Supplementary Information

DNA origami book biosensor for multiplex detections of cancer-associated nucleic acids

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Figure S1. Blueprint of a DNA origami book biosensor

Depiction of scaffold routing and staple design in a two-dimensional representation. Graphics and sequences were generated using caDNAno software package. The scaffold strand is shown in black. Staple strands labeled with Cy3, Cy5, bh2, or bbq650 are shown in orange. Strands labeled with biotin are shown in purple. Hinge staple strands are shown in red.
Figure S2. Mapping of a DNA origami book biosensor

The map of a DNA origami book biosensor showing the distance between the positioned fluorophores or quenchers.
Figure S3. TEM images of DNA origami book biosensors

Structures were self-assembled at 12 mM Mg$^{2+}$ and purified at 8 mM Mg$^{2+}$ and visualized using transmission electron microscopy (TEM) by depositing them on the carbon-coated EM grids (scale bars: 100 nm).
Figure S4. DNA origami book biosensor visualization using atomic force microscopy (AFM)

Structures were assembled with 12 mM Mg²⁺ in the closed conformation and absorbed on the mica surface. AFM measurements were performed in tapping mode with AFM NT-MDT (NTEGRA II).
Figure S5. Agarose gel analysis of DNA origami book biosensors

The presence of Mg$^{2+}$ is needed to shield the negatively charged DNA phosphate backbone, for the formation of DNA double helices during the self-assembly process of DNA origami structure.$^{1, 2}$ To determine the optimal Mg$^{2+}$ concentration for the self-assembly of the book biosensor, the structures were analyzed by agarose gel electrophoresis. Assembled structures were run on 1.5% agarose gel at 70 V together with a single-stranded M13 scaffold strand and 1 kb ladder. Gel electrophoresis analysis of the self-assembled structures with different Mg$^{2+}$ concentrations. The optimal Mg$^{2+}$ concentration for forming the structure without incorporated fluorophore was greater than 8 mM Mg$^{2+}$ (Figure S5a). Figure S5b demonstrates the gel electrophoresis analysis of the self-assembled structures in the closed state (without fluorophores) at 12 mM Mg$^{2+}$ and purified at 8 mM Mg$^{2+}$ with or without the addition of target oligonucleotides. The gel image shows the differences in the mobility of the structures between open or closed states.

![Figure S5a](image1.png)

![Figure S5b](image2.png)
In order to test the incorporation of the dyes and measure the fluorescence signal generated by DNA origami book structures, the structures were functionalized with biotin to allow their immobilization on coverslips covered by biotinylated bovine serum albumin (BSA) and neutravidin. To determine if the incorporation of labelled oligonucleotides within the structure is efficient, we first analyzed the DNA origami book structure with 1 column (5 Cy3 or Cy5), 2 columns (10 Cy3 or Cy5) and 4 columns (20 Cy3 or Cy5). Structures were imaged and the mean intensity of each structure was compared (Figure S6). The increase in the number of fluorophores incorporated within the structure resulted in a linear increase of the mean fluorescence intensity in the absence of self-quenching. This is also reported in earlier studies where it was shown that fluorescence intensity increases linearly with an increasing number of incorporated fluorophores.\textsuperscript{1,3}
To determine the optimal Mg$^{2+}$ concentration during the self-assembly and purification steps that give the maximum signal difference between open and closed states, we applied the FRET-based detection mechanism by labeling oligonucleotides with Cy3 and Cy5 fluorophores using terminal transferase (TdT) enzyme and incorporated them into DNA origami structure. Ensemble FRET analysis of the DNA origami book biosensors using fluorescence spectroscopy was performed. FRET efficiency of open and closed self-assembled structures with 5 FRET pairs at different Mg$^{2+}$ concentrations (4-16 mM Mg$^{2+}$) is shown in Figure S7a. Results indicated the optimal signal and the maximum difference between open and closed states (24%) observed when the structure is folded in the presence of 14 mM Mg$^{2+}$. After defining the optimal salt concentration during self-assembly that gives the maximum signal difference, the optimal Mg$^{2+}$ concentration for purification was determined. All structures were assembled at 14 mM Mg$^{2+}$ in the open and closed states and purified in the presence of Mg$^{2+}$ in the range of 4 mM to 14 mM (Figure S7b). The optimal purification concentration was defined as 10 mM, as it gave the maximum FRET difference between the open and closed states of the device (25%). Higher concentrations of salt are used during self-assembly to decrease repulsion between DNA helices facilitating keeping the DNA origami sensor in the closed state. On the other hand, the lower salt concentration used in the purification step increases the repulsion between DNA helices, which allows the opening of the structure upon ligand binding. We also tested if a higher number of fluorophores incorporated within the structure requires more Mg$^{2+}$. DNA origami structure with 4 columns (20 fluorophores) was assembled in open and closed states in the range of 12 to 22 mM Mg$^{2+}$ and the optimal signal was found at 16-18 mM Mg$^{2+}$ (Figure S7c), while the purification step was optimal in the range of 10-12 mM Mg$^{2+}$ (Figure S7d).
Figure S8. Single-structure analysis of the DNA origami book biosensor

