Supplementary Information

An ultrasensitive hybridization chain reaction-amplified CRISPR-Cas12a aptasensor for extracellular vesicle surface protein quantification

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Method section

Expression and purification of FnCas12a proteins

Escherichia coli Rosetta 2 (DE3) cells containing expression plasmids were used to express the FnCas12a protein according to a previous study [1]. IPTG was added to induce expression, and cells were incubated at 37 °C for 4 h. The harvested bacterial cells were centrifuged, and the precipitate was
obtained and then resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5; 1 M NaCl; 20 mM imidazole; and 10% (v/v) glycerol), lysed by sonication and purified twice using an Abiotech nickel column and an Abiotech heparin column (Jinan, China). Finally, the FnCas12a protein was eluted with elution buffer containing 20 mM Tris-HCl (pH 7.5), 200 mM NaCl and 10% (v/v) glycerol. The protein was stored at -80 °C until use.

**Effects on trans activity when nicks existed in the sequence**

To investigate whether nicks in the sequence affect the trans activity, we used the DNA Ligation High Ver. 2 reagent (TOYOBO, Shanghai, China) to ligase HCR products. Briefly, DNA fragments were mixed with ½ volume of Ligation High Ver. 2 at 16 °C for 30 min. The probes and products were then stored at 4 °C for further use. Next, HCR products with or without nicks cleaved by the Cas12a-crRNA duplex over the indicated timepoints were analyzed, and the FI was also determined using the same protocol mentioned for HCR-CRISPR detection.

**Western blotting analysis and immunofluorescence staining**

The characteristic proteins of the EVs were analyzed by Western blotting as previously reported [2]. The dilution factors of the antibodies/aptamers were as follows: CD9 (1:1,000), CD81 (1:1,000), CD63 (1:1,000), nucleolin aptamer (500nM), and PD-L1 aptamer (500nM).

Immunofluorescence staining was also conducted as reported previously [2]. Briefly, the EVs were captured from serum by a cocktail of anti-CD9/CD63/CD81 MagBeads (Genscript, Nanjing, China) after incubation overnight at 4 °C. After washing three times, the beads were incubated with biotin-labeled nucleolin or PD-L1 aptamer (100 nM) for 1 h followed by incubation with streptavidin-Cy3 (1:1000, Invitrogen, Carlsbad, USA) for 30 min at room temperature. After 3 washes, the EVs were visualized using a fluorescence microscope (Olympus, TOKYO, Japan) by two independent observers.
Results and Discussion

The effect of gaps between adjacent target sequences in H2s on Cas12a-crRNA2 cleavage events

We chemically synthesized duplicate consecutive H2 (H2-H2) and H2-10 (5'-H2-10-H2-10-3') ssDNA as activators. The results showed that a gap of 10 reduced bases did not affect the activity of Cas12a-crRNA2 (Figure S5), indicating that our gap is suitable to avoid steric hindrance.

Optimization of experimental conditions for the HCR-CRISPR assay

We next optimized the experimental conditions for our assay to obtain an improved signal response. The selection of crRNA was described above. We investigated the chemical environment of the system for improved cleavage activity. An important factor that may affect Cas12a is the concentration of Mg$^{2+}$, which is thought to induce conformational coordination between the Cas12a RuvC domain and DNA by promoting the proximity of the Cas12a RuvC active cleavage site to the DNA [3]. Therefore, we investigated the effect of the Mg$^{2+}$ concentration on cleavage activity. Cleavage activity was observed only in the presence of Mg$^{2+}$ cations (Figure S6A). Increasing the concentration of Mg$^{2+}$ ions up to 10 mM led to an enhanced fluorescence intensity (FI). Thus, an optimized Mg$^{2+}$ concentration of 10 mM was used. Considering that Na cations allow the approximation of the phosphate groups of the DNA scaffold, which contribute to higher compaction of the DNA molecule [4-6] and may prevent the Cas-crRNA target recognition-and-cleavage event, we also evaluated the effect of the Na$^+$ ion concentration on the cleavage activity (Figure S6B). Concentrations of 0, 10, 50, 100, 200 and 300 mM were tested, and an interesting result that increasing the concentration of Na$^+$ ions to greater than 50 mM prevented the cleavage activity was observed, which has not been reported before. The exact mechanism remains to be explored. Similar FIs were observed at 0, 10, and 50 mM Na$^+$ concentrations. Given that a 50 mM Na$^+$ concentration was
supported by previous studies [7, 8], we adopted this concentration for use in the following experiment. The pH condition affects the binding of enzymes and substrates by adjusting the dissociation state [9, 10], and Figure S6C shows the peak FI with pH 8.0 Tris-HCl buffer, which is similar to the previous studies [8, 11]. We also profiled the cleavage activity against the concentrations of crRNA and Cas12a. Using the control variable method, we observed that the concentration-dependent FI peaked at 500 nM crRNA and then stabilized (Figure S6D). Given that crRNA generation is relatively time consuming and expensive, we selected 500 nM crRNA for the present assay. Cas12a endonuclease at a concentration of 250 nM exhibited the most apparent and stable cleavage activity among the other groups (Figure S6E). Intriguingly, the cleavage activity decreased at a high level of Cas12a (500 nM), probably due to the steric hindrance caused by the large size of Cas12a [12]. We also attempted temperature optimization and found that the optimal temperature was 37 °C (Figure S6F), which was similar to the findings of previous studies [13].

