# **Supplementary Information**

# High-affinity five/six-letter DNA aptamers with superior specificity enabling the detection of dengue NS1 protein variants beyond the serotype identification

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**Supplementary Fig. S5:** Alignment of the random-region DNA sequences obtained by the three ExSELEX procedures, targeting DEN1-NS1.

**Supplementary Fig. S6:** Alignment of the random-region DNA sequences obtained by the three ExSELEX procedures, targeting DEN2-NS1.

Supplementary Fig. S7: Alignment of the random-region DNA sequences obtained by the three ExSELEX procedures, targeting DEN3-NS1.

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Supplementary Fig. S9: Confirmation of the presence of diol-Px in the selected clone family, D2-1.

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Supplementary Fig. S11: Differences in the amino acid sequences of DEN-NS1 proteins in the clinical samples.

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**Supplementary Fig. S15:** Differences in the amino acid sequences of DEN1-NS1 proteins in the clinical samples, PD1-1 through PD1-21.

Supplementary Fig. S16: NS1 sequence variations of dengue serotype 1 clinical samples.

Supplementary Fig. S17: Possible topologies of the G-quadruplex of AptD2-1.

**Supplementary Table S1. Patient sample information.** The recruited patients were tested and confirmed as DENV NS1 positive by routine hospital diagnostics, using the SD BIOLINE NS1 Ag rapid test, and had fevers within 3–5 days from illness onset. The DENV infection was confirmed by an RT-qPCR analysis of the samples, and the dengue serotypes were determined by an FTD dengue differentiation RT-qPCR test from Fast Track Diagnostics, using a Bio-Rad CFX96 PCR system. The samples in Figure 5 are indicated with asterisks.

| Patient S | Sample | Fever day<br>(from illness<br>onset) | Serotype<br>(RT-qPCR) | SD BIOLINE<br>Dengue NS1<br>Ag rapid test |
|-----------|--------|--------------------------------------|-----------------------|---|
| PD1-1*    | Serum  | 5                                    | D1                    | +   |
| PD1-2*    | Serum  | 4                                    | D1                    | +   |
| PD1-3*    | Serum  | 4                                    | D1                    | +   |
| PD1-4     | Serum  | 5                                    | D1                    | +   |
| PD1-5     | Serum  | 4                                    | D1                    | +   |
| PD1-6     | Serum  | 3                                    | D1                    | +   |
| PD1-7     | Serum  | 4                                    | D1                    | +   |
| PD1-8     | Serum  | 4                                    | D1                    | +   |
| PD1-9     | Serum  | 4                                    | D1                    | +   |
| PD1-10    | Serum  | 5                                    | D1                    | +   |
| PD1-11    | Serum  | 5                                    | D1                    | +   |
| PD1-12    | Serum  | 3                                    | D1                    | +   |
| PD1-13    | Serum  | 5                                    | D1                    | +   |
| PD1-14    | Serum  | 4                                    | D1                    | +   |
| PD1-15    | Serum  | 3                                    | D1                    | +   |
| PD1-16    | Serum  | 4                                    | D1                    | +   |
| PD1-17    | Serum  | 4                                    | D1                    | +   |
| PD1-18    | Serum  | 5                                    | D1                    | +   |
| PD1-19    | Serum  | 4                                    | D1                    | +   |
| PD1-20    | Serum  | 5                                    | D1                    | +   |
| PD1-21    | Serum  | 5                                    | D1                    | +   |
| PD1-22    | Serum  | 3                                    | D1                    | +   |
| PD2-1*    | Plasma | 3                                    | D2                    | +   |
| PD2-2*    | Serum  | 3                                    | D2                    | +   |
| PD2-3*    | Serum  | 5                                    | D2                    | +   |
| PD3-1*    | Serum  | 5                                    | D3                    | +   |
| PD3-2*    | Serum  | 4                                    | D3                    | +   |
| PD3-3*    | Serum  | 4                                    | D3                    | +   |
| PD4-1*    | Serum  | 3                                    | D4                    | +   |

Supplementary Table S2. ExSELEX conditions targeting each DEN-NS1 serotype. We performed ExSELEX targeting each serotype of DEN-NS1 proteins, as follows: DEN1-NS1 (D1), DEN2-NS1 (D2), DEN3-NS1 (D3), and DEN4-NS1 (D4) in the PCR cycles column. To increase the stringency of the selection conditions, we added human serum (HS) and/or BSA to the binding buffer (additives) and urea in the washing buffer in later rounds.

| ExSELEX | -1     |      |        |        |                  |        |            |                          |           |            |     |     |     |
|---------|--------|------|--------|--------|------------------|--------|------------|--------------------------|-----------|------------|-----|-----|-----|
| Round   | Mathad | DNA  | Target | Volume | Additives        | Bir    | nding      | Washing                  | Counter   | PCR cycles |     |     |     |
| Round   | Method | [nM] | [nM]   | [mL]   | Adultives -      | Buffer | Time (min) | wasiling                 | Selection | D1         | D2  | D3  | D4  |
| 1       | A      | 500  | 5      | 8      | -                | BB1    | 60         | BB1 ×3                   | -         | 18         | 18  | 18  | 18  |
| 2       | A      | 100  | 5      | 1      | -                | BB1    | 30         | BB1 ×5                   | Pre       | 10         | 12  | 10  | 12  |
| 3       | В      | 20   | 4      | 0.2    | 0.1% BSA         | BB1    | 30         | WB ×5                    | Pre       | 25         | 29  | 28  | 23  |
| 4       | В      | 5    | 4      | 0.2    | 0.1% BSA, 5% HS  | BB1    | 30         | WB ×10                   | Pre, Post | 14         | 18  | 22  | 22  |
| 5       | В      | 5    | 0.4    | 0.2    | 0.1% BSA,10% HS  | BB1    | 30         | WB ×25                   | Pre, Post | 15         | 17  | 14  | 17  |
| 6       | В      | 5    | 0.4    | 0.2    | 0.1% BSA, 20% HS | BB1    | 30         | WB (+ 20% HS) ×3, WB ×5  | Pre, Post | 12         | 15  | 12  | 14  |
| 7       | В      | 1    | 0.4    | 0.3    | 0.1% BSA, 50% HS | BB1    | 30         | WB (+ 50% HS) x3, WB x10 | Pre, Post | 13         | 16  | 16  | 18  |
| 8       | В      | 1    | 0.04   | 0.6    | 0.1% BSA, 50% HS | BB1    | 30         | WB (+ 50% HS) x3, WB x10 | Pre, Post | 20         | 21  | 23  | 23  |
| 9       | С      | 1    | 0.167  | 1      | 0.1% BSA         | BB1    | 30         | WB (+ 2M urea) ×3, WB ×2 | Pre, Post | 28         | 22  | 24  | 19  |
|         |        |      |        |        |                  |        |            |                          | Total     | 155        | 168 | 167 | 166 |

ExSELEX-2

| LYOLLY | -2     |      |        |        |                  |        |            |                             |           |     |     |       |     |
|--------|--------|------|--------|--------|------------------|--------|------------|-----------------------------|-----------|-----|-----|-------|-----|
| Pound  | Mathad | DNA  | Target | Volume | A dditives       | Bir    | nding      | Washing                     | Counter   |     | PCR | cycle | s   |
| Round  | Method | [nM] | [nM]   | [mL]   | Additives        | Buffer | Time (min) | washing                     | Selection | D1  | D2  | D3    | D4  |
| 1      | С      | 500  | 5      | 8      | -                | BB1    | 60         | BB1 x3                      | -         | 20  | 20  | 20    | 20  |
| 2      | С      | 100  | 5      | 1      | -                | BB1    | 30         | BB1 ×5                      | Pre       | 22  | 22  | 19    | 20  |
| 3      | В      | 50   | 2.5    | 0.4    | 0.1% BSA, 10% HS | BB1    | 30         | WB ×5                       | Pre       | 15  | 21  | 25    | 21  |
| 4      | В      | 10   | 1      | 0.4    | 0.1% BSA, 50% HS | BB1    | 30         | WB ×10                      | Pre, Post | 20  | 24  | 25    | 19  |
| 5      | С      | 3    | 1      | 1      | 0.1% BSA         | BB1    | 15         | BB1 (+ 3 M urea) x3, BB1 x2 | Pre, Post | 26  | 20  | 27    | 19  |
| 6      | В      | 3    | 1      | 0.4    | 0.1% BSA, 50% HS | BB1    | 30         | WB ×10                      | Pre, Post | 18  | 20  | 24    | 16  |
| 7      | С      | 3    | 1      | 1      | 0.1% BSA         | BB1    | 15         | BB1 (+ 3 M urea) x3, BB1 x2 | Pre,Post  | 24  | 18  | 27    | 18  |
| 8      | В      | 1    | 0.5    | 0.4    | 0.1% BSA, 50% HS | BB1    | 30         | WB ×10                      | Pre, Post | 23  | 23  | 25    | 21  |
| 9      | В      | 1    | 0.5    | 0.4    | 0.1% BSA,50% HS  | BB1    | 30         | WB ×20                      | Pre, Post | 23  | 24  | 27    | 21  |
| 10     | D      | 20   | 10     | 0.02   | -                | BB1    | 30         | -                           | -         | 12  | 12  | 12    | 12  |
|        |        |      |        |        |                  |        |            |                             | Total     | 203 | 204 | 231   | 187 |

ExSELEX-3

| Pound | Method | DNA  | Target | Volume | Additives        | Bir    | nding      | Washing                                    | Counter   |     | PCR | cycles |
|-------|--------|------|--------|--------|------------------|--------|------------|--|-----------|-----|-----|--------|
| Round | Method | [nM] | [nM]   | [mL]   | Additives        | Buffer | Time (min) | vvasning                                   | Selection | D1  | D2  | D3     |
| 1     | В      | 2500 | 5      | 0.8    | 0.1% BSA, 10% HS | BB2    | 30         | WB ×3                                      | -         | 21  | 22  | 20     |
| 2     | В      | 250  | 5      | 0.3    | 0.1% BSA, 10% HS | BB2    | 30         | WB ×5                                      | Pre       | 15  | 20  | 21     |
| 3     | В      | 50   | 5      | 0.3    | 0.1% BSA, 20% HS | BB2    | 30         | WB ×5                                      | Pre       | 15  | 15  | 15     |
| 4     | В      | 5    | 1      | 0.3    | 0.1% BSA, 45% HS | BB2    | 30         | WB (+2 M urea) ×3, WB ×2                   | Pre       | 24  | 27  | 23     |
| 5     | В      | 1    | 0.2    | 0.3    | 0.1% BSA, 45% HS | BB2    | 10         | WB (+2 M urea) ×3, WB ×2                   | Pre       | 24  | 25  | 28     |
| 6     | В      | 1    | 0.2    | 0.3    | 0.1% BSA, 45% HS | BB2    | 5          | WB (+50% HS) x2, WB (+ 2 M urea) x2, WB x2 | Pre       | 23  | 20  | 27     |
| 7     | В      | 0.5  | 0.2    | 0.3    | 0.1% BSA, 45% HS | BB2    | 5          | WB (+50% HS) x3, WB (+ 3 M urea) x3, WB x3 | Pre       | 25  | 25  | 29     |
|       |        |      |        |        |                  |        |            |  | Total     | 147 | 154 | 163    |

Separation of DNA-target complexes (Method):

A: Ultrafiltration (Amicon Ultra-100kDa)

B: Sandwich (Capture with mAb#D06, in 96-well plates)

C: Complex immobilization (Dynabeads™ His-Tag Isolation and Pulldown)

D: Separation by gel-mobility shift [4% PAGE (29:1 acrylamide -bisacrylamide) supplemented with 5% glycerol and 2 M urea] Buffers:

BB1: 20 mM Tris-HCI (pH 7.5), 150 mM NaCI, 1 mM MgCI<sub>2</sub>, 2.7 mM KCI, 0.005% Nonidet-P40

BB2: 20 mM Tris-HCI (pH 7.5), 150 mM NaCI, 1 mM MgCI<sub>2</sub>, 2.7 mM KCI, 2% Tween 20

WB: 20 mM Tris-HCI (pH 7.5), 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 2.7 mM KCI, 0.05% Tween 20

**Supplementary Table S3. ExSELEX conditions targeting DEN1-NS1 variant 2.** We performed ExSELEX targeting recombinant DEN1-NS1 variant 2 protein and clinical serum (PD1-13, the antigen NS1 sequence was the same as that of variant 2). To increase the stringency of the selection conditions, we added human serum (HS) and BSA to the binding buffer (additives) and urea in the washing buffer in later rounds.

