Electronic Supplementary Information

An immunoassay-like recognition mechanism-based lateral flow strategy for rapid microRNA analysis

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List of Contents：

1. Materials and reagents

Nitrocellulose (NC) membrane was purchased from Sartorius (UniSart CN140, Germany). The sample pad (SB65), absorbent pad (SX27), and PVC bottom plate (SMNF31-40) were obtained from Shanghai Kinbio Tech. Co., Ltd (Shanghai, China). S9.6 antibody and goat anti-mouse IgG (IgG) were purchased from abcam (Shanghai, China). $HAuCl_4 \cdot 3H_2O$ (≥99.9%), bovine serum albumin (BSA), streptavidin, 2-naphthalenethiol (2-NT), and 4-nitrothiophenol (4-NTP) were purchased from Sigma-Aldrich (Shanghai, China). Trisodium citrate, Tween-20, sodium borate decahydrate, 20 × PBS solution (pH 7.4), NaBr, NaCl and 20 × PB solution (200 mM, pH 7.0) were obtained from Sangon Biotech (Shanghai, China). The DNA oligonucleotides used in this study were synthesized and purified by Sangon Biotech (Shanghai, China). The RNA sequences were obtained from Takara Biotechnology (Dalian, China). The detailed nucleic acid sequences are listed in Table S1.

The detailed components of the used buffers in this study are listed below:

1 × PBS (10 mM, pH 7.4, containing 137 mM NaCl and 2.7 mM KCl)

1 × PBST (1 × PBS with 0.1% Tween-20)

Table S1. The nucleic acid sequences used in the work

Name	sequence (from 5' to 3')
let-7a	UGAGGUAGUAGGUUGUAUAGUU
miR-21	UAGCUUAUCAGACUGAUGUUGA
ssDNA capture probe for let-7a	SH-TTTTTTTTTTAACTATACAACCTACTACCTCA
ssDNA capture probe for miR-21	SH-TTTTTTTTTTTCAACATCAGTCTGATAAGCTA
control probe	SH-TTTTTTTTTTTTTTT-Biotin
miR-24	UGGCUCAGUUCAGCAGGAACAG
miR-122	UGGAGUGUGACAAUGGUGUUUG
miR-125b	UCCCUGAGACCCUAACUUGUG
miR-143	UUAAUGCUAAUCGUGAUAGGGGU
miR-181a	AACAUUCAACGCUGUCGGUGAGU
ssDNA probe (P1)	SH-TTTTTTTTTTAACTATACAAC
ssDNA1	CTACTACCTCA-Biotin

ssDNA2	Biotin -GTTGTATAGTT

Note: ssDNA probe (P1), ssDNA1 and ssDNA2 were used to build the sandwich-type hybridization-based LFA.

2. Detailed experimental procedures

Preparation of gold nanoparticles (AuNPs). AuNPs were prepared by a citate-reduction method as described literature.[S1] First, all glassware was soaked in chromic acid lotion for at least 6 hours. After washing, 100 mL of 0.01% $HAuCl_4 \cdot 3H_2O$ solution was added into the three-necked round-bottomed flask with a condenser and heated to boiling under constantly magnetic stirring. Once the solution began to reflux, 1 mL of 1% trisodium citrate aqueous solution was quickly added, and continued to be heated until the color of the solution turned red. After refluxing for 15 minutes, the solution was stopped heating but continued stirring to cool down to room temperature. Finally, the prepared AuNPs were treated by a 0.22 μm nitrocellulose membrane filtration and then stored long-term at 4 ℃ for future use. The concentration of AuNPs measured by NTA is 80 pM (ZetaView Nanoparticle tracking analyzer, Particle Matrix).

