Supplementary Data

DNA circuits compatible encoder and demultiplexer based on a single biomolecular platform with DNA strands as outputs

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## Supplementary tables

### Supplementary Table S1 Sequences of the oligonucleotides used in this work

| Name             | Sequences (5’-3’)                                                                 |
|------------------|----------------------------------------------------------------------------------|
| Molecular beacon (MB) |                                                                                 |
| MB-1             | FAM-CGGCGCGACTCCATTTGTTATCCTCGAGGGCCGCAGCG-BHQ1                                 |
| MB-2             | BHQ1-CGACTCCCCATTTGTTGAAGGGCCCTGACTGGAGTCG-HEX                                 |
| MB-3             | ROX-CGACTCCCCCTGGTTAGTCTTTTGCTCGAGGATCG-BHQ2                                   |
| MB-4             | Cy5-TGAGACTCCCCCTTCACACTAGTCATGCGACTGACTCTCA-BHQ2                              |
| Three-way junction-incorporated double hairpin unit (TJDH) |                                                                                 |
| 3-1 TJDH         | GACTCCATTGGTATCCTCGAGGAGGGCAGCTCGCCCTCTCCAGGGATAAG                             |
| 1-1 TJDH-1       | GACTCCATTGGTATCCTCGAGGAGGGCAGCTCGCCCTCTCCAGGGATAAG                             |
| 1-1 TJDH-2       | GACTCCATTGGGCACCTGTGTTAAGGCCCTGACTGCGAGCCCTTACAAATGGGCTGCTCCCTCCAGGGATAAG     |
| 1-2 TJDH         | GACTCCATTGGTATCCTCGAGGAGGGCAGCTCGCCCTCTCCAGGGATAAG                             |
| 1-4 TJDH         | GACTCCATTGGTATCCTCGAGGAGGGCAGCTCGCCCTCTCCAGGGATAAG                             |
| Input (I)        |                                                                                 |
| I-0              | GCCTGACACTCAGTATCCTTGATC                                                      |
| I-1              | GGTAAGCGGAAATAATGGATCGACAC                                                   |
| I-2              | TAAATGGATCGACACTTTATCCCTG                                                     |
| I-3              | AGTCGTTGATCGGAGGGCCGGA                                                      |
| I-4              | TAAATGGATCGACACTTTATCCCTG                                                     |
| I-5              | CTCAGATCAGTCTAGCTCTCTCAT                                                    |
| Annihilator      |                                                                                 |
| A-1              | GTATGTCTTGTGAGGGGACTCCATTGTATCCTCGAGG                                       |
| A-2              | GTATGTCTTGTGAGGGGACTCCATTGTATCCTCGAGG                                       |
| A-3              | GTATGTCTTGTGAGGGGACTCCATTGTATCCTCGAGG                                       |
| A-4              | GTATGTCTTGTGAGGGGACTCCATTGTATCCTCGAGG                                       |
| DNA logic circuits based on single-stranded gates (SSG) |                                                                                 |
| SSG-1            | GACTCCATTGGTATCCTCGAGGAGGGGACTCCATTGTATCCTCGAGG                              |
| SSG-2            | CCTCGAGGATACCAATGGGAGTC                                                       |
| SSG-3             | CAGTCAGGGCCTTCACAATGGG |
|------------------|------------------------|
| SSG-4             | AGTCGTGTGAGTCCATATTATTTGCCCTACCCGACACGAGGATAAGTCGTGTCGATTTTATCCGCTACCCGACACGACT-NH$_2$ |
| SSG-5             | TGTCGGGTAGGGCGAATAATGGATCGACACGACT |

**Exponential Amplification Reaction (EXPAR)**

| EXPAR-Template | GTGTCGATCCATTTATCCGCTACCCGACACGAGGATAAGTCGTGTCGATTTTATCCGCTACCCGACACGACT-NH$_2$ |
|----------------|--------------------------------------------------------------------------------|
|                | GTGTCGATCCATTTATCCGCTACCCGACACGACT-NH$_2$ |

**Subsequent DNA circuit of 4-2 encoder**

| And-1          | CAGTCAGGGCCTTCACAATGGGAGTCCGCTCTATGGACCTCCATTGTTATCCTCGAGG |
|----------------|-----------------------------------------------------------|
| And-2          | AGTCGTGTGAGTCCATATTATTTGCCCTACCCGACACGAGGCGACACGACT-CCTACGTAAGGGAGGACT |
| And-3          | TGTCGGGTAGGGCGAATAATGGATCGACACGACT |
| Reporter-1     | FAM-CAGACTACGCTGAGCCCCAGTCCAATCTCAACCAAGGAGTCGTGTCGATTTTATCCGCTACCCGACACGACT-GAGGAGGAGGACT |
| Reporter-2     | TCTTGGGTGAGATGGGACTGGCGCTAGCGTAGTCTG-BHQ1 |

**Left hairpin of TJDH**

| H-1            | FAM-AGTCAATGGCCCTTATGCCCTTTCAATTTAGCCATTTGACT-BHQ1 |
|----------------|-----------------------------------------------------|
| H-2            | FAM-GACTCCATTTAGGAGTCAATGGCCCTTATGCCCTTTCAATTTAGC |
|                | CATTGACTCCATGAGTC-BHQ1 |
Supplementary figures

Supplementary Figure S1. The circuit diagram and truth table of the encoder. (A) The circuit diagram of 4-2 encoder. (B) The truth table of 4-2 encoder. (C and D) The circuit diagrams for the two methods of constructing 8-3 encoder. (E) The truth table of 8-3 encoder.