### **ExSELEX-4**

|       | Selectio    | DNA  | Tar                       | get                    | Volume |                  | Bir    | nding     |                     | Counter   | PCR        |  |
|-------|-------------|------|---------------------------|------------------------|--------|------------------|--------|-----------|---------------------|-----------|------------|--|
| Round | n<br>Method | [nM] | DEN1-NS<br>variant 2 [nM] | Clinical<br>Serum [µl] | [ml]   | Aditives         | Buffer | Time(min) | Washing             | Selection | Cycle<br>s |  |
| 1     | С           | 500  | 2.5                       | -                      | 0.8    | -                | BB1    | 60        | WB1 × 3             | -         | 20         |  |
| 2     | С           | 200  | 2.5                       | -                      | 0.3    | -                | BB1    | 30        | WB1 × 5             | Pre       | 20         |  |
| 3     | В           | 50   | 2.5                       | -                      | 0.4    | 0.1% BSA         | BB2    | 30        | WB2 × 6             | Pre, Post | 22         |  |
| 4     | В           | 10   | -                         | 20                     | 0.4    | 0.1% BSA, 10% HS | BB2    | 30        | WB2 × 6             | Pre, Post | 15         |  |
| 5     | В           | 10   | -                         | 10                     | 0.4    | 0.1% BSA, 10% HS | BB2    | 30        | WB2 (+ 3M Urea) ×10 | Pre, Post | 28         |  |
| 6     | В           | 3    | 1                         | -                      | 0.4    | 0.1% BSA, 10% HS | BB2    | 30        | WB2 (+ 3M Urea) ×10 | Pre, Post | 22         |  |
| 7     | В           | 3    | -                         | 5                      | 0.4    | 0.1% BSA, 10% HS | BB2    | 30        | WB2 (+ 3M Urea) ×10 | Pre, Post | 28         |  |
|       |             |      |                           |                        |        |                  |        |           |                     | Total     | 155        |  |

### Separation of DNA-Target Complexes (Method) :

B: Sandwich (Capture with mAb#D06, in 96-well plates)

C: Complex immobilization (Dynabeads<sup>™</sup> His-Tag Isolation & Pulldown)

### Buffers :

BB1: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 2.7 mM KCl, 5 mM Imidazole, 0.005% Nonidet-P40 BB2: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 2.7 mM KCl, 5 mM Imidazole, 0.05% Tween 20 WB1: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 2.7 mM KCl, 0.005% Nonidet P-40

WB2: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl\_2, 2.7 mM KCl, 0.05% Tween 20

# **Supplementary Table S4: Summary of the sequence reads and clustering information for each enriched library.** The unnatural bases in the DNA libraries were replaced with the natural bases by replacement PCR in the presence of dPa'TP (Pa') or diol-dPxTP (Px), and the natural-base libraries were subjected to deep sequencing with an IonPGM system using the indicated chip types. "Total Read" corresponds to the sequencing reads after automated QC in the IonPGM Torrent Server, and "Extract" corresponds to the merged extracted reads after the primer sequencing trimming criteria: 5'-(full sequence of the forward/reverse primer)-[42 bases]-(complementary sequence of the last six bases of the reverse/forward primer)-3'. The merged extracted sequences were then clustered based on the sequence similarities, using in-house Perl scripts. The clustering criteria were to cluster a sequence into the same family if the mismatch between the sequence and the top sequence is less than six, and the cut-off of the family was set to 20 reads. It should be noted that the same family mainly comes from the same clone sequence, but harboring different natural base patterns at the unnatural base positions and with sequencing errors, besides similar clones with some mutations, which belong to a usual 'family" in the case of natural-base aptamers. We mainly focused on the top-ranked families for further analysis, since the enriched libraries exhibited clear binding to each target in gel-mobility shift assays (EMSA).

|              |       |         | Ic      | onPGM   | -       |         | Total c | lustered |                  | Selected                |                             |  |  |
|--------------|-------|---------|---------|---------|---------|---------|---------|----------|------------------|-------------------------|-----------------------------|--|--|
| FySFL FX     | Round | Ion BCM | Total   | Read    | Extra   | acted   | family  | number   |                  | families for further an | alysis (Pa')                |  |  |
| LIGHEN       | Round | chip    | Pa'     | Px      | Pa'     | Px      | Pa'     | Рх       | Family<br>number | Selection<br>criteria   | Covered population<br>(Pa') |  |  |
| ExSELEX-1-D1 | 9     | 314     | 103,693 | 145,765 | 39,389  | 53,596  | 89      | 93       | 17               | >500 reads (1.3%)       | 63.1%                       |  |  |
| ExSELEX-2-D1 | 10    | 314     | 103,319 | 106,064 | 56,393  | 56,411  | 26      | 21       | 6                | >500 reads (0.9%)       | 94.3%                       |  |  |
| ExSELEX-3-D1 | 7     | 316     | 496,332 | 465,076 | 269,007 | 288,202 | 294     | 275      | 14               | >2% populations         | 60.9%                       |  |  |
| ExSELEX-1-D2 | 9     | 314     | 95,511  | 131,017 | 50,663  | 75,426  | 25      | 36       | 10               | >500 reads (1%)         | 93.8%                       |  |  |
| ExSELEX-2-D2 | 10    | 314     | 90,880  | 93,077  | 66,877  | 57,383  | 11      | 12       | 8                | >500 reads (0.7%)       | 98.5%                       |  |  |
| ExSELEX-3-D2 | 7     | 316     | 425,100 | 542,125 | 293,912 | 362,713 | 93      | 121      | 5                | >2% populations         | 82.4%                       |  |  |
| ExSELEX-1-D3 | 9     | 314     | 67,232  | 117,162 | 45,611  | 79,771  | 12      | 13       | 4                | >500 reads (1.1%)       | 96.8%                       |  |  |
| ExSELEX-2-D3 | 10    | 314     | 90,946  | 70,574  | 64,544  | 46,669  | 10      | 4        | 2                | >500 reads (0.8%)       | 96.8%                       |  |  |
| ExSELEX-3-D3 | 7     | 316     | 509,598 | 432,128 | 211,895 | 158,682 | 61      | 45       | 7                | >2% populations         | 88.4%                       |  |  |
| ExSELEX-1-D4 | 9     | 314     | 58,514  | 130,996 | 39,094  | 88,975  | 16      | 15       | 8                | >500 reads (1.3%)       | 97.0%                       |  |  |
| ExSELEX-2-D4 | 10    | 314     | 104,741 | 87,325  | 43,797  | 38,672  | 31      | 29       | 10               | >500 reads (1.1%)       | 88.5%                       |  |  |
| ExSELEX-4-D1 | 7     | 314     | 99,705  | 95,668  | 43,385  | 33,387  | 9       | 10       | 2                | >500 reads (1.2%)       | 95.1%                       |  |  |

**Supplementary Table S5: Sequences of anti-DEN-NS1 DNA aptamer candidates.** The oligonucleotide sequences used for the binding analyses against each target DEN-NS1 are summarized with the results of the electrophoresis gel-mobility shift assay (EMSA) and the surface plasmon resonance (SPR) analysis. The additional complementary sequences are underlined, and the changed sequences are shown in pink. The sequences in the constant primer regions are indicated in lower-case letters. The oligonucleotides containing a mini-hairpin sequence, CGCG-(Biotin-T)-AGCG, at the 3'-terminus have an additional "h" in the aptamer candidate names. In the SPR analysis with 20 nM of each dengue NS1 protein, "specific" means that the oligonucleotide only bound to the target serotype DEN-NS1, and not to the other serotype DEN-NS1, while "less-specific" means the oligonucleotide exhibited binding to not only the target serotype NS1 but also to some of the other serotype NS1 proteins. The chemical structures of the unnatural bases, diol-Px (Px) and diol-Pa (Pa), are illustrated at the bottom of the table.