Functionalization of AuNPs. First, 1 mL of prepared AuNPs were pipetted out and centrifuged at 9000 rpm/min for 10 minutes at 4 ℃ and then dispersed into 200 μL of H_2O. After that, the 200 μL of AuNPs was incubated with 18 μL of SH-labeled ssDNA capture probe (50 μM) and 2 μL of control probe (50 μM) at ambient temperature for 12 hours. Then 80 μL of mixture containing 3 μL of 5% Tween-20, 15 μL of 200 mM PB buffer (pH 7.0) and 62 μL H_2O was added into the above aqueous system and kept at room temperature for 12 hours. Afterward, 100 μL of NaBr solution was slowly added to a final concentration of 150 mM. After 12 hours, 50 μL of 10% BSA aqueous solution was added to the system to block the vacant sites on the surface of AuNPs. Finally, the functionalized AuNPs were dispersed in 200 μL of 1 × PBST with 1% BSA buffer and kept at 4 ℃ for future use.

As to the AuNPs used in the SERS-based analysis, 10 μL of 15 mM 4-NTP or 2-NT solution was gradually added to the above solution with NaBr and incubated for 12 hours, and the subsequent blocking with BSA and storing of functionalized AuNPs are all identical to the procedures mentioned above.

Preparation of test trip. The sample pad, NC membrane and absorbent pad are assembled

as shown in Fig.1 in main text, with a scale of 2 mm × 3 mm by Numerical control cutting machine (CTS300, Shanghai Kinbio Tech.Co.,Ltd). The test and control line are respectively scribed with 1.25 mg/mL IgG and 1 mg/mL streptavidin at 50 mm/s moving speed and 0.8 μL/cm pump discharge by XYZ three-dimensional scribing and gold spraying instrument (HM3035, Shanghai Kinbio Tech.Co.,Ltd).

Standard procedures of the proposed immuno-like recognition mechanism-based LFA for colorimetric and SERS dual-mode sensing of microRNA. Firstly, 30 pM of functionalized AuNPs, 2 μL of 2 M NaCl, 10 μL of 1 × PBST, 1 μL of 0.1 mg/mL of S9.6 antibody and 4 μL of different concentration of miRNA were mixed and incubated for 2 minutes. Subsequently, the above reaction solution was mixed with 12 μL 1 × PBST and 8 μL of 10% BSA to insert into a test strip. Then the concentration of miRNA target was evaluated by the gray scale of test line on the test trip image with Image J.

For the detection of multiple miRNAs based on SERS signal readout, the immuno-like recognition of S9.6 antibody to DNA/miRNA duplexes and the solution diffusion in test trip were conducted as mentioned above. Then, the test trip was dried and monitored with the XploRA PLUS confocal Raman microscope (Horiba, France). In the SERS measurements, 638 nm/3.13 mW excitation laser, 1200 gr/mm optical grating, 1 s of acquisition time, and 10 × lens were selected. Besides, the hole was set as 200 μm and slit was set as 300 μm. All the SERS mapping images, and the averaged Raman spectra were obtained after baseline correction of the original spectra by using the Labspec6 software (Horiba, France).

Standard procedure of the sandwich-type hybridization-based LFA method. The AuNPs were immobilized as mentioned above by using P1 probe to replace the ssDNA capture probe and control probe. As for test strip, the test line is anchored with 1 mg/mL streptavidin, and the control line is immobilized with ssDNA2 by the interaction between 1 mg/mL streptavidin and 200 μM ssDNA2 in 30 μL 1 × PBS buffer for 2 hours. Then 30 pM of functionalized AuNPs was mixed with 50 nM ssDNA1 probe and different concentration of microRNA in 20 μL 1 × PBST buffer for 2 minutes to conduct the sandwich-type hybridization reaction. Subsequently, the reaction solution was mixed with 12 μL of 1 × PBST buffer and 8 μL of 10% BSA and injected to test trip. The subsequent colorimetric sensing of miRNA is identical to the mentioned above.

Procedures of cell culture and total RNA extraction from the cancer cells. The Hela cell lines were purchased from the cell bank of Chinese Academy of Sciences. And they were cultured in Dulbecco's modified Eagle's medium (DMEM, Life). All the media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2. The total RNA sample was extracted from the cells by using of RNAiso for Small RNA Kit (Takara, China) according to the manufacturer's instructions. The amount of extracted total RNA was quantified with a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA).