Supplementary Figure S2. The circuit diagram and truth table of the demultiplexer. (A) The circuit diagram of 1-2 demultiplexer. (B) The truth table of 1-2 demultiplexer. (C) The circuit diagram of 1-4 demultiplexer. (D) The truth table of 1-4 demultiplexer.
Supplementary Figure S3. Schematic illustration of possible sources of background amplification triggered by transient template hybridizations, strand replacement and the subsequent extension by DNA polymerase. (A) Hybridization at the 3’-end between two templates. (B) Hairpin formation on the 3’-end of a single template. (C) Transient interactions along the template sequence lead to a higher incidence of 3’-end hybridization. (D) The Output in the SSG reacts directly with the downstream components in a strand replacement reaction, and the involvement of polymerase accelerates this leakage. A, B and C are redrawn based on reference 27 in the main text.

Supplementary Figure S4. In addition to non-nucleic acid information (e.g. ions, small molecules, proteins, cells and microorganisms), we can also use aptamers to convert this information into a nucleic acid that can be sensed by TJDH.
Supplementary Figure S5. The secondary structure of TJDHs at 55 °C predicted by NUPACK (http://nupack.org/). The various functional areas on the TJDH are also marked out. (A) 3-1 TJDH, (B) 1-1 TJDH-1, (C) 1-1 TJDH-2, (D) 1-2 TJDH, (E) 1-4 TJDH. The secondary structure of the TJDH has been maintained as a three-way junction-incorporated double hairpin. Their main difference is between the I* and O* regions (the ring part of the two hairpins), which is related to their function - sensing different Inputs and producing different Outputs. They have a certain P-region, and the main role of the C-region is to maintain the secondary structure, which varies as needed.

Supplementary Figure S6. Verify that Input cannot open one of the TJDH hairpins directly (without polymerase). Method: The reaction system consisted of 100 nM H-1 or 100 nM H-2, 100 nM I-1, 0.5× NEBuffer 3.1 and 1× ThermoPol Reaction Buffer.
Supplementary Figure S7. Characterize the TJDH reactions and products using 1-1 TJDH-1 reaction system as an example by agarose gel electrophoresis. Lane 1: Marker. Lane 2: Background (with 1-1 TDJH-1, MB-1, Vent and Nt.BstNBI). Lane 3: Experimental group (with input, 1-1 TDJH-1, MB-1, Vent and Nt.BstNBI). Lane 4: Experimental group without Nt.BstNBI. Lane 5: Only 1-1 TJDH-1. Lane 6: Only MB-1. Lane 7: Only Output-1. Lane 8: Output-1 and MB-1. Lane 9: Marker.
Supplementary Figure S8. Construction of 1-2 demultiplexer in SSG and TJDH. (A) 1-2 Demultiplexer without address selection in SSG. (B) Structure of 1-2 TJDH. (C) Detailed reaction process of 1-2 TJDH.
Supplementary Figure S9. The fluorescence changes and the average signal-to-noise ratio at different enzyme concentrations. (A, B) Fluorescence responses of 1-2 TJDH amplification under different concentrations of Vent. The concentration of Nt.BstNBI was kept at 50 U/mL at this time. (C) The average signal-to-noise ratio at different concentrations of Vent, while maintaining the concentration of Nt.BstNBI at 50 U/mL. (D, E) Fluorescence responses of 1-2 TJDH amplification under different concentrations of Nt.BstNBI. The concentration of Vent was kept at 20 U/mL at this time. (F) The average signal-to-noise ratio at different concentrations of Nt.BstNBI, while maintaining the concentration of Vent at 25 U/mL.
Supplementary Figure S10. (A) Structure of 1-4 TJDH. (B) Detailed reaction process of 1-4 TJDH. (C-F) The fluorescence changes of 1-4 TJDH in (C) Cy5, (D) FAM, (E) HEX and (F) ROX channels.
Supplementary Figure S11. Assessment of the sensitivity of the TJDH platform. (A) Fluorescence intensity response of the 1-1 TJDH-1 to different concentrations of I-1. (B) Standard curve of the rising time to Input concentration. The Input concentration is recalculated with log2. Data represent the mean ± the standard deviation.