| Name              | EMSA -    | SI   | R .          | Sequence (5'- to -3': L = Biotin-dT, x = dDs)  |
|-------------------|-----------|--|--------------|--|
| D1-1-78           | (++) +++  | $K_{\rm D} = 132  \rm pM$                    | specific     | $\label{eq:lastgtctactgTGTGAxGTCCTACAATGGACTGGTGTxCTCGGxATGGCCATTgacaagcggagtagttagacc}$   |
| D1-1-42h          | +++       | $K_{\rm D} = 197  {\rm pM}$                  | specific     | CAGACGGACTGGTGT*CTCGG*ATGG <u>CCGTCTC</u> CGCGLAGCG  |
| D1-1-48h (AptD1)  | +++       | $K_{\rm D} = 182  {\rm pM}$                  | specific     | CCCCAGACGGACTGGTGTxCTCGGxATGG <u>CCGTCTGGGGCGCGLAGCG</u>   |
| Cont-D1-1-48h     | -         | $K_{\rm D} = 1.3 \ \mu M$                    |              | CCCCAGACGGACTGGTGTACTCGGAATGG <u>CCGTCTGGGGCGCGCGLAGCG</u>   |
| D1-2-78           | (+)       |  |              | ${\tt L} {\tt gatatggtctactgAGGAGCGCATGTCGAGATACCAACCACCAATCCATCCTTgacaagcggagtagttagacc}$   |
| D1-3-78           | (+++) +++ | $K_{\rm D} = 55  {\rm pM}$                   | non-specific | LgatatggtctactgACGCCGGGGCCCGTAXTCAGACGTATACXCATCAGGGCACATgacaagcggagtagttagacc   |
| D1-3-47           | +++       | K <sub>D</sub> = 98 pM                       | non-specific | CGAGGCCCGTAXTCAGACGTATACXCATCAGGGCCTCCCCGCGLAGCG   |
| D1-4-78           | (-)       |  |              | LgatatggtctactgCCT6CACTGCCTCTGxAGCCCAACCxACCCATCCAATCGCTCAgacaagcggagtagttagacc  |
| D1-5-61h          | ++        |  |              | GCCAGCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC   |
| D1-6-47h          | ++        |  |              | GCTCCCTxGTACCAACCCCCCTCCAATCxATTAGGCAGCCGCGLAGCG   |
| D1-7-51h          | ++        |  |              | CGTGCGACGAXGTCCAACCAGTCCCAATCXACAAGTCGCACGCGCGLAGCG  |
| Name              | EMSA      | SI   | ?R           | Sequence (5'- to -3': 1 = Biotin, L = Biotin-dT, x = dDs, d = Diol-dPa, y = Diol-dPx)  |
| D2-1-78           | (-)       |  |              | LgatatggtctactgGTCCG <mark>x</mark> CTGGGAACAAG <mark>x</mark> GGCGGGAGGGA <b>x</b> GGGTGTGGGTGCGgacaagcggagtagttagacc   |
| D2-1-96(3Ds)      | -         |  |              | $\tt tttcgcactccatgatatggtctactgGTCCG {\tt xCTGGGAACAAG {\tt xGGCGGGAGGGA {\tt xGGCTGTGGGTGCgacaagcggagtagttagaccgtcaaa}$  |
| D2-1d-97          | ++        | $K_{\rm D} = 114 \text{ pM}$                 |              | ${\tt L} {\tt tttcgcactccatgatatggtctactgGTCCG} {\tt x} {\tt CTGGGAACAAG} {\tt x} {\tt GGCGGGAGGGA} {\tt GGGTGTGGGTGCgacaagcggagtagttagaccgtcaaa}$   |
| D2-1y-96          | +++       | $K_{\rm D} = 41 \ \rm pM$                    |              | $\tt ltttcgcactccatgatatggtctactgGTCCG{\tt x} CTGGGAACAAG{\tt x} GGCGGGAGGA{\tt y} GGGTGTGGGTGCgacaagcggagtagttagaccgtcaaa$  |
| D2-1d-84          | +         |  |              | ${\tt L} {\tt tttcgcactccatgatatggtctactgGTCCG} {\tt x} {\tt CTGGGAACAAG} {\tt x} {\tt GGCGGGAGGGA} {\tt GGGTGTGGGTGCgacaagcggagtag}$  |
| D2-1d-74          | ++        |  |              | LcatgatatggtctactgGTCCG <mark>x</mark> CTGGGAACAAG <mark>x</mark> GGCGGGAGGGAdGGGTGTGGGTGCGgacaagcggagtag  |
| D2-1d-87h         | ++        | $K_{\rm D} = 105 \ \rm pM$                   | specific     | <u>GACggtcta</u> ctgGTCCG <mark>x</mark> CTGGGAACAAG <mark>x</mark> GGCGGGAGGGA <mark>d</mark> GGGTGTGGGTGCGgacaagcggagtagt <u>tagaccgtc</u> CGCGLAGCG   |
| D2-1d-77h         | ++        |  |              | <u>ggtctact</u> gGTCCG <mark>x</mark> CTGGGAACAAG <mark>x</mark> GGCGGGAGGGA <mark>d</mark> GGGTGTGGGTGCGgacaagcg <u>gagtagACC</u> CGCGLAGCG   |
| D2-1d-72h (AptD2) | ++/+++    | $K_{\rm D} = 104 \ \rm pM$                   | specific     | <u>GGctgGTCC</u> GxCTGGGAACAAG <mark>x</mark> GGCGGGAGGAdGGGTGTGGGTGCgacaag <u>cggaCCagCC</u> G <b>CGGAGCG</b>   |
| Cont-D2-1d-72h    | -         |  |              | <u>GGctgGTCC</u> GACTGGGAACAAGAGGCGGGAGGGATGGGTGGGGTG  |
| D2-1d-61h         | +++       |  |              | g <u>GTCC</u> Gx <mark>CT</mark> GGGAACAAGxGGCGGGAGGGAdGGGTGTGGGTGCGgaca <u>agcgg</u> CGCGLAGCG  |
| D2-1d-51h         | -         |  |              | gGTCCGxCTGGGAACAAGxGGCGGGAGGGAdGGGTGTGGGTGCGCGLAGCG  |
| D2-2-78           | (-)       |  |              | LgatatggtctactgGAATAACAAGTCCGTGC <b>x</b> GTCG <b>CCAATC</b> CGTG <b>xTCCAACC</b> CCgacaagcggagtagttagacc  |
| D2-2d-59h         | +         |  |              | <u>GCGGTCCG</u> TGC <b>x</b> GTCG <b>CCAATC</b> CGTG <b>d</b> TCCAACCCCgacaag <u>eggaCCGC</u> CGCGLAGCG  |
| D2-3-78           | (-)       |  |              | LgatatggtctactgGTCTACATGCAACGCTTTCGxCCAACCCGTGxTCCAATCCCCAgacaagcggggtagttagacc  |
| D2-3d-52h         | ++        |  |              | <u>GCCCGCTTTC</u> GxCCAACCCGTGdTCCAATCCCA <sub>GB</sub> AAGCGGGCCGCGLAGCG  |
| D2-4-78           | (-)       |  |              | LgatatggtctactgCTTCACGAGTCAAGG <b>x</b> CTCCAATCCGTG <b>x</b> TCCAACCAGTTTTgacaageggagtagttagace   |
| D2-4d-56h         | +         |  |              | <u>CocccgtCAA</u> GGxCTCCAATCCGTGdTCCAACCAGTT <u>TTgacGCgcg</u> CGCGLAGCG  |
| D2-5-46h          | +         |  |              | <u>GCCCGCGTG</u> CTCAACCTTACCAATCTGx <u>CACGGGGC</u> CGCGLAGCG   |
| D2-5-48h          | +         |  |              | <u>GCCCTGCGx</u> GCTCAACCTTACCAATCTGxCA <u>CGCAGGC</u> CGCGLAGCG   |
| D2-6-54h          | -         |  |              | <u>GCCCTGTCGAG</u> AxGCACCAACCAACCAACTGx <u>CTTGACAGGGC</u> CGCGLAGCG  |
| Name              | EMSA      | SI   | ?R           | Sequence (5'- to -3': L = Biotin-dT, x = dDs)  |
| D3-1-85           | (++)      |  |              | LactccatgatatggtctactgATAGTACTCC <b>x</b> GTTTAACTCTGA <b>x</b> ACTTGACGTCCATTCATAgacaageggagtagttagacc  |
| D3-2-78           | (+++) +++ | $K_{\rm D} = 41  \rm pM$                     | specific     | LgatatggtctactgAAGTGTTGCATCTAxCCTGGCCxTGTGGTACTGTACGGCTgacaagcggggtagttagacc   |
| D3-2-59h (AptD3)  | ++++      | $K_{\rm D} = 57  \mathrm{pM}$                | specific     | <u>CCCCTTGTCA</u> TCTAXCCTGGCCXTGTGGTACT5TAACGGC <u>Tgacaaccg</u> CGCLACG  |
| Cont-D3-2-59h     | -         | $K_{\rm D} = 0.19 \ \mu M$                   |              | <u>CCCUTIGICA</u> TCTAACCTGGCATGTGGTACTGTAACGCG <u>TGACAAGCGG</u> CGCGLAGCG  |
| D3-3-78           | (+)       |  |              | LgatatggtetactgGGGCTGGGTCTTGCGTTTGCGTTTGCGTGCCGGCCGGTAAgacaagoggagtagttagacc   |
| Name              | EMSA      | SE<br>K = 40 = M                             | r <b>k</b>   | Sequence (5'- to -3': L = BIOLIN-TI, X = GDS)  |
| D4-1-78           | (+++) +++ | $K_{\rm D} = 42  \text{pm}$                  | specific     |  |
| D4-1-57/11        | +++       | $\Lambda_D = 29$ pm                          | specific     |  |
| D4-2-78           | (+++)     | F = 34 mH                                    | enceifie     | August use stand so the Color Analysis Gardands to Gardan to Tanden Tadda ago Cagada use to an to an an an an an   |
| D4-3-57b (anto4)  | (+++)+++  | $\pi_{\rm D} = 34  \rm pM$                   | specific     |  |
| Cont-D4-2-57k     | ***       | $\mathbf{x}_{\mathrm{D}} = 30  \mathrm{pM}$  | specific     |  |
| D4-4-79           | (+++)     | $\mathbf{x}_{\mathrm{D}} = 2.0  \mathrm{nM}$ |              |  |
| D4-4-70           | (++)      |  |              | Tablatatotatotatanoo Neventario Control Contro |
| D4-0-10           | (++)      |  |              | MyatatyytetaetytetaetytetaAtioinamaooniioonamamaagacaagcggagtagttagacc   |

In parentheses: EMSA using 2M urea gel Relative shifted ratio (%)

- -: <10%
- +: 10-40%
- ++: 40-60%
- +++: >60%



| DEN1-NS1 | DSGCVINWKGRELKCGSGIFVTNEVHTWTEQYKFQADSPKRLSAAIGKAWEEGVCGIRSA | 60  |
|----------|--|-----|
| DEN2-NS1 | DSGCVVSWKNKELKCGSGIFITDNVHTWTEQYKFQPESPSKLASAIQKAHEEGICGIRSV | 60  |
| DEN3-NS1 | DMGCVINWKGKELKCGSGIFVTNEVHTWTEQYKFQADSPKRLATAIAGAWENGVCGIRST | 60  |
| DEN4-NS1 | DMGCVASWSGKELKCGSGIFVVDNVHTWTEQYKFQPESPARLASAILNAHKDGVCGIRST | 60  |
|          | * *** .*:********:.:::***************                        |     |
| DEN1-NS1 | TRLENIMWKQISNELNHILLENDMKFTVVVGDVSGILAQGKKMIRPQPMEHKYSWKSWGK | 120 |
| DEN2-NS1 | TRLENLMWKQITPELNHILSENEVKLTIMTGDIKGIMQAGKRSLRPQPTELKYSWKTWGK | 120 |
| DEN3-NS1 | TRMENLLWKQIANELNYILWENNIKLTVVVGDTLGVLEQGKRTLTPQPMELKYSWKTWGK | 120 |
| DEN4-NS1 | TRLENVMWKQITNELNYVLWEGGHDLTVVAGDVKGVLTKGKRALTPPVSDLKYSWKTWGK | 120 |
|          | **:**::***: ***::* *:*::.** *:: **: : * : *                  |     |
| DEN1-NS1 | AKIIGADVQNTTFIIDGPNTPECPDNQRAWNIWEVEDYGFGIFTTNIWLKLRDSYTQVCD | 180 |
| DEN2-NS1 | AKMLSTESHNQTFLIDGPETAECPNTNRAWNSLEVEDYGFGVFTTNIWLKLKEKQDVFCD | 180 |
| DEN3-NS1 | AKIVTAETQNSSFIIDGPNTPECPSASRAWNVWEVEDYGFGVFTTNIWLKLREVYTQLCD | 180 |
| DEN4-NS1 | AKIFTPEARNSTFLIDGPDTSECPNERRAWNSLEVEDYGFGMFTTNIWMKFREGSSEVCD | 180 |
|          | **:. : :* :*:**** ***. **** ***********                      |     |
| DEN1-NS1 | HRLMSAAIKDSKAVHADMGYWIESEKNETWKLARASFIEVKTCIWPKSHTLWSNGVLESE | 240 |
| DEN2-NS1 | SKLMSAAIKDNRAVHADMGYWIESALNDTWKIEKASFIEVKNCHWPKSHTLWSNGVLESE | 240 |
| DEN4-NS1 | HRLMSAAIKDQKAVHADMGYWIESSKNQTWQIEKASLIEVKTCLWPKTHTLWSNGVLESQ | 240 |
| DEN3-NS1 | HRLMSAAVKDERAVHADMGYWIESQKNGSWKLEKASLIEVKTCTWPKSHTLWTNGVLESD | 240 |
|          | •*****•**•****************************                       |     |
| DEN1-NS1 | MIIPKIYGGPISQHNYRPGYFTQTAGPWHLGKLELDFDLCEGTTVVVDEHCGNRGPSLRT | 300 |
| DEN2-NS1 | MIIPKNLAGPVSQHNYRPGYHTQITGPWHLGKLEMDFDFCDGTTVVVTEDCGNRGPSLRT | 300 |
| DEN4-NS1 | MLIPKSYAGPFSQHNYRQGYATQTVGPWHLGKLEIDFGECPGTTVTIQEDCDHRGPSLRT | 300 |
| DEN3-NS1 | MIIPKSLAGPISQHNYRPGYHTQTAGPWHLGKLELDFNYCEGTTVVITESCGTRGPSLRT | 300 |
|          | **** ******* ** ** ** *****************                      |     |
| DEN1-NS1 | TTVTGKTIHEWCCRSCTLPPLRFKGEDGCWYGMEIRPVKEKEENLVKSMVSA 352     |     |
| DEN2-NS1 | TTASGKLITEWCCRSCTLPPLRYRGEDGCWYGMEIRPLKEKEENLVNSLVTA 352     |     |
| DEN4-NS1 | TTASGKLVTQWCCRSCTMPPLRFLGEDGCWYGMEIRPLSEKEENMVKSQVTA 352     |     |
| DEN3-NS1 | TTVSGKLIHEWCCRSCTLPPLRYMGEDGCWYGMEIRPISEKEENMVKSLVSA 352     |     |
|          | ** •** • ******** **** ****************                      |     |

Amino Acid Sequence Identity (%)

|          | DEN1-NS1 | DEN2-NS1 | DEN3-NS1 | DEN4-NS1 |
|----------|----------|----------|----------|----------|
| DEN1-NS1 | 100.0    | 72.7     | 79.8     | 69.3     |
| DEN2-NS1 | 72.7     | 100.0    | 73.6     | 72.7     |
| DEN3-NS1 | 79.8     | 73.6     | 100.00   | 73.9     |
| DEN4-NS1 | 69.3     | 72.7     | 73.9     | 100.0    |

**Supplementary Fig. S1: Sequences of four serotypes of DEN-NS1 proteins used in ExSELEX.** The amino acid sequences of each serotype DEN-NS1 protein were obtained from The Native Antigen Company (DEN1-NS: Nauru/Western Pacific/1974; DEN2-NS1: Thailand/16681/84; DEN3-NS1: Sri Lanka D3/H/IMTSSA-SRI/2000/1266; DEN4-NS1: Dominica/814669/1981). The amino acid sequence alignment and identity were analyzed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

# Monoclonal antibody: Ab#D06



Supplementary Fig. S2: The kinetic binding parameters, dissociation constant ( $K_D$ ) and association and dissociation rates ( $k_{on}$  and  $k_{off}$ ) of rabbit monoclonal antibodies, Ab#D06 and Ab#D25. These kinetic binding parameters were determined by SPR analyses with various concentrations of each DEN-NS1 serotype.