3. Optimization of the experimental conditions in the immuno-like recognition mechanism-based LFA strategy

To obtain the best assay performance, the amounts of functionalized AuNPs, S9.6 antibody, IgG and the immuno-like recognition reaction time were optimized by using 10 and 100 pM let-7a.

The concentration of functionalized AuNPs has a crucial effect on the immuno-like recognition efficiency. Therefore, the optimization of functionalized AuNPs concentration was firstly conducted. As shown in Fig. S1, both the blank, 10 and 100 pM let-7a-produced signal increases with the functionalized AuNPs ascending from 5 to 45 pM. However, when the functionalized AuNPs is more than 30 pM, the signal of blank increases, causing the strong non-specific background signal. Therefore, 30 pM functionalized AuNPs is used in subsequent experiment.

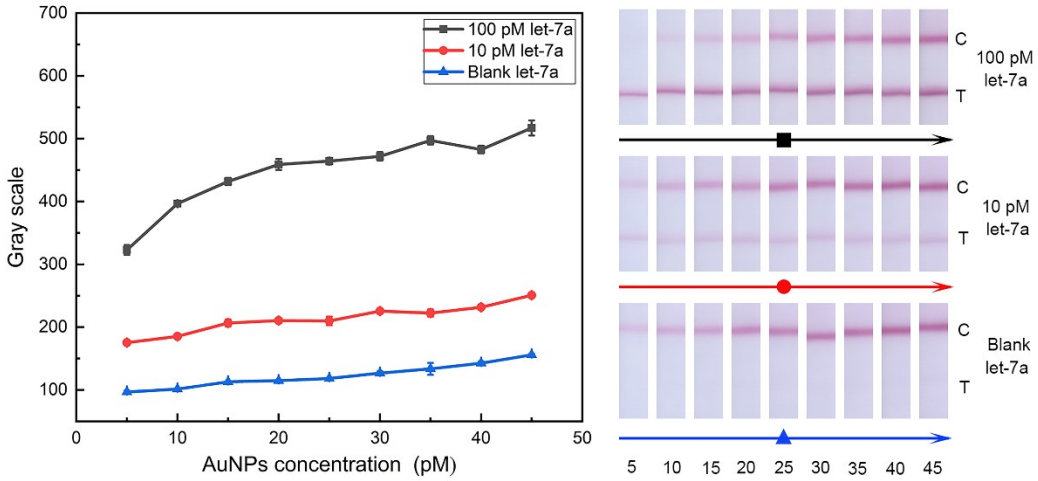

Fig. S1. Optimization of the concentration of functionalized AuNPs in the reaction system (left column) and LFA results of the control line (C) and test line (T) (right column). Other conditions: S9.6 antibody, 0.1 μg; IgG, 1.25 mg/mL; immuno-like recognition time: 2 minutes. The error bars represent the standard deviation of three replicates for each data point.

Under the optimal concentration of functionalized AuNPs, 1 μL of S9.6 antibody from 0 to 0.2 mg/mL was added to test the optimal concentration of S9.6 antibody. It can be seen from Fig. S2, the signal of blank keeps constant with the increase of S9.6 antibody. Meanwhile, both the 10 and 100 pM let-7a-produced signal increases when the concentration of S9.6 antibody ascending from 0 to 0.1 mg/mL and remains almost unchanged when the concentration of S9.6 antibody exceeds 0.1 mg/mL. Therefore, 1 μL 0.1 mg/mL (0.1 μg) of S9.6 antibody was added to obtain the best performance.

