Electronic Supplementary Material (ESI) for ChemComm.


*Supporting Information*

*For*

# Specific and relative detection of urinary microRNAs signature in bladder cancer for point-of-care diagnostics

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## Supplementary tables

### Table S1 Analysis of exponential amplification system

| Recycle No. | Products Amount |
|---|---|
| **1st** | 1 |
| **2nd** | 2 |
| **3rd** | 4 |
| **4th** | 8 |
| **5th** | 16 |
| . | . |
| . | . |
| . | . |
| **n** | $2^{n-1}$ $(n \geq 2)$ |

**Table S2 Oligonucleotide used in this study**

| Oligonucleotide name* | Sequence (5'-3') | Length |
|---|---|---|
| BSH-EXPAR | | |
| **Hsa-microRNA-126-3p** | UCGUACCGUGAGUAAUAAUGC | 21 |
| **MicroRNA-specific primer of MicroRNA 126** | GCATTATTACTCACGGTACGATGCGA | 26 |
| **Universal primer of microRNA 126** | GGGAACAAACAAGCATTATTACTCACGGTACGAAACAGACTCCAGGTCGCA | 51 |
| **Hsa-miR-182-5p** | UUUGGCAAUGGUAGAACUCACACU | 24 |
| **MicroRNA-specific primer of MicroRNA 182** | AGTGTGAGTTCTACCATTGCCAAAACGCT | 29 |
| **Universal primer of microRNA 182** | GGGAACAAACAAAGTGTGAGTTCTACCATTGCCAAAAACAGACTCCAGGAGCGT | 54 |
| **Hsa-miR-152-5p** | AGGUUCUGUGAUACACUCCGACU | 23 |
| **MicroRNA-specific primer of MicroRNA 152** | TCCAAGACACTATGTGAGGCTGAAGGCA | 28 |
| **Universal primer of microRNA 152** | GGGAACAAACAATCCAAGACACTATGTGAGGCTGAAACAGACTCCAGGTGCCT | 53 |
| TLPB | | |
| **DNA probe modified on AuNPs** | Thio/MC6-D/-GGGAACAAACAA | 12 |
| **PNA probe-T of microRNA 126** | $H_2NCO$-(Gly)-GCATTATTACTCACGGTACGACCCCCC-$NH_2$/Biotin | 27 |

| | | |
|---|---|---|
| **PNA probe-T of microRNA 182** | $H_2NCO$-(Gly)-AGTGTGAGTTCTACCATTGCCAAACCCCCC-$NH_2$/Biotin | 30 |
| **PNA probe-T of microRNA 152** | $H_2NCO$-(Gly)-TCCAAGACACTATGTGAGGCTGACCCCCC-$NH_2$/Biotin | 29 |
| **PNA probe-C of all microRNAs** | Biotin/$NH_2$-(Gly)-CCCCCCTTGTTTGTTCCC-$H_2NCO$ | 18 |
| Selectivity evaluation | | |
| **1M-5' of microRNA 126** | **C**CGUACCGUGAGUAAUAAUGC | 21 |
| **2M-5' of microRNA 126** | **CU**GUACCGUGAGUAAUAAUGC | 21 |
| **3M-5' of microRNA 126** | **CUA**UACCGUGAGUAAUAAUGC | 21 |
| **1M-3' of microRNA 126** | UCGUACCGUGAGUAAUAAUG**A** | 21 |
| **2M-3' of microRNA 126** | UCGUACCGUGAGUAAUAAU**UA** | 21 |
| **3M-3' of microRNA 126** | UCGUACCGUGAGUAAUAA**CUA** | 21 |
| **1M-M of microRNA 126** | UCGUACCGUGA**U**UAAUAAUGC | 21 |
| **2M-M of microRNA 126** | UCGUACCGUG**CU**UAAUAAUGC | 21 |
| **3M-M of microRNA 126** | UCGUACCGUG**CUG**AAUAAUGC | 21 |
| **1M-5' of microRNA 182** | **C**UUGGCAAUGGUAGAACUCACACU | 24 |
| **2M-5' of microRNA 182** | **CG**UGGCAAUGGUAGAACUCACACU | 24 |
| **3M-5' of microRNA 182** | **CGC**GGCAAUGGUAGAACUCACACU | 24 |

