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COMMUNICATION

# Duplex-specific nuclease-resistant triple-helix DNA nanoswitch for single-base differentiation of miRNA in lung cancer cells

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## Abstract

In this work, a duplex-specific nuclease (DSN)-resistant triplex-helix DNA nanoswitch was designed for assays of single-base differentiation of the let-7a family in lung cancer cells. Initially, although a 10-bp duplex stem in the nanoswitch was cleaved to pieces, a 10-bp triplex stem was resistant to DSN. Consequently, a triple-stranded DNA structure resistant to DSN was obtained. The pH-dependent formation of the triplex structure then produced the pH-related nanoswitch/miRNA hybrid, and the metastable nanoswitch generated an obvious signal increase at pH 6.8. Surprisingly, the pH condition at 6.8 for the best nanoswitch/miRNA hybrid is consistent with the optimal DSN catalysis, which paves the way for a first-rank DSN signal amplification (DSNSA) strategy for the single-base selective capacity of the homologous let-7a family with a limit of detection of 0.26 pM. The cyclic strategy based on the DSN-mediated triplex-helix DNA nanoswitch was verified in lung cancer cell samples and exhibited better discriminatory ability without user-unfriendly nucleotide modification or extra probe-mediated assistance, showing excellent potential for application in biomedical sensing and clinical diagnosis.

**Keywords** Functional nucleic acids · Biosensor · Triple-stranded DNA structure · miRNA

## Introduction

Diverse natural and artificial functional nucleic acids, termed FNAs by Lu and Li in 2009, have generated tremendous enthusiasm in the analytical community, from concept to application [1]. Besides the hereditary material in living organisms, FNAs perform other functions of catalysis (e.g. ribozymes and deoxyribozymes), ligand binding (e.g. aptamer and molecular beacon) and amplification (e.g. polymerase chain reaction) [1]. In the FNA arena, triple-stranded DNA (or triple helices or DNA triplexes) is formed when pyrimidine or purine bases occupy the major groove of the DNA duplexes via Hoogsteen pairs with purines of the Watson-Crick base pairs [2]. The unique pH response, sequence-specific recognition and flexible allosteric properties endow triple-stranded DNA with enormous advantages in signal transduction, composition of nanomaterials, drug delivery and genetic diagnostics [3, 4].

MicroRNAs (miRNAs) are endogenous, short, noncoding RNAs. They represent an abundance of biomarkers available for biomedical research and clinical diagnosis, since they play an essential regulatory role in cell proliferation, differentiation, apoptosis, and tumorigenesis [5]. To this end, although classical approaches, from northern blot, real-time reverse transcription polymerase chain reaction (RT-PCR), in situ hybridization, and microarrays to next-generation sequencing, have been employed to detect miRNAs, single-nucleotide polymorphisms (SNPs) closely related to tumor susceptibility and patient survival present a greater challenge for single-base identification among homologous family members [6].

Recently, duplex-specific nuclease (DSN) has emerged as a versatile element in SNP detection [7]. It belongs to the dsDNA and DNA/RNA endonuclease family, with DNA-

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cleaved activity, and discriminates between perfectly and imperfectly matched duplexes [8]. Different DSN amplification-based platforms for miRNA analysis, including colorimetric, fluorescence and electrochemical methods, mass spectrometry, flow cytometry and magnetic signal amplification, can be classified as long ssDNA hybridization and functional hairpin DNA methods. Interestingly, although DSN is known to incise dsDNA with at least 10 bp [4], the minimum base pairs decrease to 6 bp in a hairpin structure due to the thermodynamic and kinetic characteristics of the stem region via intramolecular hybridization [9]. Compared with an ssDNA strategy requiring nanomaterials and interfaces [10], the hairpin-based design is more likely to require user-unfriendly nucleotide modification (e.g. click chemistry, 2-Ome-MB) in the stem or extra probe-mediated complicated amplification [10, 11].

In this work, from an allosteric perspective of functional hairpins, a triple-helix DNA nanoswitch was engineered for single-base differentiation of miRNA. Because triple-stranded DNA (tsDNA) was discovered to be resistant to DSN, a novel hairpin containing a triple-helix stem was applied as a DNA nanoswitch to conduct DSN signal amplification (DSNSA) without complex chemical modification or probe assistance. Smart pH responsivity results in the pH-related hybridization between the triple-helix DNA nanoswitch and target miRNA, and the DSN-mediated circulation enabled single-base differentiation of homologous miRNA, exhibiting excellent discriminatory ability in real samples.