Fluorescence images were recorded 6 min after the addition of the target. a) A representative image of the FRET-based detection method. The image shows the emission profiles of single DNA origami book sensors after donor excitation at 532 nm; the green circle and red circle represent Cy3 and Cy5 emissions for the same DNA origami book biosensor, respectively. b) A representative image of the quenching-based detection method (Cy3+bh2). It shows the emission after excitation at 532 nm; the green circle represents a single DNA origami structure. c) A representative image of the quenching-based detection method (Cy5+bb650q). It shows the emission after excitation at 640 nm; the red circle represents a single DNA origami structure.
Figure S9. Effect of amount and position of the locks within DNA origami book biosensor on FRET efficiency

Schematic representation of the positioning of locks within DNA origami book biosensor. Four different structures with 15 FRET pairs were assembled with different combinations of locks: a) combination of all four locks, b) with 3 locks (1, 3 and 4), c) 2 locks at the edges (1 and 4) and d) 2 locks in the middle (2 and 3). Detection of 100 pM of target DNA, ODN-153 was performed using these structures. Results were plotted into histograms where red and green represent FRET efficiency distribution before (0 min) and after (6 min) the addition of target DNA.
Figure S10. Single-structure study of the limit of detection for multisensing of DNA targets

The detection limit of the book biosensor with 10 quenching pairs on both sides and with the addition of 2 DNA targets at the same time. Left: Opening at the left side of DNA origami book upon addition of ODN-153. Right: Opening at the right side of DNA origami book upon addition of ODN-342. The red and green histograms represent fluorescence intensity increase after the addition of the target of interest due to the opening of the structure. A yellow histogram represents fluorescence intensity in the closed state.
Figure S11. Terminal transferase (TdT) labeling of oligonucleotides with Cy3 and Cy5

Five oligonucleotides from each column were labeled with either Cy3 or Cy5-conjugated ddUTP using TdT enzyme. The efficiency of labeling was analyzed using HPLC. Results showed that labeling of oligonucleotides with Cy3 or Cy5 was efficient with an approximate yield of 93%.
Table S1: DNA origami book biosensor staple strand sequences

All structures were assembled using the list of oligonucleotides reported below. They are divided into different sections based on the purpose of usage.