The optimal conditions for HCR have been fully discussed, we adopted the following reaction conditions as reported by Dirks and Pierce [14]: 50 mM Na₂HPO₄/0.5 M NaCl (pH 6.8). These optimized experimental conditions were applied in the subsequent experiments.
Figures and Tables

**Figure S1.** SDS-PAGE gel of purified FnCas12a.

**Figure S2.** PAGE image of HCR products targeted by the Cas12a/CrRNA2 duplex. NHCR, nucleolin HCR; PHCR, PD-L1 HCR.
**Figure S3.** HCR products with or without ligation targeted by the Cas12a/CrRNA2 duplex.

(A) Schematic outlining the NHCR products ligated by a ligase to close the nicks.

(B) The observed fluorescence intensity of HCR-CRISPR/Cas12a using NHCR products at a 1/10 or 1/50 dilution as activators. Statistical analyses were performed using a two-tailed Student’s t-test. Error bars represent the mean ± SD, where n = 3.
Figure S4. Repetitive ssDNA activators targeted by the Cas12a/CrRNA2 duplex. The observed fluorescence intensity of CRISPR-Cas12a using 1 nM, 5 nM and 20 nM H2 ssDNA or duplicate consecutive H2 as activators. ssDNA, single-stranded DNA. Statistical analyses were performed using a two-tailed Student’s t-test. Error bars represent the mean ± SD, where n = 3.

Figure S5. The effect of gap between adjacent target sequences in H2s on Cas12a-crRNA2 cleavage events. Statistical analyses were performed using a two-tailed Student's t-test. Error bars represent the mean ± SD, where n = 3.
Figure S6. Optimization of the HCR-CRISPR assay.

Evaluation of the effect on FnCas12a collateral activity after targeting 40 nM H2 in a variety of buffers with different concentrations of Mg$^{2+}$ (A) and Na$^+$ (B) as well as different pH values (C). (D) Representative real-time fluorescence kinetic measurement of FnCas12a collateral activity after targeting 1 μL of 1/10 HCR products of different crRNA2 concentrations (250 nM Cas12a; 50 nM ssDNA reporter) and (E) different Cas12a concentrations (500 nM crRNA2; 50 nM ssDNA reporter).

(F) Temperature-dependent FI using FnCas12a. 

P values were calculated using one-way ANOVA followed by a Sidak multiple-comparisons test with the optimal group. ns, *, **, *** and **** represent $P > 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively.

Error bars represent the mean ± SD, where n = 3.
Figure S7. Sensitivity of the optimized CRISPR-Cas12a assay in detecting dsDNA and ssDNA. (A) Serial dilutions of the dsDNA template (0.01 - 40 nM) detected by the CRISPR-Cas12a assay. Assay time, 30 min. dsDNA, double-stranded DNA. (B) The concentration change in the dsDNA template is linearly related to the fluorescence intensity through fitting the following curve: \( Y = 1492X - 826.4 \) (\( R^2 = 0.9955 \)). (C) Serial dilutions of the ssDNA template (0.5 - 40 nM) detected by the CRISPR-Cas12a assay. Assay time, 30 min. ssDNA, single-stranded DNA. (D) The concentration change in the ssDNA template is linearly related to the FI through fitting the following curve: \( Y = 499.8X + 923.2 \) (\( R^2 = 0.9817 \)).

\( P \) values were calculated using one-way ANOVA followed by a Sidak multiple-comparisons test with the former group. ns, *, **, *** and **** represent \( P > 0.05 \), \( P < 0.05 \), \( P < 0.01 \), \( P < 0.001 \) and \( P < 0.0001 \), respectively.