| | | |
|---|---|---|
| **1M-3’ of microRNA 182** | UUUGGCAAUGGUAGAACUCACAC**A** | 24 |
| **2M-3’ of microRNA 182** | UUUGGCAAUGGUAGAACUCACA**UA** | 24 |
| **3M-3’ of microRNA 182** | UUUGGCAAUGGUAGAACUCAC**CUA** | 24 |
| **1M-M of microRNA 182** | UUUGGCAAUGG**A**AGAACUCACACU | 24 |
| **2M-M of microRNA 182** | UUUGGCAAUGG**AC**GAACUCACACU | 24 |
| **3M-M of microRNA 182** | UUUGGCAAUG**UAC**GAACUCACACU | 24 |
| **1M-5’ of microRNA 152** | **C**GGUUCUGUGAUACACUCCGACU | 23 |
| **2M-5’ of microRNA 152** | **CA**GUUCUGUGAUACACUCCGACU | 23 |
| **3M-5’ of microRNA 152** | **CAU**UUCUGUGAUACACUCCGACU | 23 |
| **1M-3’ of microRNA 152** | AGGUUCUGUGAUACACUCCGAC**A** | 23 |
| **2M-3’ of microRNA 152** | AGGUUCUGUGAUACACUCCGA**AA** | 23 |
| **3M-3’ of microRNA 152** | AGGUUCUGUGAUACACUCCG**GAA** | 23 |
| **1M-M of microRNA 152** | AGGUUCUGUGA**A**ACACUCCGACU | 23 |
| **2M-M of microRNA 152** | AGGUUCUGUGA**AU**CACUCCGACU | 23 |
| **3M-M of microRNA 152** | AGGUUCUGUG**CAU**CACUCCGACU | 23 |

*All DNA and RNA sequences were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China). All PNA sequences were synthesized and purified by PANAGENE, Inc. (Daejeon, Korea).

**Table S3. Comparison of sensitivities of different lateral flow assays for the detection of microRNA**

| No. | Pre-amplification | Characteristic of lateral flow assays | LOD | Working range | Reference |
|---|---|---|---|---|---|
| 1 | No amplification | Sandwich-type hybridization | 60 pM | 0 - 0.075 nM | Gao et al., 2014 |
| 2 | No amplification | Horseradish peroxidase enhancement | 7.5 pM | 7.5 pM - 75 nM | Gao et al., 2016 |
| 3 | No amplification | Molecular beacon probes | 115 pM | 500 pM - 20 nM | Kor et al., 2016 |
| 4 | No amplification | Gold nanoparticle coated silica nanorods | 10 pM | 10 pM -10 nM | Takalkar et al., 2016 |
| 5 | Target-recycled nonenzymatic amplification | Quantum dots as labels | 10 pM | 100 pM - 10 nM | Deng et al., 2017 |
| 6 | Y-shaped junction DNA and target recycling amplification | Three-line lateral flow biosensor | 0.1 pM | 0.1pM - 10 nM | Huang et al., 2016 |
| 7 | Base stacking hybridization phenomenon and exponential isothermal amplification cascade strategy | Peptide nucleic acid probes | 1 fM | 1 fM - 100 pM | This work |

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(1) Gao, X.; Xu, H.; Baloda, M.; Gurung, A. S.; Xu, L.-P.; Wang, T.; Zhang, X.; Liu, G. *Biosensors and Bioelectronics* **2014**, *54*, 578.

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(3) Kor, K.; Turner, A. P.; Zarei, K.; Atabati, M.; Beni, V.; Mak, W. C. *Analytical and bioanalytical chemistry* **2016**, *408*, 1475.

(4) Takalkar, S.; Xu, H.; Chen, J.; Baryeh, K.; Qiu, W.; Zhzo, J. X.; Liu; Guodong *Analytical Sciences* **2016**, *32*, 617.

(5) Deng, H.; Liu, Q.; Wang, X.; Huang, R.; Liu, H.; Lin, Q.; Zhou, X.; Xing, D. *Biosensors and Bioelectronics* **2017**, *87*, 931.