## Materials and methods

### Reagent and instrumentation

High-performance liquid chromatography (HPLC)-purified miRNAs were purchased from Ruibo Bio-Technology Co. Ltd. (Changzhou, China). The fluorophore/quencher-labeled triplex DNA nanoswitch sequence was synthesized and purified by Invitrogen Inc. (Shanghai, China). The sequences of these miRNAs and the triplex DNA nanoswitch are listed in Table S1 in the Electronic Supplementary Material (ESM). DSN was obtained from Evrogen JSC (Moscow, Russia). All chemicals were purchased from Sigma-Aldrich/MilliporeSigma (Saint Louis, MO, USA) and used without further purification. In order to create and maintain an RNase-free environment, the solutions used in the DSNSA method were treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved. The tips and tubes are RNase-free and did not require pretreatment to inactivate RNases. The reaction buffer consisted of 0.1 mM sodium phosphate buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 50 mM KCl at different pH levels.

### Conformational verification by circular dichroism (CD)

The advanced conformation of the double-helix DNA nanoswitch and the triple-helix DNA nanoswitch was characterized via conformational verification using the Chirascan™-plus CD spectrometer (Applied Photophysics, Surrey, UK). Before observation, both label-free sequences of nanoswitches (ESM Table S1) were dissolved in Millipore water at a concentration of 10 μM, and diluted in phosphate buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 50 mM KCl at pH 6.0, heated to 95 °C for 10 min, and allowed to cool to room temperature for 1 h. For CD observation, a total 300 μL reaction volume was prepared for the measurement.

### Verification of tsDNA resistance to DSN cleavage

Both sequences of fluorescence-labeled nanoswitches in ESM Table S1 were dissolved in Millipore water at a concentration of 10 μM. Before use, 50 nM oligonucleotide solution of the triple-helix DNA nanoswitch and double-helix DNA nanoswitch was diluted in phosphate buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 50 mM KCl at pH 6.0, respectively, heated to 95 °C for 10 min, and allowed to cool to room temperature for 1 h. This process was followed by the addition of 0.4 U DSN into the system for 1 h at 50 °C, and the fluorescence emission spectra were recorded by a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 25 °C in a 50 μL cuvette (total volume of the solution 50 μL) with excitation at 488 (±5) nm and acquisition at 517 (±5) nm.

### Feasibility confirmation and factor optimization

All oligonucleotides were dissolved in Millipore water at a concentration of 10 μM. Before use, 50 nM oligonucleotide solution of the triple-helix DNA nanoswitch was diluted in phosphate buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 50 mM KCl at pH 6.8, heated to 95 °C for 10 min and allowed to cool to room temperature for 1 h. This process was followed by the addition of 1 nM let-7a with different DSN content at different temperatures and for different reaction times. The fluorescence emission spectra were recorded by a Cary Eclipse fluorescence spectrophotometer (Agilent) at 25 °C in a 50 μL cuvette (total volume of solution 50 μL) with excitation at 488 (±5) nm and acquisition at 517 (±5) nm ($^{\triangle}F/F0$, %, $^{\triangle}F$ and $F_0$ represent the changes and initial values of fluorescence intensity).

### Detection performance analysis

All oligonucleotides were dissolved in Millipore water at a concentration of 10 μM. Before use, 50 nM oligonucleotide solution of the triple-helix DNA nanoswitch was diluted in

phosphate buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 50 mM KCl at pH 6.8, heated to 95 °C for 10 min, and allowed to cool to room temperature for 1 h. This process was followed by the addition of different concentrations of let-7a with 0.4 U DSN at 50 °C for 30 min. The fluorescence emission spectra were recorded by a Cary Eclipse fluorescence spectrophotometer (Agilent) at 25 °C in a 50 μL cuvette (total volume of solution 50 μL) with excitation at 488 (±5) nm and acquisition at 517 (±5) nm.