Supplementary Figure S12. The fluorescence changes of 1-2 TJDH at different Annihilator-1 concentrations.
Supplementary Figure S13. (A) The schematic diagram of the program that implements the 1-4 demultiplexer. (B) The corresponding truth table of the 1-4 demultiplexer.
Supplementary Figure S14. The result corresponding to the partial combination of inputs of the 1-4 demultiplexer in Fig S13. The inputs are (A) (1,0000) which means D0=1, S1=0, S2=0, S3=0 and S4=0, (B) (1,1000), (C) (1,0100), (D) (1,0010), (E) (1,0001), (F) (1,1100), (G) (1,1110) and (H) (1,1111), respectively.
Supplementary Figure S15. The reaction of the 4-2 encoder and its products were characterized using agarose gel electrophoresis. Lane 1: Marker. Lane 2: 4-2 encoder + D₀. Lane 3: 4-2 encoder + D₃. Lane 4: 4-2 encoder + D₁. Lane 5: 4-2 encoder + D₂. Lane 6: MB-1 + Output-1. Lane 7: MB-2 + Output-2. Lane 8: MB-1. Lane 9: MB-2. Lane 10: Only 1-2 TJDH. Lane 11: Only 1-1 TJDH-1. Lane 12: Only 1-1 TJDH-2. Lane 13: Marker.

The electrophoresis results showed that when D₀ is present (lane 2), almost no output is produced (band 7+8), and there is very little leakage. When D₃ is present (lane 3), there is only the product of 1-2 TJDH (band 4), and when D₁ or D₂ is present (lane 4 and lane 5) there is only the product of 1-1 TJDH (band 5 or band 6), indicating that there is very little crosstalk. In general, the 4-2 encoder has minimal leakage and crosstalk, so the background signal generated when the encoder is applied to the circuit mainly comes from the subsequent circuit.
Supplementary Discussion

Supplementary Discussion S1. The length of input and output sequences

The input length should be long enough to ensure that its melting temperature is higher than the experimental temperature so that the input could hybridize with the TJDH template. The output should be long enough to react with subsequent DNA circuit components. The minimum concentration for input in the TJDH system should be 10 nM if the threshold of the fluorescent intensity is set at 3000 a.u. with our instrument.

Supplementary Discussion S2. Characterization of the TJDH reactions and products

In Supplementary Figure S6

At 55 °C the input could not open the H-1 and H-2, so there is no increase in fluorescence, whereas when heated to 85 °C, H-1 and H-2 are all opened and fluorescence increases. This indicates that the input could not open the left hairpin of TJDH directly without polymerase at 55 °C.

In Supplementary Figure S7

Output-1 is a single-stranded DNA that is too short and does not show a band in lane 7. But the output can be characterized by MB-1 in lane 8. As shown in lane 2, when the input was added, the output could be produced and hybridized with the reporter MB-1. Without Nt.BstNBI, the output could not be released and there is no band of Output-1/MB-1 dsDNA in lane 4. This gel electrophoresis verified the reaction process shown in Figure 1.

We used ImageJ (https://imagej.nih.gov/ij/) to calculate the electrophoretic band area. From the area of the two bands in Lane 5, we could calculate the percentage of the path 1 and path 2 to be about 80% and 20%, respectively.

Supplementary Discussion S3. Enzymes in the reaction system

Vent and Nt.BstNBI are widely used for isothermal amplification of nucleic acids. Vent is a DNA polymerase with a strand-replacement activity that extends the daughter strand and displaces the previous generation of daughter strands on the template. Nt.BstNBI acts to cleave the daughter strand to a specific length and sequence. They are not irreplaceable. Nt.BstNBI can be replaced by any other nicking enzyme by simply modifying the nicking site on the template (TJDH) to the corresponding nicking enzyme. Vent can be replaced by any other polymerase with strand replacement activity. However, the reaction temperatures of the two enzymes and the melting temperatures of inputs and outputs should be matched.

Supplementary Discussion S4. The fluorescence changes and the average signal-to-noise ratio at different Vent and BstNBI

In Supplementary Figure S9, we can see that with more Vent or BstNBI, the control (without input) increased, this trend is the same for these two enzymes. The difference lines in the experiment group. With more Vent, the reaction rate increased gradually. While the reaction
rate changed a little with more BstNBI. We indicate that with the input, as its concentration is high, all the TJDH templated could be open to form the dsDNA in a very short time. As the number of nicking sites is fixed, the nicking site in TJDH may be saturated with high concentrations of BstNBI. Thus, the reaction rate did not change much. While for the Vent, the concentration we used may not be high enough to saturate the trigger strand, so more Vent could result in high reaction rate.

**Supplementary Discussion S5. Characterization of the 4-2 encoder reactions and products**

The electrophoresis results in Supplementary Figure S15 showed that when D0 is present (lane 2), almost no output is produced (band 7+8), and there is very little leakage. When D3 is present (lane 3), there is only the product of 1-2 TJDH (band 4), and when D1 or D2 is present (lane 4 and lane 5) there is only the product of 1-1 TJDH (band 5 or band 6), indicating that there is very little crosstalk. In general, the 4-2 encoder has minimal leakage and crosstalk, so the background signal generated when the encoder is applied to the circuit mainly comes from the subsequent circuit.