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**Table S4.** Recovery experiments of microRNA 126 spiked in urine of BSH-EXPAR cascade.

| Matrix | Detected (fM) | Added (fM) | Found (fM) | Recovery (%) |
|---|---|---|---|---|
| Urine | 27.64 | 100 | 119.23 | 93.41% |
| Urine | 33.13 | 200 | 228.76 | 98.13% |
| Urine | 19.98 | 500 | 542.74 | 104.38% |

## Supplementary figures

### Fig. S1

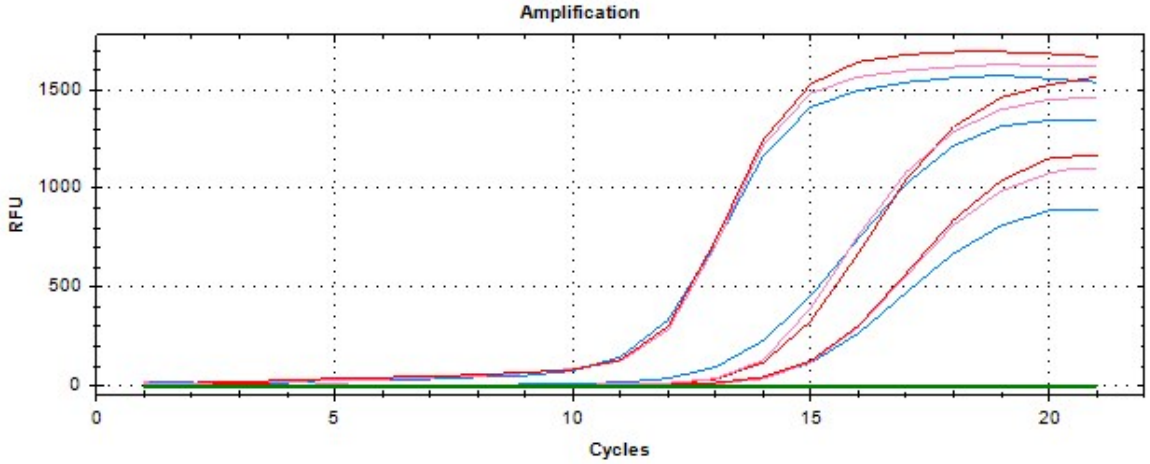


**Fig. S1** Amplification for the microRNA 126 (red curves), microRNA 182 (blue curves), microRNA 152 (pink curves), and negative control (green curves) established using high template concentration (10 pM, three left curves), medium template concentration (100 fM, three middle curves), and low template concentration (10 fM, three right curves). Each cycle lasts 1 min. A reaction time of 20 min was suitable for BSH-EXPAR.

