**Supporting Information**

# Duplex-specific nuclease-assisted isothermal signal amplification coupled with HPLC-UV for simultaneous detection of multiple microRNAs

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**Table S1.** Sequence of the oligonucleotides used in this study.

| Oligonucleotides | Sequence（5'→3') |
|---|---|
| BTDOR for miR-141 detection[a] | AAAAAAAAAAAAAAAAAAAAAAAAAAAAA<br>CCATCTTTACCAGACAGTGTTA-BioTEG |
| BTDOR for miR-21 detection[a] | GGGGGGGGGGGGGGTGGGGGGGGGGGGGG<br>TTCAACATCAGTCTGATAAGCTA-BioTEG |
| Poly A | AAAAAAAAAAAAAAAAAAAAAAAAAAAAA |
| Poly G | GGGGGGGGGGGGGGTGGGGGGGGGGGGGGT |
| Stem-loop primer for miR-141 | GTCGTATCCAGTGCAGGGTCCGAGG<br>TATTCGCACTGGATACGACTCACGC |
| Forward primer | GCGTTTGTTCGTTCGGCTC |
| Reverse primer | AGTGCAGGGTCCGAGGTATT |
| Stem-loop primer for miR-21 | GTCGTATCCAGTGCAGGGTCCGAGG<br>TATTCGCACTGGATACGACTCAACA |
| Forward primer | GCGCGTAGCTTATCAGACTGA |
| Reverse primer | AGTGCAGGGTCCGAGGTATT |
| miR-21 | UUUGUUCGUUCGGCUCGCGUGA |
| miR-21-2MT[b] | U***C***GCUUAUCAGACUGA***G***GUUGA |
| miR-21-4MT[b] | UA***C***C***TT***A***G***CAGACUGAUGUUUA |
| miR-141 | UAACACUGUCUGGUAAAGAUGG |
| miR-200a[b] | UAACACUGUCUGGUAA***C***GAUG***U*** |
| miR-200b[b] | UAA***U***ACUG***C***CUGGUAA***U***GAUG***A*** |
| miR-200c[b] | UAA***U***ACUG***C***C***G***GGUAA***U***GAUGG***A*** |

a: The color labels are biotinylated-TEG at the 3' end.

b: The bases differing from those in miR-141 and miR-21 are marked in red italic.

**Table S2.** Comparison of the proposed method with representative state-of-the-art multiplex miRNA detection methods.

| Method | Demonstrated multiplexing capacity | LOD | Assay time | Assay cost | Major instrumentation | Main features | Ref. |
|---|---|---|---|---|---|---|---|
| ICP-MS–MNAzyme | 3 miRNAs (miR-21, miR-155 and miR-10b) | 20 pM, 11 pM and 19 pM | 240 min | NR | ICP-MS | Simultaneous elemental-tag readout; good multiplexing capability, but requires lanthanide labeling and specialized instrumentation | [1] |
| Fluorescent | 2 miRNAs | 3.39 fM | 155 min | NR | Flow | One-pot and | [2] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| probe-based assay (FCM) | (miR-141 and miR-21) | | | | cytometry (FCM) | simultaneous dual-miRNA detection with very high sensitivity | |
| Fluorescent probe-based assay (LC-FLD) | 3 miRNAs (miR-210, miR-10b and miR-21) | 2.75 fM, 2.19 fM and 2.20 fM | 260 min | NR | HPLC-FLD | One-pot, simultaneous detection; dye-free fluorescent probes; no solid nanomaterials | [3] |
| DSN-HPLC | 2 miRNAs (miR-155 and miR-21) | 0.3 fM and 0.24 fM | 170 min | NR | HPLC-FLD | Highly sensitive dual-target assay based on DSN-assisted amplification and chromatographic separation | [4] |
| This work | 2 miRNAs (miR-141 and miR-21) currently demonstrated; potentially expandable to 4 | 500 fM | 2.5 h pretreatment/ reaction + 10 min HPLC detection (~160 min total) | 5.75 dollar per assay | HPLC-UV | Simultaneous detection in a one-step reaction; simple HPLC platform; low assay cost; short chromatographic readout | This work |

Notes:

NR = not reported in the cited paper.

LOD values correspond to the specific target miRNAs used in each reference.

For the present work, the multiplexing capacity is currently validated for 2 targets, while extension to 4 targets is feasible through further probe and chromatographic optimization.