Error bars represent the mean ± SD, where \( n = 3 \).
Figure S8. EV characteristics. (A) Representative TEM image of the isolated EVs from SUNE2 cells (scale bar: 200 nm). (B) NTA of isolated EVs with a peak of 168 nm and a calculated mean of 230.3 nm. (C) Western blotting demonstrating the presence of the proteins CD9, CD63, TSG101, CD81, nucleolin and PD-L1 in the EVs.
Figure S9. Comparison of the apta-ELISA, apta-HCR-ELISA and apta-HCR-CRISPR assays in detecting PD-L1+ EVs spiked in PBS. (A) Detection of PD-L1+ EVs by apta-ELISA with serial concentrations of SUNE2 EVs spiked in PBS between 64-10^6 particles/μL. (B) Detection of PD-L1+ EVs by apta-HCR-ELISA with serial concentrations of SUNE2 EVs spiked in PBS between 64-10^6 particles/μL. (C) Detection of PD-L1+ EVs by apta-HCR-CRISPR with serial concentrations of SUNE2 EVs spiked in PBS between 64-10^6 particles/μL. (D) The concentration change of PD-L1+ EVs is linearly related to the FI through fitting the following curve: $Y = 7895X - 14376$ ($R^2 = 0.9652$).

PBS served as a blank. $P$ values were calculated using one-way ANOVA followed by a Sidak multiple-comparisons test with the former group. *, **, *** and **** represent $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively.
Error bars represent the mean ± SD, where n = 3.

Figure S10. Comparison of the apta-ELISA, apta-HCR-ELISA and apta-HCR-CRISPR assay in detecting nucleolin+ and PD-L1+ EVs spiked in serum. (A) Detection of nucleolin+ EVs by apta-ELISA with serial concentrations of SUNE2 EVs spiked in 2× diluted serum from 64-10^6 particles/μL. (B) Detection of nucleolin+ EVs by apta-HCR-ELISA with serial concentrations of SUNE2 EVs spiked in 2× diluted serum from 64-10^6 particles/μL. (C) Correlation of the apta-HCR-CRISPR FI with the log EV concentration in detecting nucleolin+ EV spiked in a 2× diluted serum. (D) Detection of PD-L1+ EVs by apta-ELISA with serial concentrations of SUNE2 EVs spiked in 2× diluted serum from 64-10^6 particles/μL. (E) Detection of PD-L1+ EVs by apta-HCR-ELISA with serial concentrations of SUNE2 EVs spiked in 2× diluted serum from 64-10^6 particles/μL. (F) Correlation of the apta-HCR-CRISPR FI with the log EV concentration in detecting PD-L1+ EV spiked in a 2× diluted serum.