**Fig. S2**

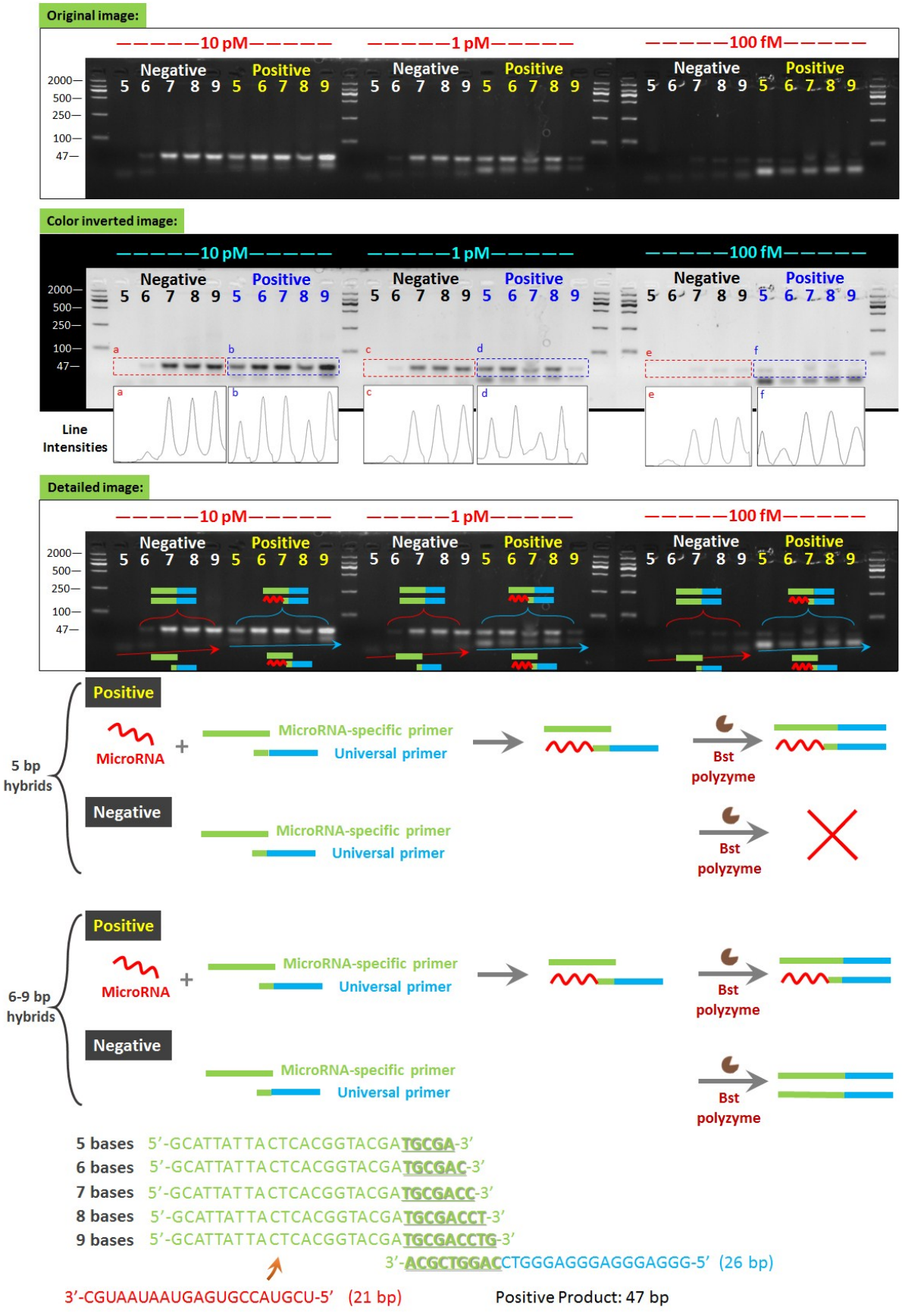
Original image:
10 pM
1 pM
100 fM
Negative
Positive
5 6 7 8 9 5 6 7 8 9
2000—
500—
250—
100—
47—
Color inverted image:
a
b
c
d
e
f
Line Intensities
Detailed image:
Positive
MicroRNA
MicroRNA-specific primer
Universal primer
Bst polyzyme
Negative
5 bp hybrids
6-9 bp hybrids
5 bases 5′-GCATTATTACTCACGGTACGATGCGA-3′
6 bases 5′-GCATTATTACTCACGGTACGATGCGAC-3′
7 bases 5′-GCATTATTACTCACGGTACGATGCGACC-3′
8 bases 5′-GCATTATTACTCACGGTACGATGCGACCT-3′
9 bases 5′-GCATTATTACTCACGGTACGATGCGACCTG-3′
3′-ACGCTGGACCTGGGAGGGAGGGAGGG-5′ (26 bp)
3′-CGUAAUAAUGAGUGCCAUGCU-5′ (21 bp)
Positive Product: 47 bp

**Fig. S2** Optimization of stacking hybridization base number. MicroRNA 126 was used as target and the concentrations were 10 pM, 1 pM, and 100 fM from left section to right section. Negative or positive detection means in the absent or present of target microRNA. Stacking hybridization base number in each section are 5, 6, 7, 8, and 9 bases. Original image was photographed under UV light. Color inverted image was obtained and measured by software named ImageJ. Detailed image was speculated according to the reaction principle.

As shown in the color inverted image, the target line intensities are positively correlated to the stacking hybrid length in negative samples (10pM, 1pM, and 100fM). In the absent of microRNA, increased base stacks created more combined opportunities. In the detailed image, the target line (47 bp) of the 5 bp hybrids show bigger difference between positive and negative samples than that of the 6-9 bp hybrids; yet the 6-9 bp hybrids generate high blank signal while signals of 5 bp hybrids are negligible. The lower band near the target line (47 bp) was a hybrid of microRNA-specific primer and universal primer in the negative samples. While in positive samples, the lower band was a hybrid of microRNA-specific primer, universal primer, and target microRNA. Based on the above observations, we designed a 5 bases DNA oligo for stacking hybridization in the following experiments.