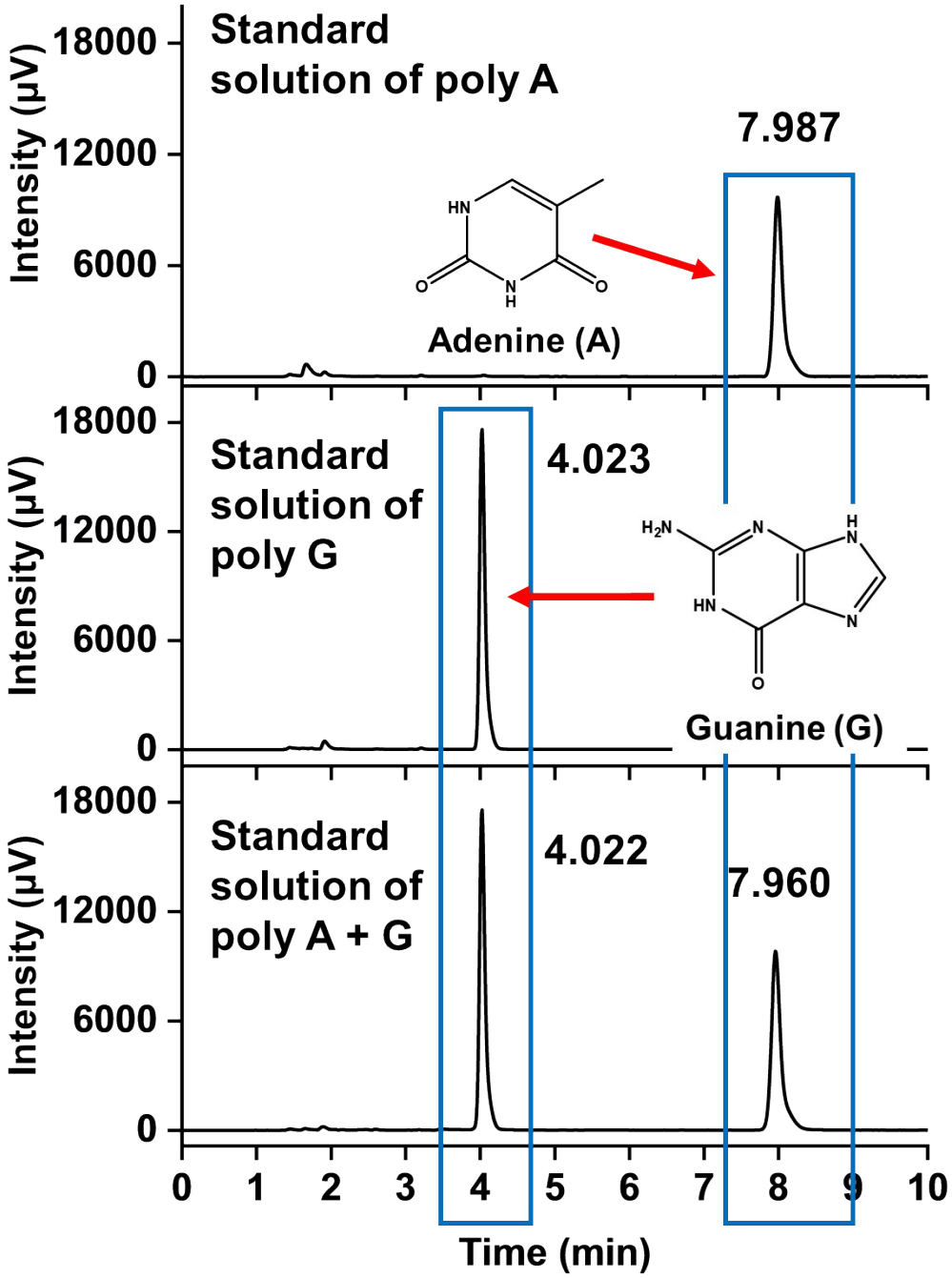


**Fig. S1.** Chromatogram of adenine and guanine released from oligonucleotide hydrolysis of polyadenine and polyguanine. The final concentrations of polyadenine, polyguanine and HCl, are 0.5 μM, 0.5 μM, and 2 M respectively.