**Supplementary Fig. S3: Quantitative PCR analysis of ExSELEX-1.** PCR amplification curves and their melting curves in ExSELEX-1 were monitored using a Bio-Rad CFX96 PCR system. For the inputs, small portions of the recovered DNA in each selection round, in the presence of each target (DEN1-NS1 (D1+): orange, DEN2-NS1 (D2+): green, DEN3-NS1 (D3+): cyan, and DEN4-NS1 (D4+): blue) or in the absence of the target (protein-: gray) were used. Amplification cycles for the large-scale PCR (Supplementary Table 2) to prepare the libraries for the next round of selection were determined from the PCR amplification curves.



Supplementary Fig. S4: Binding analysis of DNA libraries by electrophoresis gel-mobility shift assays (EMSA). The enriched DNA libraries (50 nM) in the final round of three independent ExSELEX procedures (ExSELEX-1, ExSELEX-2, and ExSELEX-3 targeting each DEN-NS1 protein) were incubated with DEN1-NS1 (NAD1), DEN2-NS1 (NAD2), DEN3-NS1 (NAD3) or DEN4-NS1 (NAD4) (25 nM as the hexamer form; total 150 nM monomeric units) in binding buffer supplemented with 0.05% Nonidet P-40 at 25°C for 30 min, The DNA–NS1 complexes were separated from the free DNA on native 4% acrylamide gels. The DNA band patterns on the gels were detected with a bio-imaging analyzer (LAS4000), after staining the DNA bands with SYBR Gold. To investigate the importance of the Ds bases in the DNA libraries, we prepared the DNA libraries without the Ds bases by replacement PCR, and performed a comparative analysis of the binding patterns (Ds vs. Ds→natural base (NB)).

| ExSELEX-Target | Family | Ratio / % (counts | ) Random and partial primer regions   |
|----------------|--------|-------------------|---|
| ExSELEX-1-D1   | D1-1   | 10.01 (3943)      | TGTGA <b>xG<mark>TCC</mark>TA<mark>CAATGG</mark>A<b>CTGGTGTxCTCGG-xATGG<mark>CCATT</mark>gacaagc<mark>gga</mark>gta</b></b>                                     |
| ExSELEX-1-D1   |        | 8.79 (3464)       | GCAGCTATGC <mark>TCAAG</mark> A <mark>TCC</mark> CTGGTGT×CTCGG-×ATG <mark>GGACTTga</mark> caagcggagta   |
| ExSELEX-1-D1   |        | 7.39 (2912)       | tactg <mark>TCCGCTT</mark> A <mark>GCx</mark> AG <mark>CT<mark>GC</mark>CTGGTGTxCTCGG-xATG</mark> GTCT <mark>GC</mark> gac <mark>aagcgga</mark> gt              |
| ExSELEX-1-D1   |        | 2.63 (1037)       | CCGTAACGTC <mark>xGG</mark> T <mark>TCG<mark>CTGGTGTxCTCGG-xATG</mark>C<mark>TGA</mark>T<mark>CC</mark>Cgacaagcg</mark>   |
| ExSELEX-1-D1   |        | 2.52 ( 994)       | atgata <mark>tggtc</mark> t <mark>actgC</mark> ACTGGTGTxCTCGG-xATGGGCAGTGACCATATTTCACTGGA   |
| ExSELEX-1-D1   |        | 3.75 (1479)       | TACTG <mark>TCCGC</mark> A <mark>TGTC<mark>x</mark>GT<mark>GCTGGTGTxCTCGG-xATG</mark>CT<mark>AC</mark>GT<mark>gaca</mark>agcggagta</mark>                       |
| ExSELEX-1-D1   |        | 1.68 ( 663)       | ggtetae <mark>tg</mark> G <mark>AG</mark> AG <b>x</b> AT <mark>GCG<mark>CTGGTGTxCTCGG-xATG</mark>CGC</mark> TCA <mark>CT</mark> TC <mark>A</mark> TTA           |
| ExSELEX-1-D1   |        | 1.57 ( 617)       | ATGGC <mark>CT</mark> A <mark>TGTCC</mark> CA <mark>xGG</mark> CTGGTGTxCTCGG-xATG <mark>CC</mark> AAA <mark>GG</mark> gacaagcgga                                |
| ExSELEX-1-D1   |        | 1.54 ( 608)       | CAAATGTCTGAGA <mark>AAACT<mark>CTGGTGTxCTCGGGxATG</mark>AGTTT</mark> Gqacaaqcqqaqt  |
| ExSELEX-1-D1   |        | 1.50 ( 591)       | GCAAG <b>xA<mark>TCCG</mark>AGCC<mark>CTTGTCA</mark>G<b>CTGGTGTxCTCGG-xA</b>ATGG<mark>Tgacaagcgga</mark>gtag</b>  |
| ExSELEX-1-D1   |        | 1.41 ( 555)       | atggtctactg <mark>GGGGGG<mark>xGCCTGGTGTxCTCGG-xATG</mark>GCTCCCCC</mark> AAAACGAT  |
| ExSELEX-1-D1   |        | 1.30 ( 511)       | AGACGTTCTG <mark>x</mark> TC <mark>ACCAC</mark> GC <b>TGGTGT×CTCGG-×ATG</b> G <mark>GTGGTg</mark> acaagcggagt   |
| ExSELEX-1-D1   | D1-2   | 10.62 (4185)      | AGGAGCGCA <mark>TGTCGAGA</mark> TACCAACCx-CCATCCAATCxTTCTTgacaagcgga  |
| ExSELEX-1-D1   |        | 2.46 ( 968)       | ctg <mark>GCTTGTC</mark> G <mark>TGCGCG<mark>CCAATCxCCCATCCAACCx</mark>T<mark>CGTGTA</mark>GG<mark>gacaagc</mark>g</mark>                                       |
| ExSELEX-1-D1   |        | 2.79 (1097)       | CA <mark>GC</mark> ATGTCACTGC <mark>GxCCAATCx-</mark> ACAGCCAACCAAA <mark>GCA</mark> AGTgacaagcg  |
| ExSELEX-1-D1   |        | 1.65 ( 650)       | a <mark>tga</mark> tatggtc <mark>tac</mark> tgTAGGGTGGGT <b>x</b> TGGGAAGG <mark>xAC</mark> TC <mark>GTA</mark> A <mark>ACCAT</mark> G <mark>TCA</mark> GTGCGga |
| ExSELEX-1-D1   |        | 1.47 ( 579)       | ATAGAATAGG <mark>CCCCG</mark> TGTT <b>xATCAGACGCAT</b> C <b>CxCAT</b> T <mark>CGGGg</mark> acaagcggag   |
| ExSELEX-2-D1   | D1-3   | 54.58 (30778)     | tactgACGCCGGG <mark>GCCC</mark> GTA <b>xTCAGACGTAT</b> A- <b>CxCAT</b> CA <mark>GGGC</mark> ACATgacaag  |
| ExSELEX-2-D1   | D1-4   | 22.71 (12808)     | CCTGCACTGCC <mark>TCTGxAGC</mark> CCAACCxCCCATCCAATCGCTCAgacaagcggag  |
| ExSELEX-2-D1   |        | 11.73 ( 6615)     | TCTAA <mark>x</mark> GTCATGAGCCCAACCxCCCATCCAATCGCGATTATAgacaagcg   |
| ExSELEX-2-D1   |        | 2.14 ( 1206)      | tactgGCCGG <mark>x</mark> AGT <mark>CGCTA<mark>CCAATC</mark>TAC-<mark>CCAACC</mark>ATGCGxCATGCACgacaag</mark>   |
| ExSELEX-2-D1   |        | 1.75 ( 988)       | CT <mark>GC</mark> TTTGTCACA <mark>GGA</mark> G <b>x-CAATC</b> TAG- <mark>CCAACC</mark> G <mark>TCC</mark> GCACTG <mark>gacaagc</mark> gga                      |
| ExSELEX-2-D1   |        | 1.34 ( 758)       | actgA <mark>TTGTC</mark> C <mark>ATA</mark> ×TCGGTGGCA×TGGCAAGGTT×AGGT <mark>TAT</mark> CCG <mark>gacaa</mark> g  |
| ExSELEX-3-D1   | D1-5   | 7.88 (21188)      | GCGAC <b>xG<mark>CGCGTC</mark>GA<mark>TTG</mark>xCCAATCTTAGCCAACC<mark>CAA</mark>AATT<mark>gac</mark>aa<mark>gcg</mark>ga</b>                                   |
| ExSELEX-3-D1   |        | 8.23 (22148)      | atg <mark>gtctac</mark> tgCAGCTAA <mark>GT</mark> CCAACCCACGCCAATCxACGC <mark>GGCTGC</mark> xAT <mark>GTAgac</mark> a   |
| ExSELEX-3-D1   | D1-6   | 7.05 (18969)      | ACGA <mark>GCTT</mark> AGGA <mark>CCT</mark> xGTA <b>CCAACCCCCTCCAATC</b> xATT <mark>AGG</mark> gac <mark>aagc</mark> ggag                                      |
| ExSELEX-3-D1   |        | 2.22 ( 5985)      | GTATGAA <mark>GCT</mark> xGA <mark>CAAC</mark> GGxCCAACCCCCCCCCAATCTTA <mark>GTTg</mark> aca <mark>agc</mark> gg  |
| ExSELEX-3-D1   |        | 4.11 (11054)      | G <mark>TCC</mark> ATTGAACTGAA <mark>xAC<mark>CCAATC</mark>ACCTCCAACCx<mark>GT</mark>TGAAGga<mark>caa</mark>gc<mark>gga</mark>g</mark>                          |
| ExSELEX-3-D1   |        | 2.75 ( 7408)      | CTACGGTTGGCGGAT <mark>xTTA</mark> CCAACCTCCTCCAATCxTAGTGCgacaagcggag  |
| ExSELEX-3-D1   | D1-7   | 6.10 (16398)      | GACGG <mark>TTGT</mark> T <mark>AAC</mark> GA <mark>xGT</mark> CCAACCAGTCCCAATCx <mark>AC</mark> AA <mark>GTT</mark> gacaagc                                    |
| ExSELEX-3-D1   |        | 3.31 ( 8905)      | TCCG <mark>Tx</mark> AAGGT <mark>TGTGCAC</mark> CCAACCAGCTCCAATC <mark>GTx</mark> GCACAgacaag <mark>cgga</mark> g   |
| ExSELEX-3-D1   |        | 3.20 ( 8613)      | CTCTGTTTGT <mark>x</mark> AGGA <mark>GAGCCAACCAGT-CCAATC</mark> CTCACAACTg <mark>acaa</mark> gcg <mark>gag</mark>   |
| ExSELEX-3-D1   |        | 5.89 (15835)      | GGTAGCGCG <mark>GGA</mark> GGC <mark>xGT</mark> CCAACCTAT-CCAATCx <mark>AC</mark> AG <mark>TCC</mark> Ggacaagc  |
| ExSELEX-3-D1   |        | 3.22 ( 8664)      | ATACG <b>x</b> ATTGACAA <mark>GGCC</mark> xCCAAA-CCAAACCACCGGCCGgacaagc   |
| ExSELEX-3-D1   |        | 2.64 ( 7115)      | G <mark>TCCGC</mark> GAA <mark>GTCCC</mark> C <b>xACCAACCx</b> GCGC <b>CCAATC</b> AAACCA <mark>GGgac</mark> aa <mark>gcgga</mark> g                             |
| ExSELEX-3-D1   |        | 2.15 ( 5797)      | GGTAGCATGTT <mark>TTCT</mark> xGC <mark>CCAATC</mark> TCCCC <mark>CCAACCx</mark> GC <mark>GAGAA</mark> gacaagcggag  |
| ExSELEX-3-D1   |        | 2.11 ( 5667)      | GCGAGCAGGC <mark>xATGCGAC</mark> CCAATCTAG-CCAACCx <mark>GTCGCGT</mark> Aqacaaqcqqaq  |