### Real sample analysis

BEAS-2B human lung normal bronchial epithelial cells and A549 human lung cancer cells (American Type Culture Collection, Manassas, VA, USA) were selected as the test samples. The cell culture and total RNA extraction were carried out according to the Stromberg method, as described previously [12]. Total RNA extracts were detected using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and diluted with reaction buffer. The quantity of let-7a in cell samples was validated according to the previously prepared standard curve with different concentrations of the standard by reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. The RT-PCR quantification was conducted with a Bio-Rad CFX96 PCR system (Hercules, CA, USA) with specific primers (ESM Table S1) under an established protocol [13]. For the nanocircuit assay, the same procedure as with the fluorescence experiments was followed.

## Results and discussion

### tsDNA resistance to DSN

Firstly, the assumption that tsDNA is resistant to DSN cleavage was verified by designing a double-helix DNA nanoswitch and a triple-helix alternative. As presented in the schematic diagram (Fig. 1a), the 10-bp duplex-stem region in the double-helix DNA nanoswitch is cleaved into smaller fragments by DSN, while the 10-bp triplex-stem domain in the triple-helix DNA nanoswitch is perfectly maintained in the presence of DSN (for DNA sequences see ESM Table S1). Circular dichroism (CD) spectroscopy (see ESM Fig. S1) provided the information regarding the duplex and triplex structures. An obvious negative peak at a wavelength of 210 nm (inset ESM Fig. S1) indicates the DNA triplex conformation in the tsDNA nanoswitch. The duplex structure in the dsDNA nanoswitch is confirmed by a positive band at 280 nm and a negative band at 245 nm. The contrast of the duplex and triplex conformation in response to DSN was then verified by the fluorescence spectrum (see ESM Fig. S2). Although the fluorescence signal for the duplex nanoswitch displayed