2× serum was derived from healthy participants without detectable target protein expression and served as a blank. P values were calculated using
one-way ANOVA followed by a Sidak multiple-comparisons test with the former group. *, **, *** and **** represent $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. Error bars represent the mean ± SD, where $n = 3$. 
| Name           | Sequence (5’-3’)                                                                 |
|----------------|---------------------------------------------------------------------------------|
| Nucleolin H0  | AGTCTAGGATTCGGCGTGGTGGATTATTTTTTTTTGG TGGTGGTGGTTGGTGGTGGTGGTGGTGGTGGTGGTGGG |
| PD-L1 H0      | AGTCTAGGATTCGGCGTGGTGGTTATTTTTTTTTTTTAATTTTTTTTTTTTTAC GCTCGGATGCCACTCACAGGGGCCCACATCACT CATTGATAGACAATCGTCCACTGCCTGGCTCTCATG GACGTGCTGGTGGAC |
| H1            | TTAGGCTGGCGTGGGTTAACACGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| H2            | AGTCTAGGATTGGCGTGGTGGTTATTTTTTTTTTTTTTTACAGGATTGGCGTGGTGGTTAACACGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA-F    | GAAATTAATACGACTCACTATAGGG                                                        |
| T7-crRNA1-R   | TTGGGCTGGGTTAACACGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA2-R   | ATGGGCTGGGTTAACACGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA3-R   | GATGGGCTGGGTTAACACGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA4-R   | GGTAGATCTAGATTCGGGCGTGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA5-R   | TTAACCCACGCACATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA6-R   | AGTCTAGGATTCGGCGTGGTGGTTATTTTTTTTTTTTTTTACAGGATTGGCGTGGTGGTTAACACGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA7-R   | AAGTATGCTAGATTCGGGCGTGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA8-R   | AAAAACTACACGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| T7-crRNA9-R   | TAACCCACGCACGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| NS-T7-crRNA10-R | TTGCTGTATGGTGGGCGTGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| NS-T7-crRNA11-R | TCTGAAATAGTGGTGGGCGTGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| NS-T7-crRNA12-R | TTGCTGTATGGTGGGCGTGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| NS-T7-crRNA13-R | TACCAGTGCGATGCTAGTGGCGCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| H2-R          | CAAAGTATGCTAGATTCGGGCGTGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC |
| Primer          | Sequence                                                                 |
|----------------|--------------------------------------------------------------------------|
| H2-H2-F        | AGTCTAGGATTCGGCGTGGTTAAACACGCCTAAGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCTAGACCTAGACTACTTTG |
| H2-H2-R        | CAAAGTAGTCTAGGATTCGGCGTGGTTAAACACGCCTAAGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCTAGACCTAGACTACTTTG |
| H2-10          | AGTCTAGGATTCGGCGTGGTTAAACACGCCTAAGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCTAGACCTAGACTACTTTG |
| H2-10-H2-10    | AGTCTAGGATTCGGCGTGGTTAAACACGCCTAAGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCTAGACCTAGACTACTTTG |
| Nucleolin-apt-biotin | AGTCTAGGATTCGGCGTGGTTAAACACGCCTAAGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCTAGACCTAGACTACTTTG |
| PD-L1-apt-biotin | AGTCTAGGATTCGGCGTGGTTAAACACGCCTAAGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCTAGACCTAGACTACTTTG |
| Biotin-H1      | Biotin-TACCAGTGCGATGCTCAGTGCCGTATCTACACTTGAGAAATTACCCTATAGTGAGTCGTATTAATTTC-biotin |
| Biotin-H2      | Biotin-TACCAGTGCGATGCTCAGTGCCGTATCTACACTTGAGAAATTACCCTATAGTGAGTCGTATTAATTTC-biotin |
| ssDNA-FQ reporter | HEX-TTATT-BHQ1                                                           |

Yellow highlighted bases indicate 5’ PAM sequences, and the optimal sequence is marked in red; bold sites represent the targeted sequences; NS, non-specific.
Table S2. Information on the NPC serum samples used in Figure 6B/C.

| Patient ID | Group   | Sex    | Age | Pathological stage<sup>a</sup> | EBV-DNA, copy/ml<sup>b</sup> | VCA-IgA<sup>b</sup> | EA-IgA<sup>b</sup> |
|------------|---------|--------|-----|---------------------------------|-------------------------------|---------------------|-------------------|
| 1          | Early   | Male   | 60  | T1N0M0, I                       | 0                            | Missing            | Missing          |
| 2          | Early   | Male   | 34  | T2N1M0, II                      | 0                            | 40                  | 10               |
| 3          | Early   | Male   | 58  | T1N0M0, I                       | 0                            | 0                   | 0                |
| 4          | Early   | Male   | 64  | T2N1M0, II                      | 2480                         | 1280                | 320              |
| 5          | Early   | Female | 42  | T2N1M0, II                      | 0                            | 0                   | 0                |
| 6          | Early   | Male   | 46  | T2N1M0, II                      | 194                          | 80                  | 0                |
| 7          | Early   | Male   | 41  | T1N1aM0, II                     | 0                            | 0                   | 0                |
| 8          | Early   | Female | 26  | T2N0M0, II                      | 189                          | 0                   | 40               |
| 9          | Early   | Male   | 67  | T1N0M0, I                       | 0                            | 0                   | 40               |
| 10         | Early   | Female | 45  | T2N1M0, II                      | 0                            | 160                 | 320              |
| 11         | Advanced| Male   | 50  | T3N2M0, III                     | 11500                         | 320                 | 1280             |
| 12         | Advanced| Male   | 52  | T3N2M0, III                     | 1750                         | 80                  | 320              |
| 13         | Advanced| Male   | 62  | T3N1M0, III                     | 705                          | 160                 | 640              |
| 14         | Advanced| Male   | 57  | T3N2M0, III                     | 585                          | 40                  | 160              |
| 15         | Advanced| Female | 40  | T3N2M0, III                     | 580                          | 0                   | 40               |
| 16         | Advanced| Male   | 53  | T3N1M0, III                     | 530                          | 160                 | 640              |
| 17         | Advanced| Male   | 44  | T3N1M0, III                     | 520                          | 160                 | 40               |
| 18         | Advanced| Male   | 53  | T3N1M0, III                     | 0                            | 160                 | 40               |
| 19         | Advanced| Male   | 50  | T3N1M0, III                     | 940                          | 640                 | 160              |
| 20         | Advanced| Male   | 43  | T3N1M0, III                     | 90                           | 160                 | 20               |