**Supplementary Fig. S5: Alignment of the random-region DNA sequences obtained by the three ExSELEX procedures, targeting DEN1-NS1.** The sequences were obtained by deep sequencing through replacement PCR, by using an intermediate unnatural-base substrate, dPa'TP. Partial constant sequences, flanking the 42-nucleotide random region, are indicated in lower-case letters. The unnatural-base positions, indicated by a red "x", were predicted from the mutation spectra (natural-base composition rates) after replacement PCR. The ratio (%) of each family was calculated from the total read counts (replacement with Pa') clustered in the same family (shown in parentheses) against the total extracted reads for the analysis (replacement with Pa', Supplementary Table S4). Several representative family sequences, from D1-1 to D1-7, were chosen for binding analyses by EMSA and SPR (summarized in Supplementary Table S5). The complementary sequences forming two or more consecutive A–T and G–C/T pairs are shown in yellow, and the consensus sequences found among the families are shown in different colors.

| ExSELEX-Target | Family | Ratio / % (counts) | Random and partial primer regions  |
|----------------|--------|--------------------|--|
| ExSELEX-1-D2   | D2-1   | 34.41 (17433)      | gatat <mark>ggtctact</mark> gG <mark>TCCG</mark> xCTGGGAACAAGxGGCGGGAGGGAxGGGT-GTGGGTGCgacaag <mark>cgga</mark> gt <mark>agt</mark> tagaccg  |
| ExSELEX-1-D2   |        | 7.66 ( 3800)       | at <mark>ggtctact</mark> gAGGGAGTAG <b>x</b> GAGGAC <b>CAAGxG</b> -C-GGA- <b>GGAxGG</b> CG <b>-G</b> C <b>GGGTG</b> Ggacaagcggagt <mark>agt</mark> t <mark>agacc</mark> g          |
| ExSELEX-1-D2   |        | 1.88 ( 951)        | at <mark>ggtctact</mark> gATTATGG <mark>CTC</mark> xAGT <b>CAAGxGGC</b> AGTT <b>GGGAxGGGT</b> T <b>GTGGGT</b> gacaagcg <mark>gag</mark> t <mark>agt</mark> t <mark>agacc</mark> gt |
| ExSELEX-1-D2   |        | 17.36 ( 8797)      | ggtctactgTGGGAAGTCA <b>x</b> GGGGGC <b>x</b> TGGTGTAGTCGG <b>x</b> AGACGGGAGTTgacaagcggagtagt  |
| ExSELEX-1-D2   |        | 1.13 ( 570)        | ggtctactgAC <mark>GTC</mark> *AGG <mark>TTG</mark> GAAAGG*ACGGCCGT*AATACATGCA <mark>CAA</mark> G <mark>gac</mark> aagcggagtagt   |
| ExSELEX-1-D2   |        | 20.75 (10512)      | ggtctactgCTCTAACCAACAGAGGC <mark>CAG<mark>xCCAATCTTA-CCAACCx</mark>CC<mark>CTG</mark>gacaagcggagtagt</mark>  |
| ExSELEX-1-D2   |        | 2.85 ( 1442)       | ggtctactgAGTGTCGAC <mark>GGGGAG<mark>xCCAATC</mark>CGC-<mark>CCAACCx</mark>CC<mark>CTCCCC</mark>TAgacaagcggagtagt</mark>   |
| ExSELEX-1-D2   |        | 3.54 ( 1794)       | ggtetaet <mark>gCT</mark> A <mark>CGTGA</mark> AG <b>x</b> T <b>CCAATC</b> TCTA <b>CCAACC</b> TGT <mark>xTCACG</mark> A <b>x</b> AGTgaca <mark>age</mark> ggagtagt                 |
| ExSELEX-1-D2   |        | 2.79 ( 1413)       | ggtctactgATAGCCTG <mark>TCCGCTT</mark> x <mark>GTC</mark> GC <b>CCAACCCGTGxTCCAATC</b> CAA <mark>gacaagcgga</mark> gtagt   |
| ExSELEX-1-D2   |        | 1.40 ( 709)        | ggtctactgAAAGG <mark>CGC</mark> GT <b>x</b> GTAA <mark>CTGT</mark> CCAATCCGCGxTCCAACC <mark>ACAG</mark> Agacaa <mark>gcg</mark> gagtagt  |
| ExSELEX-2-D2   | D2-2   | 23.26 (15556)      | ggtctactgGAA <mark>TAAC</mark> AAG <mark>TCCG</mark> TGC <mark>xGTC</mark> G <b>CCAATCCGTGxTCCAACC</b> CC <mark>gac</mark> aag <mark>cgga</mark> gta <mark>gtta</mark> gaccg       |
| ExSELEX-2-D2   | D2-3   | 21.67 (14490)      | ggtctactgGT <mark>CTAC</mark> A <b>x</b> GCAA <mark>CGCTTTC</mark> G <b>xCCAACCCGTGxTCCAATC</b> CCA <mark>ga</mark> c <mark>aagcg</mark> ga <mark>gtag</mark> t                    |
| ExSELEX-2-D2   | D2-4   | 11.97 ( 8003)      | ggt <mark>ctactgCTT</mark> CACGAGTC <mark>AAGG<mark>x</mark>CT<mark>CCAATCCGTGxTCCAACCAGTTTT</mark>gac<mark>aagc</mark>gga<mark>gtag</mark>t</mark>                                |
| ExSELEX-2-D2   |        | 6.97 ( 7781)       | ggt <mark>ctact</mark> gA <mark>CCGC</mark> GAAAGTA <mark>GGC</mark> xT <b>CAACCCGTGxTCCAATC</b> CGC <mark>GCC</mark> GAgacaa <mark>gcggagtag</mark> t_                            |
| ExSELEX-2-D2   |        | 10.59 ( 7079)      | ggtct <mark>act</mark> gG <mark>CGCT</mark> CGGC <mark>TCGCGC</mark> xT <b>CAATCCGTGxTCCAACC</b> CC <mark>GCGCGA</mark> gaca <mark>agcg</mark> gagt <mark>agt</mark>               |
| ExSELEX-2-D2   | D2-5   | 11.63 ( 5530)      | ggtctactgTGGCTGGGCCA <mark>xGCGTG</mark> CT <b>CAACCTTACCAATC</b> TG <mark>x</mark> CACG <mark>C</mark> Ggacaagcggagtagt   |
| ExSELEX-2-D2   | D2-6   | 8.27 ( 4661)       | ggt <mark>ctact</mark> gTAGAT <mark>xTTGTCGAGAX</mark> G <mark>CA</mark> CCAACCAATC <mark>TGxCTTGA</mark> ga <mark>caa</mark> gcgg <mark>agtag</mark> t                            |
| ExSELEX-2-D2   |        | 4.16 ( 2785)       | ggtctactgAC <mark>CCG</mark> TCGAC <mark>CTCT</mark> CACCCAACCATC <b>CCAATCx</b> AGCATA <mark>AGAg</mark> acaag <mark>cgg</mark> agtagt  |
| ExSELEX-3-D2   | D2-1   | 65.94 (193819)     | tat <mark>ggtctact</mark> gG <mark>TCCG</mark> xCTGGGAACAAGxGGCGGGAGGGAxGGGTGTGGGTGCgacaag <mark>cgga</mark> gt <mark>agt</mark> t <mark>agacc</mark> g                            |
| ExSELEX-3-D2   |        | 7.99 ( 23478)      | ggt <mark>ctact</mark> gAGACG <mark>CGC</mark> AGGA <mark>CT</mark> A <mark>xGA</mark> CCAATCTTACCAACCAxCTCAAGGgacaa <mark>gcggagtag</mark> t                                      |
| ExSELEX-3-D2   |        | 2.96 ( 8712)       | ggt <mark>ctact</mark> gC <mark>CCGAx<mark>TTG</mark>CC<mark>TGCCCC</mark>xCCAACCAGCCAATC</mark> CAT <mark>GGGGCG</mark> ga <mark>caagcggagtag</mark> t                            |
| ExSELEX-3-D2   |        | 2.84 ( 8354)       | ggtctactgC <mark>CTTG</mark> xTA <mark>TGTCTTC</mark> TACCAACCAACCCAATCTAx <mark>GAAGACA</mark> ga <mark>caag</mark> cggagtagt   |
| ExSELEX-3-D2   |        | 2.65 ( 7781)       | ggt <mark>ctact</mark> g <mark>CTGG</mark> C <b>x</b> GGGGCGAGGGCCAACCAGACCAATCCC <b>x</b> CGAGA <mark>CCAg</mark> acaagcgg <mark>agtag</mark> t                                   |

**Supplementary Fig. S6: Alignment of the random-region DNA sequences obtained by the three ExSELEX procedures, targeting DEN2-NS1.** The sequences were obtained by deep sequencing through replacement PCR, by using an intermediate unnatural-base substrate, dPa'TP. Partial constant sequences, flanking the 42-nucleotide random region, are indicated in lower-case letters. The unnatural-base positions, shown by the red "x", were predicted from the mutation spectra (natural-base composition rates) after replacement PCR. The ratio (%) of each family was calculated from the total counts (replacement with Pa') clustered in the same family against the total extracted reads for the analysis (replacement with Pa', Supplementary Table S4). Several representative family sequences, from D2-1 to D2-6, were chosen for binding analyses by EMSA and SPR (summarized in Supplementary Table S5). The complementary sequences forming two or more consecutive A–T and G–C/T pairs are shown in yellow, and the consensus sequences found among the families are shown in different colors.