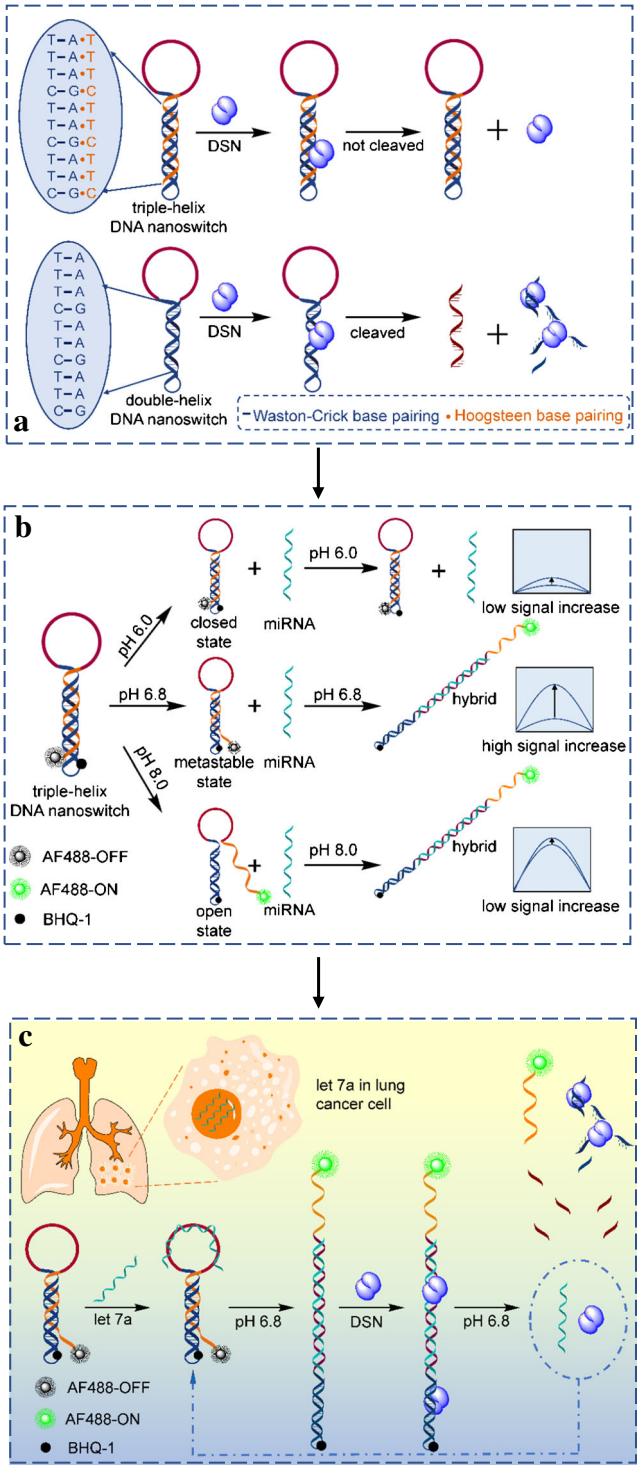


**Fig. 1** Schematic illustration of the duplex-specific nuclease-resistant triple-helix DNA nanoswitch for single-base differentiation of miRNA. (**a**) Assumption and verification that tsDNA is resistant to DSN cleavage: schematic diagram of the design of a triple-helix DNA nanoswitch and a double-helix DNA nanoswitch in the presence of DSN. (**b**) Schematic conceptualization of the pH-related hybridization of the triple-helix DNA nanoswitch with miRNA. (**c**) Schematic diagram of the DSNSA strategy based on the DSN-resistant triple-helix DNA nanoswitch for single-base differentiation of miRNA

an almost sixfold increase at 517 nm, that of the triplex nanoswitch was scarcely changed.

## pH-related hybridization

The pH-related hybridization between the triple-helix DNA nanoswitch and target miRNA was then demonstrated by the pH responsivity of the triplex nanoswitch. With fluorescence modification, the fluorescence intensity of the triplex nanoswitch gradually increased with the increase in pH from 6.0 to 8.0 (see ESM Fig. S3), which suggests that the disruption of Hoogsteen bonds caused the deconstruction of the triplex configuration. Based on this, the triplex nanoswitch presented closed, metastable and open states at pH 6.0, 6.8 and 8.0, respectively. Furthermore, specific states emerged as different allosteric effects. As shown in Fig. 1b, in the closed state at pH 6.0, the triplex structure is too tense to be opened by target miRNA, exporting a low signal increase. In the open state, the hybrid nanoswitch/miRNA produces limited signal gain, because the fluorescence signal has been leaked due to the disassembly of the triplex configuration at pH 8.0. In the metastable state (pH 6.8), negligible dissolution of the tsDNA stem produces low signal background. Once the loop region of the nanoswitch hybridizes with the target through molecular identification, the separation of AF488/BHQ-1 generates significant signal enhancement. As a proof of the concept, fluorescence analysis was conducted with let-7a selected as the model target (see ESM Fig. S4). Compared with the small signal increase at pH 6.0 and 8.0, the triple-helix DNA nanoswitch showed an obvious signal variation with the addition of let-7a at pH 6.8, which was compatible with optimal DSN activity.

## The DSNSA strategy

Importantly, the proposed DSNSA strategy with the metastable triplex nanoswitch was performed without complex chemical modification or probe assistance. As presented in Fig. 1c, let-7a, a biomarker in lung cancer cells, hybridizes with the specific loop to mediate the allosteric effect for the first signal amplification, from the hairpin structure with a triplex stem to the duplex configuration comprising the miRNA/DNA and dsDNA domain at pH 6.8. Under DSN digestion, the dsDNA hybrid is digested into pieces, and the miRNA/DNA hybrid releases the intact miRNA to identify another triplex nanoswitch for cyclic signal amplification.

## Feasibility and optimization

To verify the DSNSA concept, the fluorescent intensity was monitored to characterize the cyclic amplification effect (Fig. 2a). The system in the presence of DSN resulted in a sixfold increase compared with that in the absence of DSN, which proved the tremendous cyclic amplification of DSNSA. Before the investigation of detection performance, several components were optimized. (i) *Temperature* (Fig. 2b). The signal response increased steadily, then fell dramatically, reaching its peak at 50 °C, which was selected as the optimal reaction temperature. (ii) *DSN content* (Fig. 2c). The fluorescence intensity increased with the increase in DSN content from 0.008 U to 0.8 U, reaching a plateau beyond 0.4 U DSN. Thus, 0.4 U DSN was employed in the subsequent studies. (iii) *Reaction time* (Fig. 2d). The fluorescence response increased when the reaction time was prolonged from 0 min to 30 min and reached its highest value at 30 min. Consequently, 30 min was chosen for the examination of detection performance.