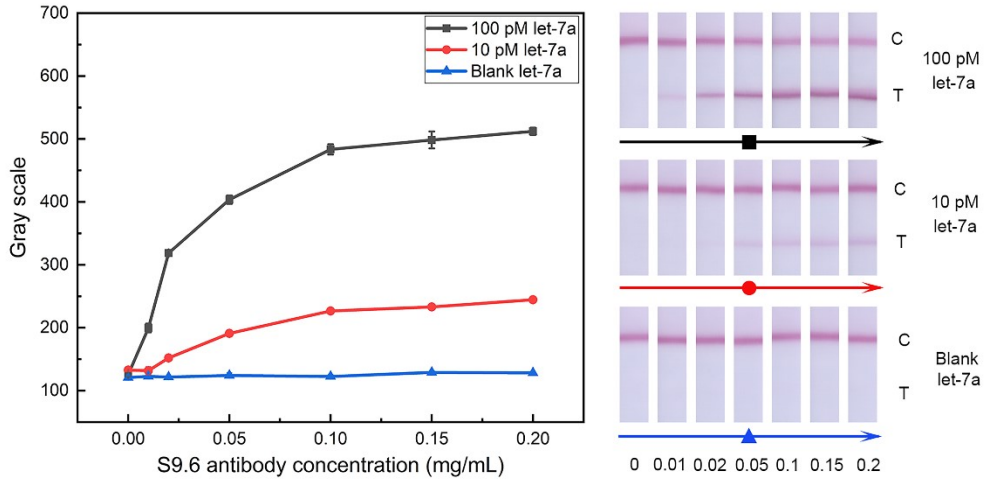

Fig. S2. Optimization of the concentration of S9.6 antibody (left column) and LFA results of the control line (C) and test line (T) (right column). Other conditions: functionalized AuNPs, 30 pM; IgG, 1.25 mg/mL; immuno-like recognition time: 2 minutes. The error bars represent the standard deviation of three replicates for each data point.

The concentration of IgG influences the combination efficiency of immuno-like complexes on the test line. Therefore, the concentration of IgG was also optimized. As shown in Fig. S3, both the 10 and 100 pM let-7a-produced signal increases with increase of IgG and keeps almost constant until 1.25 mg/mL IgG was introduced. Meanwhile, the signal of blank keeps almost unchanged. Therefore, the optimal concentration of the IgG is 1.25 mg/mL.

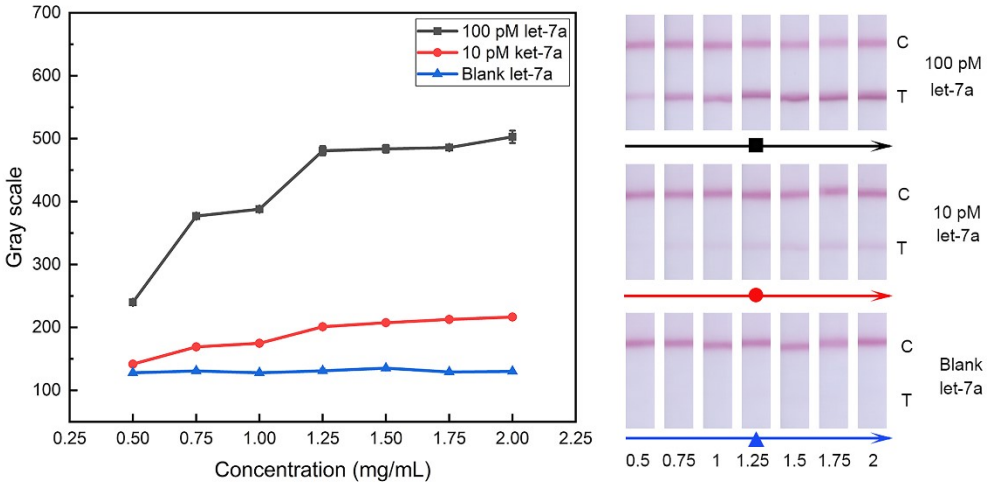

Fig. S3. Optimization of the concentration of IgG (left column) on the LFA results of the control line (C) and test line (T) (right column). Other conditions: functionalized AuNPs, 30 pM; S9.6 antibody, 0.1 μg; immuno-like recognition time: 2 minutes. The error bars represent the standard deviation of three replicates for each data point.