| ExSELEX-Target | Family | Ratio / % (counts) | Random and partial primer regions   |
|----------------|--------|--------------------|---|
| ExSELEX-1-D3   | D3-1   | 89.07 (40625)      | gtctactg <b>ATAG<mark>TACTCC</mark>xGTTTAACTCTGAxACTTGACGTCCATTCATA</b> gacaagc <mark>ggagta</mark> gttagac   |
| ExSELEX-1-D3   |        | 3.09 ( 1409)       | gtctactgCTGAAG <mark>CTCCG</mark> TGCG <b>xCCCC-GCGGTTTGTT-AA</b> AxCCCTTTAgacaag <mark>cggag</mark> tagttag  |
| ExSELEX-1-D3   |        | 2.64 ( 1202)       | atatggtctactg <mark>CTT</mark> GC <b>xCCCCCCGCGGTTTGTTTAA</b> TGG <b>x</b> AGAATTCTGTT <mark>AAg</mark> acaagcgga   |
| ExSELEX-1-D3   |        | 2.00 ( 911)        | atatggt <mark>ctact</mark> gGT <mark>CGCTT</mark> TGC <b>xCCCC</b> GTC <b>CGGT</b> GAC <b>T</b> GA <b>AA</b> ATA <b>x</b> CACGTCAGgac <mark>aagcg</mark> g <mark>agtag</mark> t |
| ExSELEX-2-D3   | D3-2   | 81.23 (52430)      | atggt <mark>ctact</mark> gAAGTG <mark>TTGTCA</mark> TCTA <b>x</b> CCTGGCC <b>x</b> TGTGGTACTGTAACGGC <mark>Tgacaa</mark> gegg <mark>agtag</mark> tta                            |
| ExSELEX-2-D3   | D3-3   | 15.56 (10040)      | atggt <mark>ctact</mark> gGGGCT <b>x</b> GGT <mark>CTTG</mark> CGT <b>x</b> TGCAGATTA <b>ACTTGC</b> GTGCCAGTAAga <mark>caag</mark> cgg <mark>agtag</mark> tta                   |
| ExSELEX-3-D3   | D3-1   | 34.13 (72313)      | gtctactg <b>ATAG<mark>TACTCC</mark>×GTTTAACTCTGA×ACTTGACGTCCATTCATA</b> gacaagc <mark>ggagta</mark> gttagac   |
| ExSELEX-3-D3   |        | 27.21 (57653)      | atggt <mark>ctact</mark> gTA <mark>CC<mark>ACTTG</mark>CAATGGACGCG<b>x</b>CGTATGGTGG<b>x</b>TCGGGGAATGga<mark>caag</mark>c<mark>ggagtag</mark>tta</mark>                        |
| ExSELEX-3-D3   |        | 8.17 (17312)       | tatggtctactgGTGTAA <mark>TCCG</mark> G <mark>TTGT</mark> GAGACGGCGGAATG <b>x</b> ATTTGGAAAGGCg <mark>acaa</mark> g <mark>cgga</mark> gtagt                                      |
| ExSELEX-3-D3   |        | 8.07 (17097)       | atatggt <mark>ctact</mark> gCG <mark>CGCTTG</mark> GG <b>x</b> GACGAATTTGTACAGCGTATAT <u>CCA</u> xCGACGga <mark>caagcg</mark> g <mark>agtag</mark> t                            |
| ExSELEX-3-D3   |        | 4.85 (10283)       | gatat <mark>ggt</mark> ctact <mark>gC</mark> T <mark>CTG</mark> TGCC <b>GTCGAG×G</b> ACCCTTAGGTTC <mark>×CAG</mark> CT <mark>GC</mark> TA <mark>ACC</mark> gacaagcggagt         |
| ExSELEX-3-D3   |        | 2.83 ( 6003)       | gatat <mark>gg</mark> t <mark>ctactgCGTG<mark>GTCGAGxG</mark>GGCGT<b>GTTTAA</b>ACGCAT<mark>CAC</mark>A<mark>GC</mark>C<mark>GTAGCC</mark>gacaagc</mark>                         |
| ExSELEX-3-D3   |        | 3.14 ( 6662)       | tatggtct <mark>actgAC</mark> A <mark>CCGTCT</mark> xTGTATGTCGCATTCCTGAC <b>TCTAxCC</b> CCGAC <mark>Aga</mark> caag <mark>cgg</mark> a <mark>gtagt</mark> t                      |

**Supplementary Fig. S7: Alignment of the random-region DNA sequences obtained by the three ExSELEX procedures, targeting DEN3-NS1.** The sequences were obtained by deep sequencing through replacement PCR, by using an intermediate unnatural-base substrate, dPa'TP. Partial constant sequences, flanking the 42-nucleotide random region, are indicated in lower-case letters. The unnatural-base positions, indicated by the red "x", were predicted from the mutation spectra (natural-base composition rates) after replacement PCR. The ratio (%) of each family was calculated from the total counts (replacement with Pa') clustered in the same family against the total extracted reads for the analysis (replacement with Pa', Supplementary Table S4). Several representative family sequences, from D3-1 to D3-3, were chosen for binding analyses by EMSA and SPR (summarized in Supplementary Table S5). The complementary sequences forming two or more consecutive A–T and G–C/T pairs are shown in yellow, and the consensus sequences found among the families are shown in different colors.

| ExSELEX-Target | Family | Ratio / % (counts) | Random and partial primer regions  |
|----------------|--------|--------------------|--|
| ExSELEX-1-D4   | D4-1   | 39.19(15319)       | tactgTCTCAAC <mark>GGTTGT</mark> CAA <b>ACG</b> G <b>xTATCA</b> CGGC <mark>x</mark> AC <mark>ACC</mark> TGCGgacaagcggagtagt                          |
| ExSELEX-1-D4   |        | 13.74( 5371)       | GAAAACA <mark>GCTT</mark> TATCATATAA <b>ACGC×TATCA</b> CGgac <mark>aagcg</mark> gagtagt  |
| ExSELEX-1-D4   |        | 2.38( 929)         | actgCGGTA <mark>xCGCT</mark> A <mark>AAGG<b>A</b></mark> C <mark>GG</mark> xTATCAAATTA- <b>AAACA<mark>CCT</mark>CCTTgaca<mark>agcg</mark>gagtagt</b> |
| ExSELEX-1-D4   |        | 9.18( 3590)        | actgTAAGAACAG <mark>CGCTGTGA<b>A</b>C<mark>GC</mark>×TATCAAA</mark> TC-×AAACA <mark>GCT</mark> TC <mark>gac</mark> a <mark>agcg</mark> gagtagt       |
| ExSELEX-1-D4   |        | 2.88( 1125)        | tactgTCCTCTAAA <mark>GCTGTCCG<mark>CGC</mark>xTATCAAA</mark> GGA <b>xAAACA</b> GC <mark>CG</mark> Agacaagcggagtagt                                   |
| ExSELEX-1-D4   |        | 4.40( 1719)        | actgTGGGG <b>x</b> GCGTGA <mark>GCTTG</mark> GC <mark>AAGGGxTTGGxTAGGGCTGGCAA</mark> ga <mark>caagc</mark> ggagtagt                                  |
| ExSELEX-1-D4   | D4-2   | 23.92( 9350)       | ggtctac <mark>tgTCAC</mark> A <b>x</b> ATCGCCGTAAAG <b>x</b> CGAAGAGCTGCGGAATCTAAG <mark>GTgaca</mark> agcggagt                                      |
| ExSELEX-1-D4   |        | 1.29( 506)         | gtctact <mark>gGTC</mark> CCTCGTCCAACCGTGCC <b>x</b> ACTCTCACT <b>xGA<mark>GACC</mark>CAATCgacaagcggagt</b>  |
| EXSELEX-2-D4   | D4-3   | 21 96 ( 9619)      | netnennneenen) <mark>079</mark> 338 <b>4779369-666</b> x0766667767-x <mark>03</mark> 0667769663875eto  |
| ExSELEX-2-D4   |        | 17.04 (7461)       | actgTGGCGCGAGGGAATC <b>xACGCx-TATCAAATAxAAA-CAGCT</b> AATgacaaggggggtag  |
| ExSELEX-2-D4   |        | 8.60 ( 3766)       | actgTCGCACGTTAAACGC <b>xACGGx-TATCAAAT</b> C <b>xAAA-CACCT</b> GAGgacaagcggagtag   |
| ExSELEX-2-D4   |        | 1.78 ( 780)        | actgGGCACCCA <mark>TTGTC</mark> TCA <b>ACGC×</b> A <b>TATCAAAT</b> C <b>×AAA-CA</b> GCTAGC <mark>gacaa</mark> gcggagtag                              |
| ExSELEX-2-D4   |        | 1.43 ( 627)        | actgGGCACCCA <mark>TTGT</mark> CTCA <b>ACGCx-TATCAAAT</b> C <b>xAAA</b> A <b>CAGCT</b> GAg <mark>acaa</mark> gcggagtag                               |
| ExSELEX-2-D4   | D4-4   | 23.35 (10228)      | tactgTATAA <mark>TCCGC<mark>xTT</mark>C<mark>GTC</mark>ATGT<b>GGxTTGG</b>ATCT-<b>GGG</b>T-<b>CTGGCA</b>gacaagcgga</mark> gtagt                       |
| ExSELEX-2-D4   |        | 7.28 ( 3189)       | ctactgCATAGCGGCA <b>x</b> CG <mark>GTCG</mark> GT <b>GGGxTTGG</b> C <b>x</b> GTG <b>GGGC-TGGCA<mark>C</mark>gacaagcggagtagt</b>                      |
| ExSELEX-2-D4   | D4-5   | 3.50 ( 1535)       | gtctactgCCCAA <mark>xCTT</mark> GTCTGT <b>AAGGGxTTGGxTAGGGC-TGGCAAA</b> A <mark>AAg</mark> acaagcggag  |
| ExSELEX-2-D4   |        | 2.09 ( 915)        | gtctactgGTC <mark>TGT</mark> GGGG <b>xC<mark>TTG</mark>AAGGGxTTGGxTAGGGC-TGGCAAACAA<mark>gaca</mark>agcggag</b>                                      |
| ExSELEX-2-D4   |        | 1.51 ( 660)        | tctactgGTTTT <b>x</b> GGTTAGTTCTTT <b>GGGxTTGG</b> CA <b>x</b> C <b>GGG</b> C <b>TGGC</b> GTGgacaagcggagtag  |

**Supplementary Fig. S8: Alignment of the random-region DNA sequences obtained by the three ExSELEX procedures, targeting DEN4-NS1.** The sequences were obtained by deep sequencing through replacement PCR, by using an intermediate unnatural-base substrate, dPa'TP. Partial constant sequences, flanking the 42-nucleotide random region, are indicated in lower-case letters. The unnatural-base positions, indicated by the red "x", were predicted from the mutation spectra (natural-base composition rates) after replacement PCR. The ratio (%) of each family was calculated from the total counts (replacement with Pa') clustered in the same family against the total extracted reads for the analysis (replacement with Pa', Supplementary Table S4). Several representative family sequences, from D4-1 to D4-5, were chosen for binding analyses by EMSA and SPR (summarized in Supplementary Table S5). The complementary sequences forming two or more consecutive A–T and G–C/T pairs are shown in yellow, and the consensus sequences found among the families are shown in different colors.

## A. Enriched DNA libraries targeting DEN-2 NS1 through ExSELEX (Round 7 in ExSELEX-3)



**Supplementary Fig. S9: Confirmation of the presence of diol-Px in the selected clone family, D2-1.** (A) Schematic illustration of the experiment flow to identify a diol-Px position in the family D2-1 isolate. (B) Gel mobility shift patterns support the binding of the aptamer strand to the target DEN2-NS1, while the D2-1-96(3Ds), in which the predicted unnatural base positions are all Ds (see Supplementary Table S5), did not bind to the target. (C) The Cy5-labelled DNA band patterns, shown in red on the gel image, indicate that the aptamer strand that was PCR-amplified from the isolated clone should contain a Px base, while PCR using an initial Ds-DNA library only produced the aptamer strand without Px bases (no Cy5 incorporation) and the complementary strand with Px bases (Cy5 incorporation). (D) The DNA band patterns on the gel images indicate that the aptamer strand was cleaved during a four-hour incubation at 55°C under basic conditions (concentrated ammonia), due to the presence of the Px base, but not the Ds base, at the specific position corresponding to the predicted third Ds base (position 57 of the aptamer strand).



| 10 μl in assays  | DEN1-NS | S1 (ng/ml) | DEN2-NS | S1 (ng/ml) | DEN3-N | S1 (ng/ml) | DEN4-NS1 (ng/ml) |      |  |
|------------------|---------|------------|---------|------------|--------|------------|------------------|------|--|
|                  | Buffer  | HS         | Buffer  | HS         | Buffer | HS         | Buffer           | HS   |  |
| LOD (blank+ 3δ)  | 1.60    | 2.22       | 1.86    | 1.99       | 2.36   | 3.31       | 1.19             | 1.14 |  |
| LOQ (blank+ 10δ) | 4.77    | 6.97       | 5.84    | 6.91       | 7.34   | 11.06      | 3.92             | 4.10 |  |

Supplementary Fig. S10: Limit of detection (LOD) and limit of quantification (LOQ) targeting each dengue serotype NS1 by a sandwich-type ELISA. UB-DNA aptamers and an anti-DEN-NS1 monoclonal antibody (Ab# D06) were used as capture agents and the primary detector agent, respectively. For the target binding process, 10  $\mu$ l of serially diluted NS1 (0 to 100 ng/ml) was used in buffer with and without control human serum (PD0-1, 10%) in each 100- $\mu$ l binding solution. The sample size is two per each combination set, and the data are from two independent experiments. The error bars represent one average deviation. The bars with wavy lines indicate that the signal in at least one of the two sample wells was saturated (OD<sub>450</sub> > 4.000).