<sup>a</sup> The 8<sup>th</sup> AJCC staging system;

<sup>b</sup> The data were collected from clinical records.
Table S3. Baseline characteristics of patients treated with anti-PD-1 monoclonal antibody.

| Patient ID | Sex | Age | Tumor type | Clinical stage | Endpoint |
|------------|-----|-----|------------|----------------|----------|
| 1          | male | 31  | NPC        | TxNxM1IV       | SD       |
| 2          | female | 66  | lung adenocarcinoma | T2N2M1cIVb | PD       |
| 3          | female | 53  | NPC        | TxNxM1IV       | PR       |
| 4          | male | 39  | NPC        | TxNxM1IV       | PR       |
| 5          | male | 34  | NPC        | T4N1M1IVb      | PR       |
| 6          | male | 66  | lung adenocarcinoma | T4N3M1IV | PD       |
| 7          | male | 53  | NPC        | TxNxM1IV       | SD       |
| 8          | male | 57  | NPC        | TxNxM1IV       | PD       |
| 9          | male | 47  | NPC        | T3N3M0IVb      | PR       |
| 10         | male | 48  | NPC        | TxNxM1IV       | PD       |

* NPC, nasopharyngeal carcinoma; b Endpoint, SD, stable disease; PR, partial response; PD, progressive disease.
Table S4. EV-derived protein detection method comparison

| Detection method | LOD<sup>a</sup> | Dynamic range | Target | Recognition elements | Ref |
|------------------|----------------|--------------|--------|----------------------|-----|
| Thermophoretic aptasensor | 3.3 x10<sup>3</sup> particles/μL | 10<sup>3</sup>-10<sup>7</sup> particles/μL | CD63, PTK7, EpCAM, LZH8, HER2, PSA, CA125, Lib | aptamer | [15] |
| nPLEX | 3,000 counts | 10<sup>3</sup>-10<sup>7</sup> counts | EpCAM, CD24, CA125, MUC18, EGFR, HER2 | antibody | [16] |
| Dual-signal amplification based on RCA and endonuclease | 10<sup>2</sup> particles/μL | 10<sup>3</sup>-10<sup>5</sup> particles/μL | nucleolin | aptamer | [17] |
| ExoProfile | 21 particles/μL | 10-10<sup>6</sup> particles/μL | EGFR, Her2, CA125, EpCAM, CD24, FRα, CD9, CD63 | antibody | [18] |
| ExoPCD-chip | 43.9 particles/μL | 7.61x10<sup>-10</sup>-10<sup>5</sup> particles/μL | CD63 | aptamer | [19] |
| g-C3N4 NSs-CD63 aptamer | 13.52 x10<sup>5</sup> particles/μL | 0.19x10<sup>-5</sup>-3.38x10<sup>7</sup> particles/μL | CD63 | aptamer | [20] |
| α-CD9 antibodies electrode | 2 x10<sup>2</sup> particles/μL | 10<sup>2</sup>-10<sup>6</sup> particles/μL | CD9 | antibody | [21] |
| Electrochemical aptasensor based on a Hemin/G - Quadruplex - Assisted Signal Amplification | 9.54 x 10<sup>2</sup> particles/mL | 4.8 x 10<sup>3</sup> to 4.8 x 10<sup>6</sup> particles/mL | MUC1 | aptamer | [22] |
Table S5. Recovery tests for nucleolin⁺ and PD-L1⁺ EVs in 50% FBS (n = 3)

| Group               | Added EVs (particles/µL) | PBS* | 50% FBS* | Rate of recovery (%) | RSDb (%) |
|---------------------|--------------------------|------|----------|----------------------|----------|
| nucleolin⁺          |                          |      |          |                      |          |
| 1                   | 320                      | 5566 | 4661     | 83.7                 | 9.1      |
| 2                   | 8000                     | 16065| 14951    | 93.1                 | 9.0      |
| 3                   | 10⁶                      | 36996| 35015    | 94.6                 | 4.2      |
| PD-L1⁺              |                          |      |          |                      |          |
| 1                   | 320                      | 4430 | 5121     | 115.6                | 9.0      |
| 2                   | 8000                     | 15340| 14801    | 96.5                 | 7.4      |
| 3                   | 10⁶                      | 37770| 38606    | 102.2                | 2.8      |

a LOD: limit of detection

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