To obtain the best performance, the immuno-like recognition time was optimized. As shown in Fig. S4, with the increase of reaction time (2 to 30 minutes), the signal of blank, 10 and 100 pM let-7a keeps almost unchanged. Therefore, 2 minutes is enough to form stable immuno-like complexes, which also meets the requirements for efficient and rapid miRNA detection in practical application.

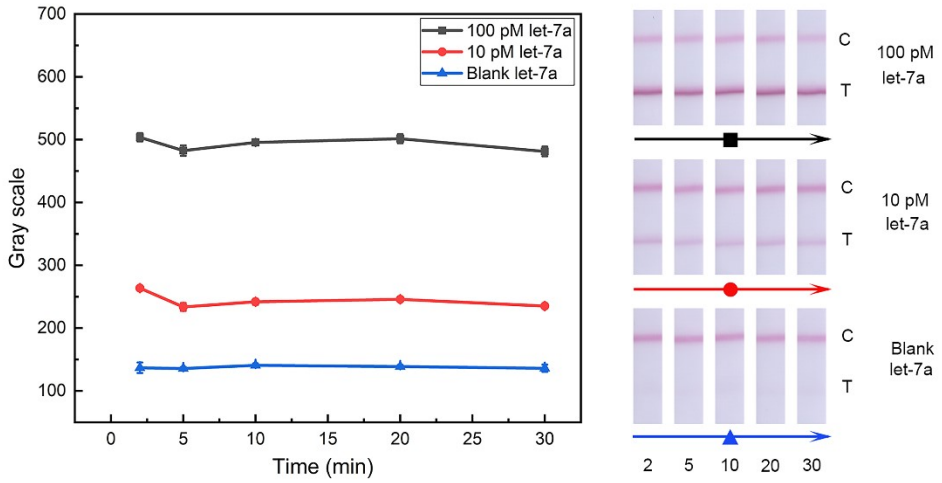

Fig. S4. Optimization of the immuno-like recognition time (left column) and LFA results of the control line (C) and test line (T) (right column). Other conditions: functionalized AuNPs, 30 pM; S9.6 antibody, 0.1 μg; IgG: 1.25 mg/mL. The error bars represent the standard deviation of three replicates for each data point.

4. Comparison of representative LFA-based assays for miRNA analysis under different signal readout modes

Table S2. Comparison of representative LFA-based assays for miRNA analysis under different signal readout modes.

Sensing strategy	***Analytical technique for signal readout***	***Assay time***	***Introduction of nucleic acid signal amplification***	***Detection limit/The lowest detectable concentration***
DNA-gold nanoparticle (DNA-GNP) based lateral flow nucleic acid biosensor	*Colorimetric*[S2]	*20 min*	*No*	*60 pM*
Combining rolling circle amplification with AuNPs-based lateral flow strip (LFS-RCA)	*Colorimetric*[S3]	*Within 3 min*	*Yes*	*20 pM*
Combining multichannel paper chips with a portable gas pressure meter	*Gas pressure*[S4]	*Within 1 h*	*No*	*7.5 pM*
A duplex-specific nuclease (DSN)-assisted	*Fluorescence*[S5]	*2 h*	*Yes*	*0.16 pM*

target recycling signal amplification-based fluorescent lateral flow assay				
A method integrated rolling circle amplification (RCA) with fluorescent microspheres (FM) based lateral flow assay	*Smartphone (fluorescence)*[S6]	*within 3 h*	*Yes*	*27.89 pM*
Synthesized palladium-gold (Pd-Au) bimetallic nanoplates with high photothermal effect combined locked nucleic acid (LNA) detection probe to develop a photothermal lateral flow locked nucleic acid biosensor (P-LFLNAB)	*Smartphone (thermal signal)*[S7]	*20 min*	*No*	*0.094 pM*
A surface-enhanced Raman scattering (SERS)-lateral flow assay (LFA) strip by catalytic hairpin assembly as a signal amplification strategy	*SERS*[S8]	*30 min*	*Yes*	*2.18 pM*
A fluorogenic oligonucleotide-templated reaction carried out directly on paper	*Fluorescence*[S9]	*within 10 min*	*No*	*9.05 nM*
A rapid immunoassay-like recognition-based lateral flow strategy for colorimetric and surface-enhanced Raman scattering dual-mode biosensing	*Colorimetric*	*Within 10 min*	*No*	*1.24 pM (This work)*