## Detection performance

Under these optimal conditions, detection performance was investigated. (i) *Sensitivity* (Fig. 2e). The fluorescent intensity increased gradually with the let-7a concentration from 0.1 pM to 150 pM. Specifically, a linear correlation is fitted from 1 pM to 20 pM of let-7a, with the calibration curve of $Y = 11.866X + 25.607$ ($R^2 = 0.9969$, where Y is the fluorescence intensity and X is the concentration of let-7a (pM). Based on the 3σ rule (mean fluorescence of blank+3σ (standard deviation of the blank)), the detection limit is calculated as 0.26 pM. (ii) *Specificity* (Fig. 2f). Even with one-base variation, the signal response of trace let-7a (20 pM) remains much higher than those of high concentrations of let-7e and let-7f (5 nM). Similarly, both let-7d (20 pM) with a two-base variation and let-7i (20 pM) with a four-base difference present insignificant fluorescent intensity. Other high levels of interferences (20 pM) from different miRNA families (miRNA-141, miRNA-200b, miRNA-15a and miRNA-21) gave negligible signals. This suggests that the DSN-assisted triplex nanoswitch enables single-base differentiation of homologous miRNAs. (iii) *Uncertainty and reproducibility* (see ESM Table S2). Standard deviation and relative standard deviation, in a range of 0.64–4.01 and 2.99%–6.78%, respectively, indicated quite good uncertainty and reproducibility. (iv) *Comparison with other methods* (see ESM Table S3). The sensitivity of the triple-helix DNA nanoswitch is comparable to both the classical RT-PCR method and rolling-circle amplification (RCA) method. The single-base selectivity rivals other DSN-mediated amplification methods, and the one-step detection time of 30 min has potential for rapid and straightforward on-site analysis.

## Real sample test

This study challenges the DSN-resistant triple-helix DNA nanoswitch for single-base differentiation of let-7 family members in cell lysates. As shown in Table S4 (see ESM), excellent agreement is obtained between the results achieved