**Fig. S3**

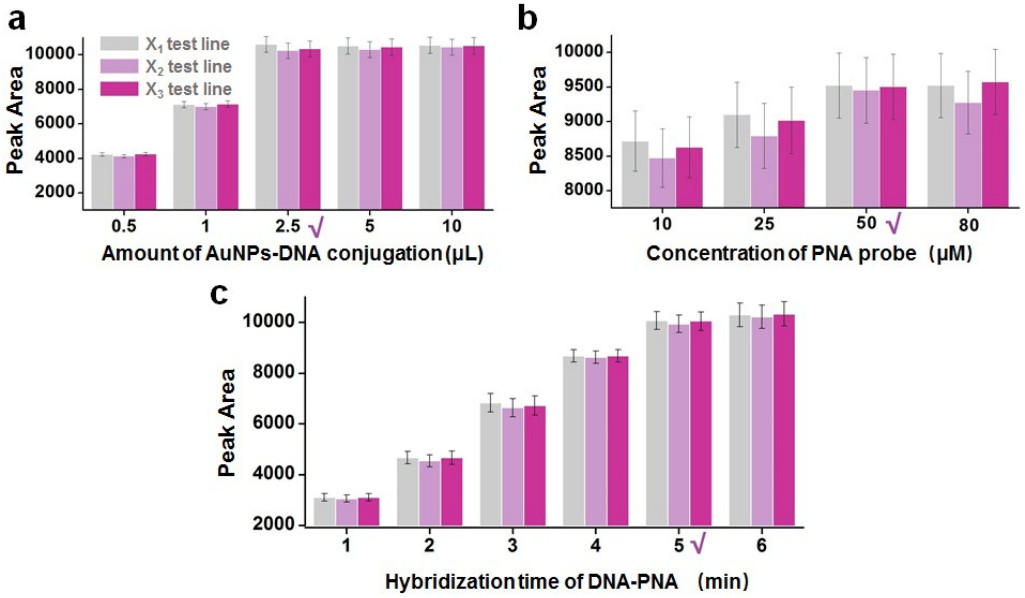


**Fig. S3** Optimization of TLPB. (a) Effect of the amount of AuNPs-DNA conjugation; (b) Effect of the concentration of PNA probe; (C) Effect of the hybridization time of DNA-PNA on lines. Target concentration was 10 fM.

**Fig. S4**

**Comparison of proposed method and qRT-PCR detection methods**

Expression levels of microRNA 126 and microRNA 182 in bladder cancer patients (No.1-3) and 3 healthy donors (No.4-6) were simultaneously quantified by our method and qRT-PCR. The results obtained with the proposed assay are in good agreement with those obtained by qRT-PCR on the same samples.