| Start | End | Core staples |
|-------|-----|--------------|
| 0[55] | 2[40] | GTCTATCATCGGAACCGAAAGGAAGTGCTTTC |
| 0[87] | 2[72] | CGTGGACTCAACGTCCGTAGGACTATAGGG |
| 0[111] | 2[104] | GAACAAGATGCCTACCGCCCG |
| 0[151] | 2[136] | AAGAATAGCCCGAGATGGCGGTTTCGTCG |
| 0[183] | 2[168] | TCCGAAATCGGCAAATTCTTTCTTGGCT |
| 0[215] | 2[200] | CCCAGCAGCCGAAAAATTGCCCTCTAATGAG |
| 1[216] | 0[216] | TGGCCCTGAGAGAGTTGTCCACGCTGTTTGC |
| 2[135] | 4[128] | GCCGCTGGGTAAAGTGTCGCA |
| 2[231] | 4[216] | CATAAAGTGGTGCCGGATCAGACGATCCAGCG |
| 3[40] | 6[40] | AGGGATTTAGTGAAGATGGCAAACCTGAAAGC |
| 3[72] | 6[72] | TCCTGAGATTCTTCTGATACATTTTCTGGCC |
| 3[104] | 6[104] | ACCGAGTATCCATCATATTACACCCAGTC |
| 3[136] | 6[136] | CTCCTCTACCGGGGTTTCTTTGCTAGAG |
| 3[168] | 6[168] | GCTCGAATCCAGAATGTGGTGTGTGCTTTCG |
| 3[200] | 6[200] | TCCTGTGTCTGCGCGCGATGCCGGGGTGTCCA |
| 4[55] | 1[63] | CTCTGCTGTAAGACCGGCCTATACGGAAGAAGCGAAA |
| 4[87] | 1[87] | AGCAATACAGTGTGTATTAGGGGCTCCTGCA |
| 4[151] | 1[151] | CGGTCATACAGTGGAGTCCGGAAAGCTATTG |
| 4[183] | 1[183] | TGCTGGTGCTGAATAGTGGCAGCAGTGA |
| 4[215] | 1[215] | CAGTGTCAGAAATTGTTGGGGTGCTCACCGCC |
| 5[88] | 3[103] | ATCGTCGTGCGCCGCATCCGGGTGTCCCA |
| 6[39] | 8[24] | GTAAGAATTGGTCAGTAGCCTACCTTGGCTGA |
| 6[135] | 8[128] | CCGGCTCGAGATGAGTAAAACCGGCT |
| 6[230] | 8[216] | CACGCAACAGCAGTTGACTCGACATATAAAA |
| 7[10] | 10[40] | GTCTTTAAAAATCTAATTGCAAATTATTAAAT |
| 7[72] | 10[72] | CATCGCAAGTGCCACAGGTTATCTTATTAGAC |
| 7[104] | 10[104] | GCAGAAGAGTPCGAGGCAACTAATAGTCAATAG |
| 7[136] | 10[136] | CGGCCAGATCCGGCGACCTTCTAAACGTAC |
| 7[168] | 10[168] | CGGACTGTTGAGGGTGAAAGGGAACCGGA |
| 7[200] | 10[200] | CTGGTGCTAGAAAAATTTATAATTGGCGCAAG |
| 8[55] | 5[55] | CAAATGAATGGCGCAGAATTCCTTCAGGAAAAC |
| 8[87] | 5[87] | CCGAATACATTTAAATTGAGCACGACGCTCA |
| 8[151] | 5[151] | GATTGCCTGCCACATTCCGCGGTGCTGTCG |