A CLUSTAL O(1.2.4) multiple sequence alignment

В

| D1 tarret       | DSCCVTNWKCPF                                      | TROCSCIE                 | TNEVHTWTE                         | OVEROADS                     |                       |                              | CALCIDEN             | TOTEN        | MAKOTSN                 | FINHTIFN                      | MKETTATA                     | 90  |
|-----------------|---|--------------------------|-----------------------------------|------------------------------|-----------------------|------------------------------|----------------------|--------------|-------------------------|-------------------------------|------------------------------|-----|
| pn1-1           | •S••VIN•KG••                                      |                          | WINE                              | e K e ADe                    | •KD•SA                | . GK .WFF                    | .V.                  |              | MSN                     | HT . T N                      | DMKFOVVV                     | 90  |
| PD1-2/PD1-3     | •S••VIN•KG••                                      |                          | TINE                              | ••K••AD•                     | .KR.SA                | • GK •WFF                    | • V • • • • • • •    | • • T. • • T | M SN                    | I HT . T. N                   | DMKF VVV                     | 90  |
| D2 target       | • S• • VVS • KN• •                                |                          | ITDN                              | ••K••PE•                     | •SK•AS                | • • OK • HEE                 | • I • • • • • V      | ••1.••1      | MeeeTF                  | •••HI•S•N                     | EVKL. IMT                    | 90  |
| PD2-1           | • S• • VVS • KN• •                                |                          | ITDN · · · · ·                    | •• K •• PE •                 | • SK • AS             | • OK • OEF                   | • I • • • • V        | ••L••]       | MTE                     | • • • HI • <mark>A</mark> • N | EVKL • IMT                   | 90  |
| PD2-2/PD2-3     | • S • • VVS • KN • •                              |                          | ITDN · · · · · ·                  | •• K •• PE •                 | •SK•AS                | • OK • HEF                   | • I • • • • • V      | ••L••1       | M•••TF                  | • • • HI • T • N              | EVKLOIMT                     | 90  |
| D3 target       | •M• •VIN•KG• •                                    |                          | TNE                               | ••K••AD•                     | •KR•AT                | • AG •WEN                    | •V••••T              | • •M• • 1    | L AN                    | • • YI • W•N                  |                              | 90  |
| PD3-1           | • M • • VVN • KG • •                              |                          | 7TNE                              | • • K • • AD •               | •KR•AT                | • AG • WEN                   | •V••••T              |              |                         | •••YT•W•N                     | NTRT. • VVV                  | 90  |
| PD3-2           | •M• •VTN •KG• •                                   |                          | TINE                              | • • <b>R</b> • • AD •        | .KR.AT                | • AG •WEN                    | •V••••               | • • M • • 1  |                         | • • • YT • W • N              | NTKT. • VVV                  | 90  |
| PD3-3           | •M• •VTN •KG• •                                   |                          | TTNE                              | • • K • • AD •               | •KR•AT                | • AG • WEN                   | •V••••               | • • M • • 1  | .T. • • • • AN          | • • • YT • W • N              | NTKT. • VVV                  | 90  |
| D4 target       | • M• • VAS • SG• •                                |                          | VVDN•••••                         | ••K••PE•                     | •AR•AS                | ••LN•HKD                     | •V••••T              | ••L••V       | 7M • • • • TN           | •••YV•W•G                     | GHDL • VVA                   | 90  |
| PD4-1           | • M • • V <mark>V</mark> S • <mark>N</mark> G • • |                          | VDN•••••                          | •• K •• PE •                 | • AR• AS              | •• LN • HKT                  | •V••••T              | • • L • • V  | 7M • • • • TN           | • • • YV • W • G              | GHDL • VVA                   | 90  |
|                 |   |                          |                                   |                              |                       |                              |                      |              |                         |                               |                              |     |
| D1 target       | GDVSGILAQGKK                                      | MIRPOPME                 | KYSWKSWGK                         | AKIIGADV                     | QNTTFI                | IDGPNTPE                     | CPDNQRAW             | NIWEVE       | DYGFGIF                 | TTNIWLKLR                     | DSYTQVCD                     | 180 |
| PD1-1           | • • VA • ILAQ • • KI                              | MIR• OPMEH               | s                                 | • KIIGADV                    | _<br>Q•TT•I           | ••••N•P•                     | ••DDQ•••             | • IW • • •   |                         | ••••L•LR                      | DSYTQV                       | 180 |
| PD1-2/PD1-3     | • • AN • ILTO • • KI                              | MIR • OPMEN              | H••••s•••                         | • KIIGAD                     | Q•TT•I                | •••• <mark>D</mark> •P•      | ••DDQ•••             | • IW • • •   |                         | ••••L•LR                      | DSYTOM••                     | 180 |
| D2 target       | ••IK•IMQA••R                                      | SLR•QPTEI                | LT                                | • KMLSTES                    | H•QT•L                | •••• <mark>E</mark> •A•      | • • NTN • • •        | •SL•••       | •••• <mark>v</mark> •   | ••••L•LK                      | EKQDVF •                     | 180 |
| PD2-1           | ••IK•IMQA••R                                      | SLR • QPTEI              | ьт                                | • KMLSTES                    | H•QT•L                | ••••E•A•                     | • • NTN • • •        | •SL•••       | ••••v•                  | ••••L•LK                      | EKQD <mark>A</mark> F••      | 180 |
| PD2-2/PD2-3     | ••IK•IMOA••R                                      | SLR • OPTEI              | БХ                                | • KMLSTE                     | H•HT•L                | ••••E•A•                     | ••NTN•••             | •SL•••       | ••••v•                  | ••••L•LK                      | ERODVS · ·                   | 180 |
| D3 target       | • TL • VLEO • R                                   | TLT • OPMEI              |                                   | •KIVTAET                     | 0.55.1                | ••••N•P•                     | ••SAS•••             | • VW • • •   | • • • • • V             | ••••L•LR                      | EVYTOL .                     | 180 |
| PD3-1           | • TL • VLEO • R                                   | TLT • OPMEI              |                                   | • KIVTAET                    | 0.33.1                | ••••N•P•                     | •• SAS ••••          | • VW • • •   | ••••v•                  | ••••L•LR                      | EVYTOL .                     | 180 |
| PD3-2           | . TT . TLEO . R                                   | TLT • OPMEI              |                                   | • RIVTAET                    | 0.55.1                | ••••N•P•                     | NAS                  | • VW • • •   |                         | ••••L•LR                      | EMYTOL •                     | 180 |
| PD3-3           | • TF • VLEO • R                                   | TLT • OPMET              |                                   | • KIVTAETO                   | 0•55•T                | ••••N•P•                     | •• SAS •••           | • VW • • •   | v .                     | • • • • T • TR                | EVYTOM••                     | 180 |
| D4 target       | • • VK • VLTK • R                                 | ALT • PVSDI              |                                   | •KIFTPEA                     | R•ST•L                | ••••D•S•                     | • • NER • • •        | •SL •••      | • • • • • M•            | ••••M•FR                      | EGSSEV                       | 180 |
| PD4-1           | • • VK • VL TK • • R                              | ALT • PVNDI              | G                                 | • KIFTPEA                    | R•ST•L                | ••••D•S•                     | • • NER • • •        | • FF • • •   | • • • • M •             | ••••M•FR                      | EGSSEV.                      | 180 |
|                 |   |                          |                                   |                              |                       |                              |                      |              |                         |                               |                              |     |
| D1 target       | HRLMSAAIKDSK                                      | AVHADMGY                 | I ESEKNETW                        | KLARASFI                     | EVKTCI                | WPKSHTLW                     | SNGVLESE             | MIIPK        | IYGGPI SÇ               | HNYRPGYFT                     | TAGPWHL                      | 270 |
| PD1-1           | HR I.SK   |                          | ••••EK•ET•                        | KLAR • F •                   | •••T•I                | • PKS • • • •                | S••••E               | • I • • KI   | IYG••I•                 | ••Y•P••F•                     | • TA • • • • •               | 270 |
| PD1-2/PD1-3     | HR I.SK   |                          | ••••EK•ET•                        | KLAR ·· F ·                  | •••T•I                | • P <mark>R</mark> S • • • • | S••••E               | • I • • KI   | IYG••I••                | ••Y•P••F•                     | • TA • • • • •               | 270 |
| D2 target       | SK ••••I••NR                                      |                          | •••AL•DT•                         | KIEK • F •                   | • • •N • H            | • PKS • • • •                | S••••E               | • I • • KI   | ILA • • V • •           | ••¥•P••H•                     | • IT • • • • •               | 270 |
| PD2-1           | SK · · · · I · · NR                               |                          | •••AL•DT•                         | KIEK••F•                     | •••N•H                | •PKS ••••                    | s••••E               | • I • • KM   | ILA••V••                | ••Y•P••H•                     | • 1 <mark>4</mark> • • • • • | 270 |
| PD2-2/PD2-3     | SK····I··NR                                       |                          | •••AL•DT•                         | KIEK ·· F·                   | ••• <mark>5</mark> •H | •PKS••••                     | S••••E               | • I • • KM   | FA••V••                 | ••Y•P••H•                     | • <u>TA</u> • • • • •        | 270 |
| D3 target       | HRV.ER  |                          | •••QK•GS•                         | KLEK .L.                     | •••T•T                | •PKS • • • •                 | <b>T</b> • • • • • D | • I • • KS   | SLA••I••                | ••¥•P••H•                     | TA                           | 270 |
| PD3-1           | HR••••V••ER                                       |                          | •••QK•GS•                         | KLEK · L ·                   | •••••••               | •PKS ••••                    | <b>s</b> •••••D      | • I • • KS   | SLA••I••                | ••Y•P••H•                     | • TA • • • • •               | 270 |
| PD3-2           | HR••••V••ER                                       |                          | ····QK ·GS ·                      | KLEK ·· L ·                  | •••T•T                | •PKS ••••                    | <b>s</b> •••••D      | • I • • KS   | SLA••I••                | •• <b>H</b> •P••H•            | • TA • • • • •               | 270 |
| PD3-3           | HR••••V••ER                                       |                          | ••••QK•GS•                        | KLEK .L.                     | •••T•T                | • <b>A</b> KS••••            | <b>s</b> •••••D      | • I • • KS   | SLA••I••                | ••Y•P••H•                     | • TA • • • • •               | 270 |
| D4 target       | HR••••I••QK                                       |                          | •••SK•QT•                         | QIEKL.                       | •••T•L                | • PKT • • • •                | S • • • • • • Q      | • L• • KS    | SYA••F••                | ••Y•Q••A•                     | • TV • • • • •               | 270 |
| PD4-1           | HR • • • • I • • QK                               |                          | ••••s <mark>r</mark> •QT•         | QIEK ·· L ·                  | •••T•L                | • PKT • • • •                | s•••••Q              | • L• • R     | SYA••F••                | ••Y•Q••A•                     | • TV • • • •                 | 270 |
|                 |   |                          |                                   |                              |                       |                              |                      |              |                         |                               |                              |     |
| D1 target       | GKLELDFDLCEG                                      | TTVVVDEHO                | CGNRGPSLRT                        | TTVTGKT II                   | HEWCCR                | SCTLPPLR                     | FKGEDGCW             | YGMEII       | RPVKEKEE                | NLVKSMVSA                     | 352                          |     |
| PD1-1           | •K••L••DL•E•                                      | •••VVD•H                 | •GN • • • • • •                   | • • VT • • <mark>I</mark> II | HE••••                | •••L••••                     | FR                   | ••••         | • VK • • • •            | •L•K•M•S•                     | 352                          |     |
| PD1-2/PD1-3     | •K••L•• <mark>N</mark> L•E•                       | •••VVD•H                 | •GN • • • • • •                   | ••VT•• <mark>I</mark> II     | HE••••                | •••L••••                     | FR                   |              | • VK • • • •            | •L• <mark>R</mark> •M•S•      | 352                          |     |
| D2 target       | •K••M••DF•D•                                      | •••VVT•D                 | GN • • • • • •                    | ••AS••LI                     | TE····                | •••L••••                     | YR•••••              |              | • LK • • • •            | •L•N•L•T•                     | 352                          |     |
| PD2-1           | • K• • M• • DF • D •                              | ••• <mark>T</mark> VT•D  | •G <mark>3</mark> • • • • • • • • | ••AS••L <mark>V</mark>       | TE••••                | •••L••••                     | YR•••••              |              | • LK • • • •            | •L•N•L•T•                     | 352                          |     |
| PD2-2/PD2-3     | • <mark>R</mark> ••M••DF• <mark>E</mark> •        | •••• VVT • D             | GN                                | ••AS••LI                     | TE••••                | •••L••••                     | YR•••••              |              | ·LK····                 | •L•N•L•T•                     | 352                          |     |
| D3 target       | •K••L••NY•E•                                      | •••VIT•S                 | GT                                | ••VS••LI                     | HE••••                | •••L•••                      | <b>YM••••</b>        |              | • IS • • • •            | •M•K•L•S•                     | 352                          |     |
| PD3-1           | •K••L••NY•E•                                      | ···VIT·S                 | GT                                | ••VS••LI                     | не••••                | •••L••••                     | үм•••••              |              | •IS••••                 | •M•K•L•S•                     | 352                          |     |
| PD3-2           | •K••L••NY•E•                                      | •••VIT•N                 | GT                                | ••VS••LI                     | HE••••                | •••L••••                     | үм•••••              |              | ••I <mark>N</mark> •••• | •M•K•L•S•                     | 352                          |     |
| PD3-3           | •K••L••NY•E•                                      | •••VIT• <mark>N</mark> • | GT                                | ••VS••LI                     | HE••••                | •••L••••                     | үм•••••              |              | • IS • • •              | •M•K•L•S•                     | 352                          |     |
| D4 target       | •K••I••GE•P•                                      | •••TIQ•D                 | DH                                | ••AS••LV                     | TQ····                | • • • M • • • •              | FL····               |              | ·LS····                 | •M•K•Q•T•                     | 352                          |     |
| PD4-1           | •K••I••GE•P•                                      | ••• <mark>AIR</mark> •D• | DH                                | • • AS• • LV                 | TQ••••                | •••M••••                     | FL····               | ••••         | •LS••••                 | •M•K•Q•T•                     | 352                          |     |
|                 |   |                          |                                   |                              |                       |                              |                      |              |                         |                               |                              |     |
|                 |   |                          |                                   |                              |                       |                              |                      |              |                         |                               |                              |     |
| Mutated Amino A | CIOS  | 4                        | 13                                | 7                            |                       | 12                           |                      | 4            | 11                      | 5                             |                              | 9   |