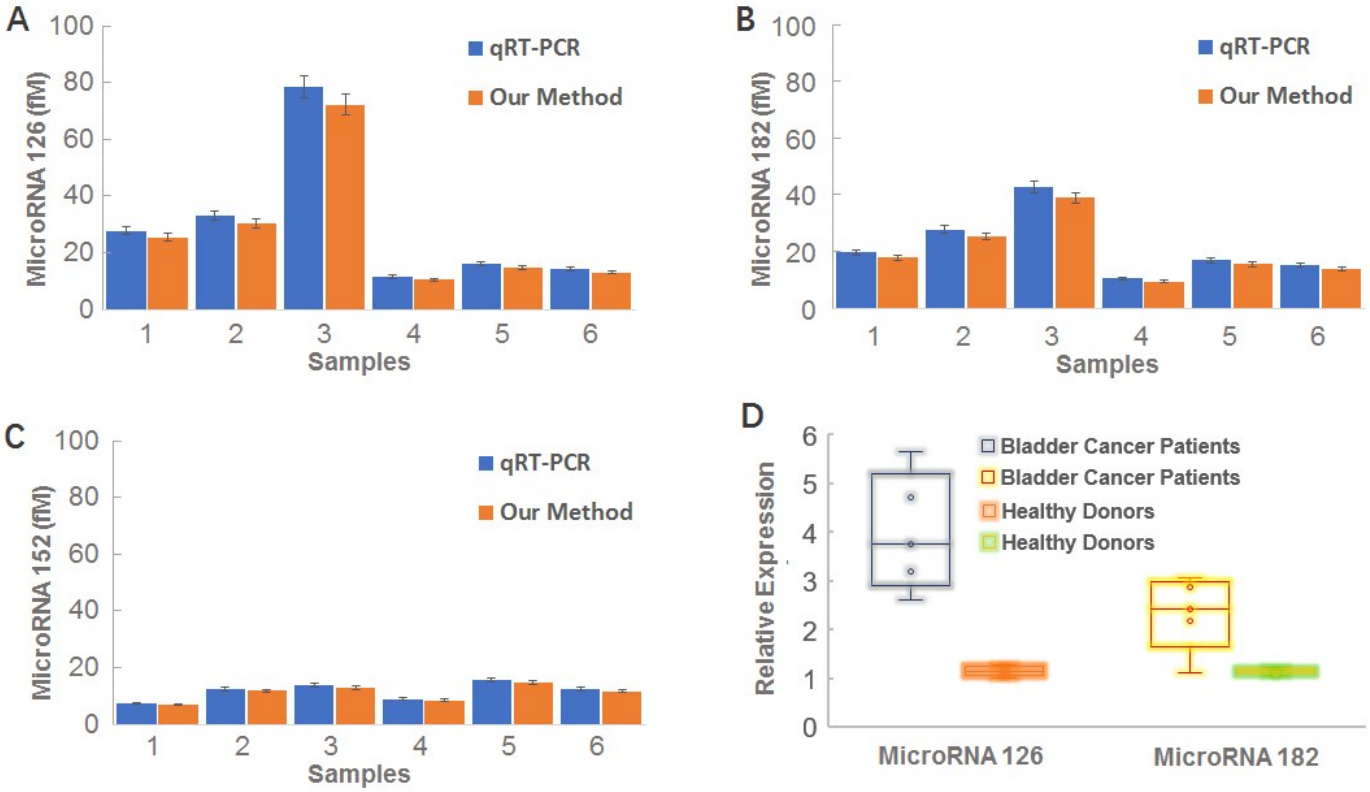


**Figure S4.** (A-C) Bars represent the expressions of microRNA 126, microRNA 182, and microRNA 152 in urine samples detected by the proposed method (orange bars) and qRT-PCR (blue bars), respectively. Error bars represent standard deviations for measurements taken from three independent experiments. (D) Responses comparison of target microRNA 126 and microRNA 182 in urine samples of bladder cancer patients and healthy donors. Error bars represent the standard error in data collected from three samples.

## Materials and methods

### *1.1 Chemicals and materials*

Chloroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), trisodium citrate, sodium citrate dihydrate, Triton X-100, mycose, sodium dodecyl sulfonate(SDS), NaCl, $Na_3PO_4 \cdot 12H_2O$, Tween-20, sucrose, deoxyadenosine triphosphate (dATP), bovine serum albumin (BSA), phosphate buffered saline (PBS, pH 7.4, 0.01 M), sodium chloride–sodium citrate (SSC) buffer (20 concentrate, pH 7.0), and borate buffer (BB, pH 9.0, 0.1 M) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Streptavidin was purchased from Invitrogen (Carlsbad, CA, USA). Double distilled water (ddwater) was used in all experiments. Backing cards (HF000MC100), glass fiber sample pads (CFSP001700), conjugation pads (GFCP000800), nitrocellulose membranes (135s) and absorbent pads (CFSP001700) were purchased from Millipore (Bedford, MA, USA).

### *2.2. BSH-EXPAR procedure*

The reaction mixtures for the BSH-EXPAR were prepared separately on ice as part A and part B. Part A consisted of microRNA-specific primer, universal primer, RNase inhibitor, and the microRNA target (5 μL); part B consisted of ThermoPol buffer, Nt.BstNBI, Bst. polymerase, and DEPC-treated water. Parts A and B were mixed immediately before being placed in a conventional metal heating block.

BSH-EXPAR assays were performed in a 50 μL total reaction mixture containing the microRNA-specific primer (1 μM), universal primer (1 μM), each dNTPs (400 μM), Nt.BstNBI (0.4 U $\mu L^{-1}$), Bst. polymerase (0.05 U $\mu L^{-1}$), RNase inhibitor (0.8 U $\mu L^{-1}$), and 1× ThermoPol buffer (20 mM Tris–HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X-100; Tris=2-amino-2-hydroxymethylpropane-1,3-diol). We normalized the primer concentration for each set of primers so that the reaction time for each set was the same for a set quantity of template DNA.[1] The mixture was incubated at 65 °C for 20 min in a conventional metal heating block. BSH-EXPAR assays were carried out in triplicate for each microRNA target, and a no template control contained water instead of the template.