| 8[183] | 5[183] | TTTAGTGATAGAACGTTGCAGGCTCAGCAA |
| 8[215] | 5[215] | AAATCCCGCCAGAAACCGGGCTGAGTTACCTG |
| 9[88] | 7[103] | CTTAGGAAGTAAATAACCGGAACGACACCA |
| 10[135] | 12[128] | AGGCCAACATCTGTAGCTGTTGGCC |
| 10[231] | 12[216] | CACGACGTGTCGCTGGGCTCGCTAGCGTAACC |
| 11[40] | 14[40] | TATCAATTTATAATGAGTAATATCAAGAAG |
| 11[72] | 14[72] | AGGAGCGGTGAAATATCGGGAGATAACCTGAG |
| Start | End | foot staples |
|-------|-----|--------------|
| 1[128] | 35[127] | CGGGGAGAAGGTTGAGTGTTGGAAGGATA |
| 3[112] | 1[127] | AAAGATGTAATACACACACCGGCAACCGG |
| 4[127] | 34[120] | GCACCGGTAAATCACCATTTCGCTC |
| 5[120] | 3[135] | CTGGTAATTAAATCTCCCGTCCGAGT |
| 7[112] | 5[119] | TAAATCTCTGAGATTTTGACCG |
| 8[127] | 32[120] | CCGTTTTTTCACTC AAAAGGTAG |
| 9[112] | 7[135] | GATTACCGAATTGTGCTGCGTCAGTGC |
| 12[127] | 30[120] | GAAACCGGAAGTACACACCGGCAACCGG |
| 16[127] | 28[120] | CTATTTTGGGATAGTTTAGTCAAT |
| 17[112] | 13[119] | TTTAGTAATACCGGGAAATAATTGCTTACCAAGCGT |
| 20[127] | 26[120] | ATATGCAATAAGTAAAGAACAACAA |
| Start  | End   | hinge staples                      |
|--------|-------|------------------------------------|
| 25[104]| 24[96]| TACAGAGATTTTGAAGCCTTAAT            |
| 33[137]| 31[135]| ACAACGCTGAAAATCTCCAAAAAAAAGGAGC    |
| 35[96] | 33[103]| TAAGAGGGTGAGACTCCCCGAATGGGAAACGCGGCTTGA |
| 35[128]| 33[136]| GGATTAGCCGGGGTTTTACCCTGAACACTGAGTGTACAAACT |
| 25[136]| 24[128]| GCCTTGAGTCAATACATAACGC            |
| 27[104]| 25[103]| AAGGAACCGGCATTAGACGGGAGACGCGCTT |
| 27[136]| 25[135]| CGGAACGACCTGACGAGAAACCGTGAAATTG |
| 29[104]| 27[103]| GTTTGAGGATACCCAAAAGAACTGTTACCAAG |
| 29[136]| 27[135]| GGACTAAAGTCGAAATCCGCGACCTACTTAGC |
| 31[104]| 29[103]| TCATCGGCAATATTCAATTAAAGATTCAACC |
| 31[136]| 29[135]| CTAAAAATTGGGAACGAGGGTAGCAAGTCCTTAGG |
| 33[104]| 31[103]| TATTCCATATTAGCGTTTGCCATCTGCGGTTT |