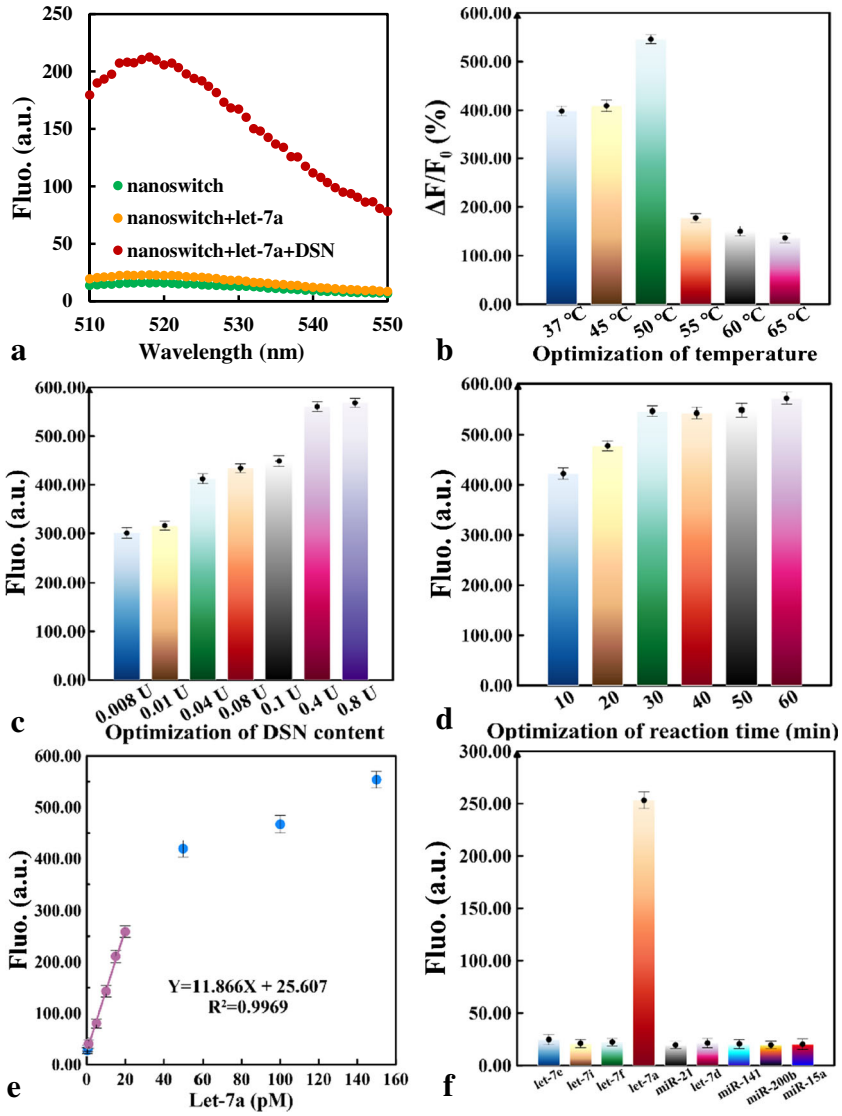


**Fig. 2** Fluorescence feasibility test (**a**) and the optimization of temperature (**b**), DSN content (**c**) and reaction time (**d**) of the DSNSA strategy based on the DSN-resistant triple-helix DNA nanoswitch (experimental details in ESM), and detection performance of the DSN-resistant triple-helix DNA nanoswitch for single-base differentiation of miRNA. Let-7a was detected to verify the ability for quantitative analysis (**e**) and selectivity (**f**) of the biosensor. Error bars represent the standard deviation ($N = 5$)

by RT-qPCR, and the proposed nanoswitch indicated that the let-7a expression level in A549 lung cancer cells is lower than that in BEAS-2B normal lung cells. This proves that the proposed sensing strategy holds great promise for practical application in miRNA detection, with exceptional accuracy and reliability.

## Versatility discussion

With respect to the universality of our triple-helix DNA nanoswitch, the consistent length of miRNA targets (22 nt) generates an identical length of loop domain and uniform conformation of the nanoswitch. Due to the short 10-bp stem length, the hybrid between the loop region and triplex-forming codes barely exists based on its Delta G close to zero. For targeting miRNA-21 and miRNA-200b as examples, codes of the stem domain consistently maintained the triplex-DNA characteristic, and the loop sequence was substituted for 5'-TCAACATCAGTCTGATAAGCTA-3′ for miRNA-21 and 5'-TCATCATTACCAGGCAGTATTA-3′ for miRNA-200b, respectively. Based on analysis using the OligoAnalyzer software program (Integrated DNA Technologies; https://sg.idtdna.com/calc/analyzer), the melting temperature ($T_m$) of the miRNA-21/nanoswitch and miRNA-200b/nanoswitch is 28.5 °C and 30.1, respectively. Compared with the $T_m$ (26.7 °C) of the let-7a/nanoswitch hybrid, the similar $T_m$ will give rise to the analogous properties of structural alteration for sensitive response. Generally, the similar formation and allostery of the triple-helix DNA nanoswitch can contribute to the detection of a wide range of miRNA targets. In a weakly acidic environment represented by pH 6.8, the triple-helix DNA nanoswitch can achieve an optimal allosteric response, and DSN can maintain more than 85% maximum activity. This supports the first-rank

DSNSA strategy for the sensitive and single-base selective detection of miRNA targets.

## Summary and conclusion

In summary, compared with other DSN-mediated SNP detection, the triple-helix DNA nanoswitch demonstrates several advantages: (1) Although a 10-bp duplex stem in the nanoswitch is cleaved to pieces, a 10-bp triplex stem in the nanoswitch is resistant to DSN. This provides an optimized hairpin strategy with stable thermodynamic and superior kinetic characteristics. (2) The pH-responsive formation of the triplex structure gives rise to the pH-related nanoswitch/miRNA hybrid, and the metastable nanoswitch generates an obvious signal increase at pH 6.8 that is beneficial for optimal DSN activity. (3) The DSNSA strategy based on the triple-helix DNA nanoswitch reinforces the signal amplification in accordance with the best nanoswitch/miRNA response and DSN catalysis at pH 6.8, which enables single-base differentiation of the homologous let-7a family with a limit of detection of 0.26 pM. (iv) The cyclic strategy of the DSN-resistant triple-helix DNA nanoswitch is simply designed and avoids user-unfriendly nucleotide modification and extra probe-mediated assistance, exhibiting better discrimination in real samples.

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**Authors' contributions** Jingjing Tian conducted the experiments and wrote the draft, Huashuo Chu completed the scientific visualization, Liye Zhu guided the cell experiments and corresponding data analysis, and Wentao Xu provided the idea and manuscript modification.

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## Compliance with ethical standards

Research involving human participants and/or animals: Not applicable.

All the authors, Jingjing Tian, Huashuo Chu, Liye Zhu and Wentao Xu, approved of the manuscript submitted for publication.

**Conflict of interest** The authors declare that they have no conflict of interest.

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