Wallet, J. G. Trevino and S. J. Hughes, *Lab. Invest.*, 2015, **95**, 1331-1340.
2. S. Han, Z. Huo, K. Nguyen, F. Zhu, P. W. Underwood, K. B. G. Basso, T. J. George and S. J. Hughes, *Proteomics*, 2019, **19**, e1800394.