| Start  | End   | staples with biotin               |
|--------|-------|----------------------------------|
| 21[216]| 19[223]| ACCGGAAGAGGTGGCATAATAAC         |
| 2[39]  | 4[32] | CTCGTTAGGCCAGCCAAACTCAA        |
| 10[39] | 12[24] | CTTTGCCTAACGCTTTGCAGTAAACAG    |
| 21[56] | 19[71] | AATAGATAAAATAAGTTCTAACGATTAAA  |
| 2[199] | 4[184] | TGAGCTAACGGCATCTGTGCACCTGTGGA  |
| 10[199]| 12[184]| CTTCAGGAAACAAACGGGGAGCACGAGCAT |

| Start  | End   | endcap staples                   |
|--------|-------|---------------------------------|
| 1[10]  | 1[24] | CCGCAGGGAAGGAAAC                 |
| 2[245] | 2[232]| CCCCCGAGCCGGAAAG                 |
| 5[10]  | 5[24] | CCCCATCCGAAACAA                  |
| 6[245] | 6[231]| CCGTGTTGCCATCC                  |
| 9[11]  | 9[23] | CCCCCAAACACCTCAA                |
| 10[245]| 10[232]| CCCCCGTTTCCCCAGT            |
| 11[232]| 11[245]| GTAACGCCAGGCCCC                |
| 12[23] | 12[10]| AAATAAAGAAACCCCC                |
| 13[11] | 13[23]| CCCCCTTGCGTAGATT                |
| 14[245]| 14[232]| CCCCCAAACAGGAAGA                |
| Start | End | Cy3_staples column 1 |
|-------|-----|---------------------|
| 28[39]| 26[40]| CATAAAGGGAATAGCAATAGCTAATCAGAG |
| 30[39]| 28[40]| ACCAGTATGTTATTTTGTCACAATCAATACATA |
| 32[39]| 30[40]| GCCGCCACCATCGATAACGCACCAGCAAAATC |
| 34[39]| 32[40]| AGTGATCACCAGAAACCACCACCACCCCTAG |
| 35[32]| 34[40]| CGTATAAACAGTATTAGATACAGG |
|       |      | **Cy3_staples column 2** |
| 24[71]| 25[55]| CAGCTACAATTTATCCTGAAATCAAATCCAA |
| 25[56]| 27[55]| TAAGAAAAGTATTGAGCCTAATTCTTACCG |
| 27[56]| 29[55]| AAGCCCTTTTAGCAAACGTTAGAAAAATAGAAA |
| 29[56]| 31[55]| ATTCATATGGGAATTAGAGCCAGTAATCAG |
| 31[56]| 33[55]| TAGGCACCCACCAGAACCACAGCCTAAGAGCCGC |
|       |      | **Cy3_staples column 3** |
| 28[71]| 26[72]| GCAGTATGTTAAGAAAGTAAAGGAACAAAG |
| 30[71]| 28[72]| GAGCCATTGGTTACCAGCCACCTATTAC |
| 32[71]| 30[72]| CAGAGCCGAAATCAGTTTGGCCTACCGACTT |
| 34[71]| 32[72]| AAGCCCTTGCAGGAGGTTAAGGCAACCG |
| 35[56]| 34[72]| GCCATATTCGGAACCTATTATTTTGCTTCCAGT |
|       |      | **Cy3_staples column 4** |
| 24[95]| 25[87]| CAAGATTAGTGGCTATGTCAAAAA |
| 25[88]| 27[87]| TGAAAAATAATTACGAAACCACCCTAGATAGC |
| 27[88]| 29[87]| CGAACCAAGGCATATGAAGGTCAAGACAA |
| 29[88]| 31[87]| AGGGCGACGTAATTACCGCTTACGAG |
| 31[88]| 33[87]| AGACTGATTTTCATAAATCAATCGGAGGTC |

**Note:** The table above represents the sequences for Cy3 staples columns 1 through 4. Each column contains sequences that align with specific positions in the DNA or RNA sequence.
### Cy3_staples column 5 (FRET) or Cy5_staples column 4 (quenching)

| Start | End  |
|-------|------|
| 25[152] | 27[151] |
| 27[152] | 29[151] |
| 29[152] | 31[151] |
| 31[152] | 33[151] |
| 33[152] | 35[151] |

ATTTCAACAGTAATAAGGCTTGGCGCGCAGA
CGGTCAACTTCGCTGATAAAAATGTACTTTTT
CATGAGGAAGCGAAGACGAGCATCGTATCGGT
TTATCAGCAATAAATTTTTTCAGTCTGTAGCA
TTCCACAGCAATAGGAACCCATGGCTCAGTA

### Cy3_staples column 6 (FRET) or Cy5_staples column 3 (quenching)

| Start | End  |
|-------|------|
| 28[167] | 26[168] |
| 30[167] | 28[168] |
| 32[167] | 30[168] |
| 34[167] | 32[168] |
| 35[152] | 34[168] |

TTGTATCACATAAGGGAACCAGAAACAAGACT
CCCAGACAGTTTCCATTAACGCGAGGAGAT
TTGCGAATTTCGCTGCGGCGAGATCGTCA
TAGCAAGCAGCCCCCTCATAGTTAAAAAGGAA
CCAGGGGATAAGTGCGCAGATTCAGGGGA

### Cy3_staples column 7 (FRET) or Cy5_staples column 2 (quenching)

| Start | End  |
|-------|------|
| 25[184] | 27[183] |
| 27[184] | 29[183] |
| 29[184] | 31[183] |
| 31[184] | 33[183] |
| 33[184] | 35[183] |

TGCGATTACCCAAATCAACGTATGACCAA
CTTTGAAAGGCAACAAAGTACATGAAAAAT
ACGTAATGAGGCCGCTTTTGCGGTTTCTTA
AAGCAGTTTAGAAAGGAAACACCAGGAAC
GATCTAAAGCCACCCCCCTCATGGGTTGGA