### *2.3 LFPNAB preparation*

#### *2.3.1 Synthesis of Au-NPs*

Au-NPs were synthesized following a previous method with a little modification.[2,3] Briefly, a 50 mL aqueous solution of $2.4 \times 10^{-4}$ M $HAuCl_4$ was heated to boiling, and then 1.67 mL of 1% trisodium citrate was added. The boiling solution was stirred for another 30 min, and then stored at 4°C prior to use. TEM images showed the diameter of such Au-NPs ~15 nm, and the UV-vis spectrum exhibited a characteristic plasmon absorption band with a maximum at 524 nm (as shown in Fig. S5a and S5b). Based on the formula of Beer–Lambert law, the concentration of Au-NPs was about 2.5 nM.

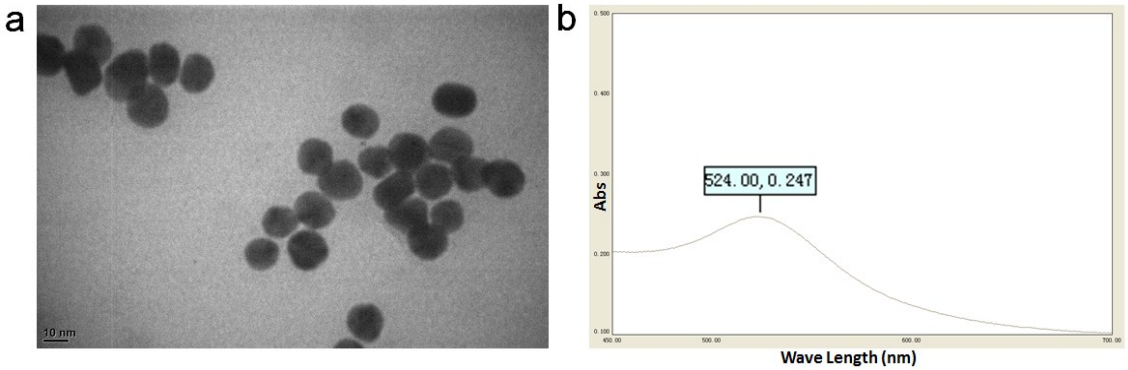


Fig. S5 Characterization of AuNPs by means of TEM and UV-vis spectrum

### *2.3.2 Modification of Au-NPs by DNA probes*

Au-NPs modified by DNA probes were prepared by a modified method according to the literature.[2,3] Procedures were as follows: 30 μL of 100 mM of dATP was added into 1 mL of concentrated Au-NP solution to protect Au-NPs from salt-induced aggregation. The mixture was incubated at room temperature for 20 min. 15 μL of 1% of SDS was slowly added into the mixture, and incubated on a shaker for 10 min. 20 μL of 1 M NaCl was dropped into the mixture at a rate of 5 μL/10 min. Then, 0.5 OD of each thiolated DNA probe which is required was added, and the mixture was incubated for 4 hours in metal bath at 60°C. After the incubation, the mixture was centrifuged at 12,000 rpm for 15 min, and the supernatant was discarded. Then, the deposited Au-NPs were washed with 1 mL of PBS and dispersed in 20 μL of storage buffer (containing 20 nM of $Na_3PO_4 \cdot 12H_2O$, 5% BSA, 0.25% Tween-20 and 10% sucrose). TEM image (Fig. S6a) of DNA probes modified AuNPs also showed a bright circle around AuNPs, which indicatd the functionalization of DNA probes. Fig. S6b showed the UV-vis absorption spectra of Au-NPs modified by the DNA probes after centrifugation, from

which the monodispersion of Au-NPs and the surface plasmon absorption of Au-NPs slightly shifted to 527 nm were observed.

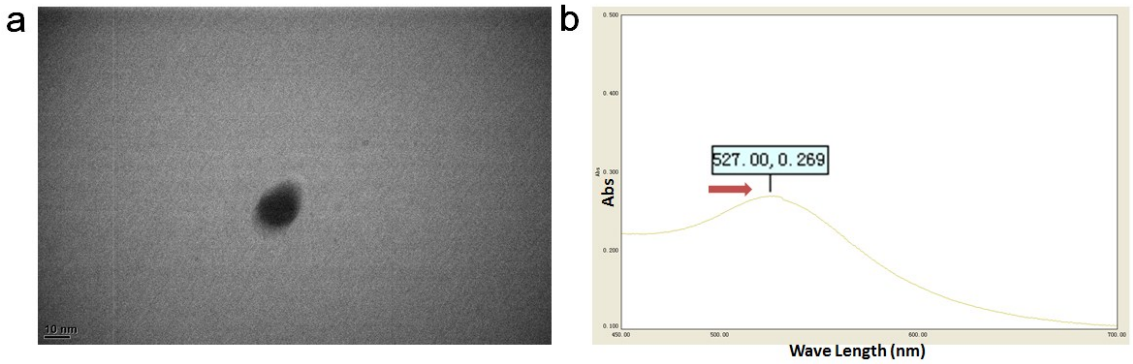


Fig. S6 Characterization of DNA probes modified AuNPs by means of TEM and UV-vis spectrum