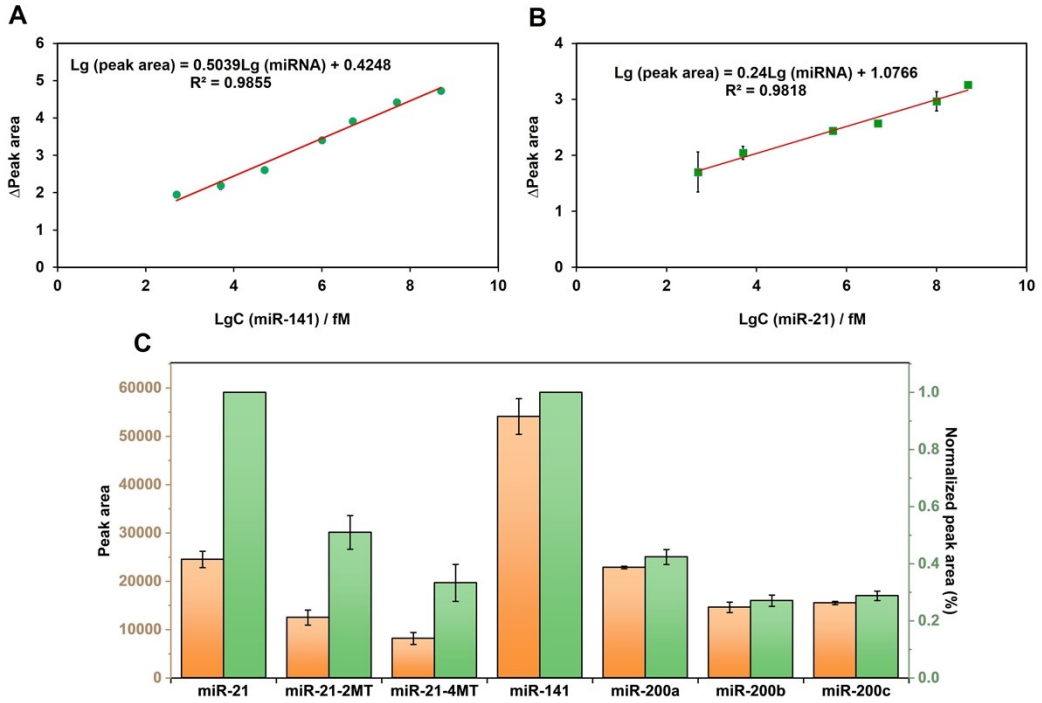


**Fig. S2.** Calibration curve of the duplex-specific nuclease-assisted isothermal signal amplification (DSNSA) coupled with HPLC-UV for simultaneous detection of miR-141 and miR-21 at varying concentrations ranging from (A) 500 fM–500 nM and (B) 500 fM–500 nM. (C) Selectivity of this system in different mismatches (2MT, and 4MT) and homologous family for miRNA detection. Error bars: SD, $n = 3$.

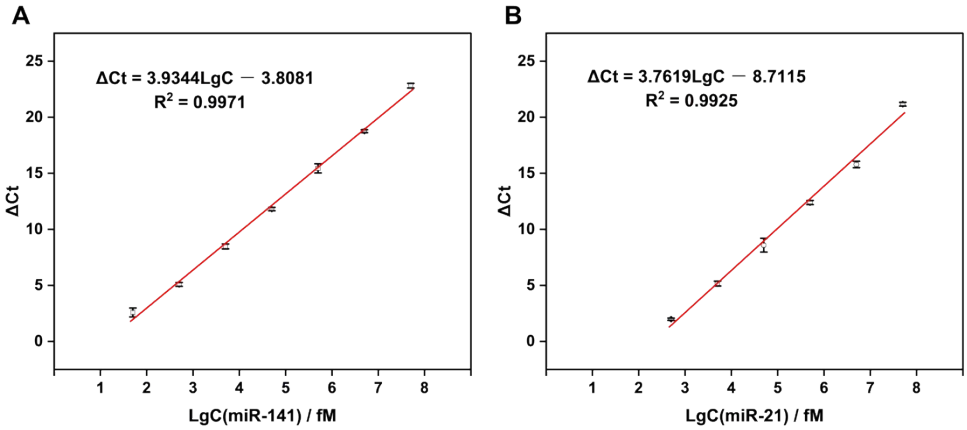


**Fig. S3.** Calibration curve of the logarithm of miR-141 (50 fM to 50 nM) and miR-21 (500 fM to 50 nM) concentration vs. ΔCt value. Error bars: SD, n = 3.

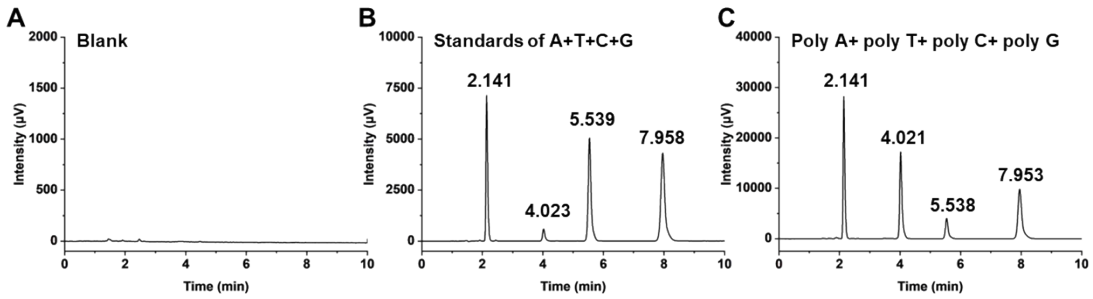


**Fig. S4.** Chromatographic analysis of the hydrolysates of four homopolymeric oligonucleotides. (A) Blank; (B) Standards of the four nucleobases; (C) Hydrolysate solution of the homopolymeric oligonucleotides. The final concentrations of four nucleobases (adenine, thymine, cytosine and guanine), homopolymeric oligonucleotides (poly A, poly T, poly C and poly G) and HCl, are 0.5 μM, 0.5 μM, and 2 M respectively.

## References

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