### Cy3_staples column 8 (FRET) or Cy5_staples column 1 (quenching)

| Start | End  |
|-------|------|
| 28[199] | 26[200] |
| 30[199] | 28[200] |
| 32[199] | 30[200] |
| 34[199] | 32[200] |
| 35[184] | 34[200] |

TACCAAGCGAGGAGATGAACGGGAACCGGA
GGAGTTAACCACTACGAAGGCACCAGCGATTA
GAGTAGGAGATCGACATCAGTTGGCGCTTGC
CCCTCAGAGTTTGTCCTTTCTTCTTCCACG
TATAAGTATAGCCCGGAATAAGTGAACCGGA

### Cy5_staples column 1 (FRET) or bh2_staples column 1 (quenching)

| Start | End  |
|-------|------|
| 5[24] | 3[39] |
| 13[24] | 11[39] |
| 17[24] | 15[39] |
| 9[24] | 7[39] |
| 21[24] | 19[39] |

ATATTCAACATGAACAGACACGGCGATTAA
TTTGTTCAAACATTTAGTATAAACATCA
TGCGGTCTTCAAGTGTTAAAGTTTGAGGTAAC
CTTAGGTTAAATACATCAATTGATTACC
TCAATACACGTGGCATTGGGATGCTATTA

### Cy5_staples column 2 (FRET) or bh2_staples column 2 (quenching)

| Start | End  |
|-------|------|
| 2[71] | 4[56] |
| 6[71] | 8[56] |
| 10[71] | 12[56] |
| 14[71] | 16[56] |
| 18[71] | 20[56] |

TTTGGTTGGAAATACCTTAGTATAAACATCA
AAACAGAGATGGAGCTGAGACGACGAGAG
TTTCAAAATTATCGGAGGGGTAGAAACC
CAAAAAGAAATCAAAAGCGCTGAGAAGATC
CGGATGTAGTCTGTGATAAGAGAAATAAA
| Column 3 (quenching) | Column 4 (FRET) or bh2_staples | Column 4 (quenching) | Column 5 (FRET) or bb650q_staples | Column 4 (quenching) | Column 6 (FRET) or bb650q_staples | Column 3 (quenching) | Column 8 (FRET) or bb650q_staples | Column 1 (quenching) |
|---------------------|--------------------------------|----------------------|-----------------------------|----------------------|-------------------------------|---------------------|-------------------------------|---------------------|
| 5[56] 3[71]         | GCTCATGGACGAGCAACGGTACGCCAGA  | 9[56] 7[71]          | GAAAGGAATAGAACCCTGTAGACCTTAAAA | 13[56] 11[71]        | CAGTACCTCAATTCGAAGAACCACCAAGA | 17[56] 15[71]       | CTGATGCAGATGATGACATAAATCAATATA | 21[56] 19[71]       |
| 11[56] 9[71]        | GAAAGGAATAGAACCCTGTAGACCTTAAAA | 15[56] 13[71]        | CAGTACCTCAATTCGAAGAACCACCAAGA | 19[56] 17[71]        | AATAGATAAAATAAGTCTCTTACCAGTATAA |

Cy5_staples column 4 (FRET) or bh2_staples column 4 (quenching)

| Column 6 (FRET) or bb650q_staples | Column 3 (quenching) | Column 7 (FRET) or bb650q_staples | Column 2 (quenching) |
|-----------------------------------|---------------------|-----------------------------------|---------------------|
| 1[152] 0[152]                     | GGGCGCCAGGGTGGTTTATCCCTTTATATTAAATCAA | 2[167] 4[152]                  | ACTGCCCGCCTTTACACCGGCGGCGTTTTTCAGC |
| 5[152] 3[167]                     | TAAACATCCTTTTCAGGATCCCCCGGTACCGA  | 6[167] 8[152]                   | ACTCAATCGCTCTCACAAAGTTAAACGATGCT |
| 9[152] 7[167]                     | AAGGGATACGCCGGGCTCATAACGGAACGATGCT | 10[167] 12[152]                 | TAACCTCAAGGCGATCCACCAGGCCGAG |
| 13[152] 11[167]                   | TAAATGTGCCGGAAACGGAAGGGCGATCGGTCG | 14[167] 16[152]                 | GTTAAATCGGCGGAGATCCACCCTTCTAGCTG |