### *2.3.3 Conjugation of streptavidin-biotinylated PNA*

The streptavidin solution and probe solution were prepared by dissolving the appropriate amount of streptavidin and biotinylated PNA in solubilizing buffer (PBS containing 0.5% mycose). Briefly, 30 μL of 1 mg/ml of streptavidin solution was mixed with 30 μL of 100 μM PNA probe solution. The mixture was incubated at room temperature for 1 h and stored at 4℃ until be used.

### *2.3.4 Assembling of trident-like LFPNAB*

The LPB consisted of the following five components: a sample pad, conjugate pad, nitrocellulose membrane, absorbent pad, and backing. The sample pad (17 mm × 30 cm) and conjugate pad (8 mm × 30 cm) were made from glass fiber and saturated with a buffer (pH 8.0) containing 0.25% Triton X-100, 0.1 M BB, and 0.15 M NaCl. Then, the pad was dried at 37°C for 2 h and stored in a desiccator. A test line (1 μL/cm) and control line (1 μL/cm) were prepared by dispensing 30 μL of streptavidin-biotinylated PNA conjugates at different locations on the nitrocellulose membrane (25 mm × 30 cm) using a BioDot BioJet BJQ 3000 dispenser (Irvine, CA). The distance between each line was approximately 5 mm. The nitrocellulose membranes were then dried overnight at 37°C and stored at 4°C. The different pads were assembled on backing (60 mm × 30 cm) with an overlap between them of approximately 1-2 mm to ensure that the solution could migrate through the biosensor. LFPNABs were cut at a width of 3 mm using a Bio-Dot Paper Cutter module CM4000 (Irvine, CA).

Finally, three LPBs with different test lines were assembled together, like a trident, using the sasmple pad as linker and either used immediately or sealed and stored under dry conditions at room temperature until use (Fig. S7).

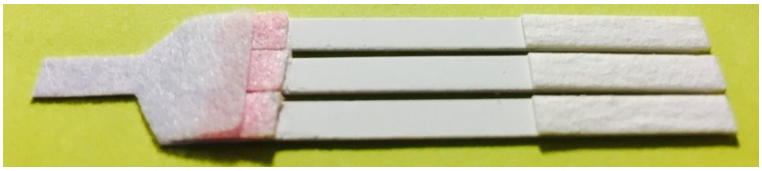

Fig. S7 Typical image of the trident-like LFPNAB

***2.4 Detection of microRNA***

In a typical test, 50 μL of running buffer (4 concentrate SSC containing 2% BSA and 0.05% Tween-20) was mixed with 50 μL of the amplified product solution; then, the mixture was applied to the sample pad of the trident-like LFPNAB and migrated up by capillary force. The lines were evaluated visually within 5 min. The naked-eye detection was performed simply by observing the color change of the three test lines, which differed between target microRNA and non-target microRNA samples. For quantitative measurements, the LFPNAB was inserted into the portable "strip reader" instrument DT2050 purchased from Shanghai Goldbio Tech. Co., Ltd. (Shanghai, China), and the optical intensities of the red lines were recorded with "Goldbio strip reader" software, which could determine parameters, such as peak height and area integral. Each sample was detected 3 times, and the average value of three measurements was used.

***2.5 qRT-PCR***

First, total RNA was extracted from urine samples using miRNeasy RNA extraction kit (Qiagen, Limburg, NL) according to the manufacturer's protocol. The extracted RNA was quantitated by measuring its optical density (OD) at 260 nm using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, USA). The RNA was reversed transcribed into cDNA. The reverse transcription was performed in a DNA Thermal Cycler 4800. The reaction was carried out in a water bath kettle. Real-time PCR was performed by using a Quantitative Real-

Time PCR Kit (TaKaRa) an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, CA).

**References**

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