5. Quantification of let-7a in the total small RNA by RT-PCR method

The stem-loop reverse-transcription PCR (RT-PCR) protocol is referred to the methods in the literatures with some modifications.[S10] The detailed experimental steps are listed as

follows:

Reverse transcription reaction. The reverse transcription reaction was carried out in the mixture with 1 μL target miRNA (or total small RNA sample), 1.2 μL of RNase-free water, 1 μL of 5 × RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 1 μL of 2.5 mM dNTPs, 0.2 μL of 200 U/μL ProtoScrip II reverse transcriptase, 0.5 μL of 1 μM stem-loop RT-Probe (see detailed sequence in Table S2), and 0.1 μL of 40 U/μL RNase inhibitor. The 5 μL mixture was treated with following conditions: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at 4 °C.

Quantitative real-time PCR analysis. 5 μL transcription product was added into the PCR reaction mixture with a final volume of 10 μL. The PCR reaction mixture consists of 200 nM forward primer and 200 nM reverse primer, 250 μM dNTPs, 0.4 × SYBR Green I, 0.5 U JumpStartTM Taq DNA Polymerase, and 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001(w/v) gelatin, pH 8.3). The 10 μL PCR reaction mixture was incubated in a StepOne Real-Time PCR System (Applied Biosystems, USA) according to the following thermal cycling conditions: hot start at 95 °C for 2 min, followed by 50 cycles of 95 °C for 15 s, and 60 °C for 1 min.

Table S3. Nucleic acid sequences used in stem-loop RT-PCR method

name	sequence (from 5’ to 3’)
stem-loop RT-Probe	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACAACTA
forward primer	GCCGCTGAGGTAGTAGGTTGTA
reverse primer	GTGCAGGGTCCGAGGT

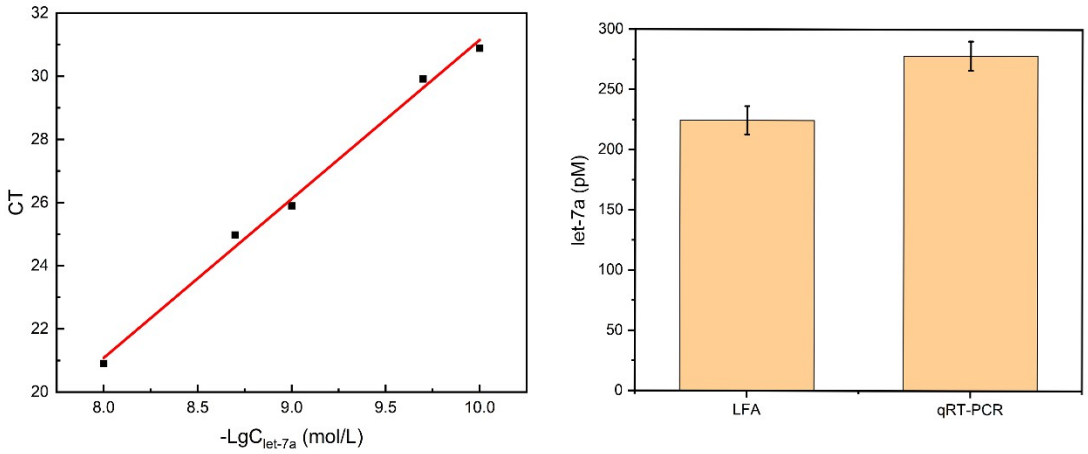

Fig. S5. Standard calibration curve of the stem-loop RT-PCR protocol for the detection of let-7a, and the comparison of two methods for detecting let-7a in the same batch of total small RNA extracted from Hela cells.

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