Cy5_staples column 5 (FRET) or bb650q_staples column 4 (quenching)

| Column 8 (FRET) or bb650q_staples | Column 1 (quenching) |
|-----------------------------------|---------------------|
| 5[184] 3[199]                     | ATCGTAACTCACATTAGTCTAGTCTAGT |
| 9[184] 7[199]                     | GAAACACGGGGTGTACACGTGAGTCTAGT |
| 13[184] 11[199]                   | CTCGTTGGGTTGAGTACACGTGAGTCTAGT |
| 17[184] 15[199]                   | GCAAGGATGTTAAAAATCGGAAAAACTAGCA |
| 21[184] 19[199]                   | CGCGTTTTTTACATTAGTTTGGACGACCAAGA |

Start End Locks for ODN-153 (blue) and ODN-342 (orange)
| Sequence | Description |
|----------|-------------|
| AGCCCCCGATTAGAGCTTTTGATCACTTTTGTGACTATGCAA | Locks for miR-21 (left side) |
| CTATCGGCCTTGGCTGGTAATTTGATCACTTTTGTGACTATGCAA | |
| GGTCTGAGAGACTACCTTTTTGATCACTTTTGTGACTATGCAA | |
| CTGTCCAGACGACGACAATTTGATCACTTTTGTGACTATGCAA | |
| CTGTTTAGCTATATTTTCCAACGTTGCGATTTCGTTGACTAGA | |
| AGTCACAAAAGGTGATCAGTGCCTTGAGTAACAGTGC | Locks for let-7a (right side) |
| ATCAGACTGATGTTGATGCCAGTTACAAAATAAACAGACACAGAGTTAAGAAGGTAT | |
| ATCAGACTGATGTTGATAATAAGAGCAAGAAACAATTGGCAACA | |
| ATCAGACTGATGTTGAGCCACCACCCTCAGAGCCGCGGTAATAAGTTTTAAC | |
| ATCAGACTGATGTTGACAGTGCCTTGAGTAACAGTGC | |

Table S2: List of the target key oligonucleotide sequences
| miRNAs (or DNA analogs) | Sequence                      |
|-------------------------|-------------------------------|
| ODN-342                 | TCTCACACAGAAATCGACCGT (GC%-52.2) |
| ODN-153                 | TTGCATAGTCACAAAAGTGATC (GC%-40.9) |
| let-7a                  | UGAGGUAGUAGGUUGUAUAGU (GC%-36.4) |
| miR-21                  | UCAACAUCAUGCUAAAGCUA (GC%-36.4) |
| non-target              | CATCATAATTAAAACATAAT (GC%-14.2) |

References

(1) Selnihhin, D.; Sparvath, S. M.; Preus, S.; Birkedal, V.; Andersen, E. S. Multifluorophore DNA Origami Beacon as a Biosensing Platform. *ACS Nano* 2018, 12 (6), 5699-5708.

(2) Stephanopoulos, N. Strategies for Stabilizing DNA Nanostructures to Biological Conditions. *Chembiochem* 2019, 20 (17), 2191-2197.

(3) Schmied, J. J.; Raab, M.; Forthmann, C.; Pibiri, E.; Wunsch, B.; Dammeyer, T.; Tinnefeld, P. DNA origami-based standards for quantitative fluorescence microscopy. *Nat Protoc* 2014, 9 (6), 1367-1391.

(4) Sorensen, R. S.; Okholm, A. H.; Schaffert, D.; Kodal, A. L.; Gothelf, K. V.; Kjems, J. Enzymatic ligation of large biomolecules to DNA. *ACS Nano* 2013, 7 (9), 8098-8104.