| (in 352 Amino Acids) |        | 4            | 15    |        |       | 12    |        | 4            |              | 9            |        | 9     |
|----------------------|--------|--------------|-------|--------|-------|-------|--------|--------------|--------------|--------------|--------|-------|
|                      | D1     | <b>DD1 1</b> | PD1-2 | D2     | BD2 4 | PD2-2 | D3     | <b>DD2 4</b> | <b>BD2.2</b> | <b>DD2 2</b> | D4     | PD4.4 |
|                      | target | PD1-1        | PD1-3 | target | FU2-1 | PD2-3 | target | PD3-1        | PD3-2        | PD3-3        | target | FD4-1 |
| D1_target            | 100.0  | 98.9         | 96.3  | 72.7   | 72.2  | 73.3  | 79.8   | 80.1         | 79.6         | 79.8         | 69.3   | 68.8  |
| D2_target            | 72.7   | 73.0         | 72.7  | 100.0  | 98.0  | 96.6  | 73.6   | 74.4         | 73.9         | 73.6         | 72.7   | 72.2  |
| D3_target            | 79.8   | 79.8         | 79.8  | 73.6   | 73.3  | 73.6  | 100.0  | 98.9         | 96.9         | 98.6         | 73.9   | 73.3  |
| D4_target            | 69.3   | 69.3         | 68.8  | 72.7   | 73.0  | 72.4  | 73.9   | 74.2         | 73.0         | 73.9         | 100.0  | 97.4  |

Supplementary Fig. S11: Differences in the amino acid sequences of DEN-NS1 proteins in the clinical samples. (A) Alignment of the amino acid sequences of DEN-NS1 proteins in clinical samples and each recombinant DEN-NS1 protein used in aptamer generation as the target. The common amino acids in the sequences are denoted with asterisks. Each serotype is colored: DEN1-NS1 is dark orange, DEN2-NS1 is green, DEN3-NS1 is blue, and DEN4-NS1 is purple. Amino acids that are different from those in each targeted serotype NS1 protein are highlighted in light blue. (B) Summary of the sequence identity of the NS1 sequences, with mutation numbers, compared with each target NS1 protein sequence. The samples in which we successfully detected NS1 with the ELISA format, using the specific UB-DNA aptamers, are highlighted with each serotype's color.



Supplementary Fig. S12: Binding analysis of the enriched DNA library and 19D1F1 to DEN-NS1 variant 2 by EMSA. The enriched DNA library in the final round of ExSELEX-4 (A) and the isolated clone 19D1F1 (B) were incubated with DEN1-NS1 variant 2 (SIN), DEN1-NS1, DEN2-NS1, DEN3-NS1 and DEN4-NS1 from The Native Antigen Company, in binding buffer supplemented with 0.05% Nonidet P-40, at 25°C for 30 min. The DNA–NS1 complexes were separated from the free DNA on a native 4% acrylamide gel (A) and a 4% acrylamide gel in the presence of 2 M urea (B). DNA: 50 nM, DEN-NS1: 25 nM as the hexamer form; total 150 nM monomeric units.

5'-tttcgcactccatgatatggtctactg- $[N_{42}]$ -gacaagcggagtagttagaccgtcaaa-3'

|          |                 | Random region  |
|----------|-----------------|--|
| Family 1 | 40,282 (92.85%) | AGCGAGACGATGCTGCTAAAXTACGCCGTGGTXACGAAGACA   |
|          | 34,541 (79.62%) | AGCNAGNCGATGCTGCTAAA <b>x</b> TACGCCGTGGT <b>x</b> ACGAAGACA                         |
| 19D1F1   | 24,890          | AGC <b>G</b> AG <b>A</b> CGATGCTGCTAAA <b>x</b> TACGCCGTGGT <b>x</b> ACGAAGACA       |
|          | 5,699           | AGC <b>A</b> AG <b>A</b> CGATGCTGCTAAA <b>x</b> TACGCCGTGGT <mark>x</mark> ACGAAGACA |
|          | 2,589           | AGC <b>A</b> AG <b>T</b> CGATGCTGCTAAA <b>x</b> TACGCCGTGGT <b>x</b> ACGAAGACA       |
|          | 800             | AGC <b>T</b> AG <b>A</b> CGATGCTGCTAAA <b>x</b> TACGCCGTGGT <b>x</b> ACGAAGACA       |
|          | 418             | AGC <b>G</b> AG <b>T</b> CGATGCTGCTAAA <b>x</b> TACGCCGTGGT <b>x</b> ACGAAGACA       |
|          |                 |  |
| Family 2 | 965 (2.22%)     | ACGCAXGCGGGGGGGATAGGTAXAGGGGCGTGGTTXGGGTACG  |

В

С

|                   |                       | ELISA detection                          |           |  | SPR       |  |  |  |  |  |  |  |
|-------------------|-----------------------|--|-----------|--|-----------|--|--|--|--|--|--|--|
| DNA               | Preparation           | OD <sub>4</sub><br>reparation recombinar |           | OD <sub>450</sub> for<br>recombinant DEN-NS1 |           | Sequence   |  |  |  |  |  |  |
|                   |                       | None                                     | variant 2 | variant 1<br>(NAD1)                          | variant 2 |  |  |  |  |  |  |  |
| 19D1F1(isolate)   | DCD                   | 0.117                                    | >4.000    | 0.116  | 9.1 pM    | ItttogcactocatgatatggtotactgAGCGAGACGATGCTGCTAAAxTACGCCGTGGTxACGAAGACAgacaagoggagtagttagacogtgaaa                        |  |  |  |  |  |  |
| 19D1F1:Ds→NB      | FUR                   | 0.105                                    | 0.391     | 0.101  | N.D.      | $\tt ItttegcactccatgatatggtctactgAGCGAGACGATGCTGCTAAA\texttt{NTACGCCGTGGT\texttt{NACGAAGACAgacaagcggagtagttagaccgtgaaa}$ |  |  |  |  |  |  |
| 19D1F1-1          |                       | 0.104                                    | 0.119     | 0.099  | N.A.      | C66CC6AT6CT6CTAAAxTAC6CC6T6GTxAC6AA6ACAgacaagcggagtagttagacc66CC <u>6C6C6JA6C</u> 6                                      |  |  |  |  |  |  |
| 19D1F1-2          | 1                     | 0.102                                    | 0.101     | 0.096  | N.A.      | GCGCCAAAxTACGCCGTGGTxACGAAGACAgacaagcggagtagttGGCGC <u>CGCGLAGCG</u>   |  |  |  |  |  |  |
| 19D1F1-3 (AptD1b) | Chemical<br>synthesis | 0.078                                    | 3.592     | 0.078  | 27 pM     | <u>gcactcc</u> atgatatggtctactgA6CGAG6ACGATGCTGCTAAAxTACGCCGTGGTxACGAAGACAgacaagc <u>ggagt gt</u> <u>CGCGLA6CG</u>       |  |  |  |  |  |  |
| 19D1F1-4          | 0,110,000             | 0.079                                    | 0.080     | 0.079  | N.A.      | <u>gcactec</u> <u>gtet</u> actgA6CGAGACGATGCTGCTAAAXTACGCCGTGGTXACGA <u>AGAC</u> <u>ggagt gt</u> <u>CGCGLA6CG</u>        |  |  |  |  |  |  |
| 19D1F1-5          |                       | 0.079                                    | 0.083     | 0.078  | N.A.      | gcactcc g_ctactqAGCGAGACGATGCTGCTAAAxTACGCCGTGGTxACGAAGACAg_cqqaqt_qtCCCCLAGCG   |  |  |  |  |  |  |



**Supplementary Fig. S13: The UB-DNA aptamer generation targeting DEN1-NS1 variant 2.** (A) Alignment of the random-region DNA sequences obtained by ExSELEX-4 (Supplementary Table S3). The unnatural-base positions, indicated by "x" in red, were predicted from the mutation spectra (natural-base composition rates) after replacement PCR. The ratio (%) of each family was calculated from the total counts categorized in the same family against the total extracted reads for the analysis. (B) DNA aptamer candidates targeting DEN1-NS1 variant 2. 19D1F1 variants, used for the binding analyses (ELISA and SPR) against the DEN1-NS1 variant 2 are summarized. (C) Possible secondary structures of Apt1Db (19D1F1-3), predicted from the binding activities of its variants. The description of each nucleotide is similar to that in Figure 2.



Supplementary Fig. S14: Binding analysis of UB-DNA aptamers, 19D1F1-3 (AptD1b) and 19D1F1 (isolate), to each target by a Biacore T200 SPR system at 25°C. The SPR analysis was performed using the DEN1-NS1 variant 2 (SIN DEN1-NS1) (A) and the DEN1-NS1 from The Native Antigen Company (NAD1) (B). Running buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl<sub>2</sub>, 2.7 mM KCl, and 0.05% Tween 20. Flow rate: 30  $\mu$ l/min. Injection (association) time: 150 sec. Dissociation time: 600 sec (general) or 1,200 sec for determination of kinetic parameters. The kinetic parameters, association rates ( $k_{on}$ ), dissociation rates ( $k_{off}$ ), and dissociation constants ( $K_D$ ), were determined through 1:1 global curve fitting with the BIAevaluation software version 3.0, by using the double-reference subtraction method. Representative association and dissociation curves with fitting (gray lines) are shown. Regeneration was performed with a 5-sec injection of 50 mM NaOH, followed by a 10-min equilibration with running buffer.

| NAD1  | DSGCVINWKGRELKCGSGI | FVTNEVHTWTEQYKFQADS                              | SPKRLSAAIGKAWEEGVCGI                | RSA 60     |
|---|---------------------|--|-------------------------------------|------------|
| PD1-1/4/5/6                                 |                     |  |                                     |            |
| PD1-7                                       |                     |  |                                     |            |
| PD1-8                                       |                     |  |                                     |            |
| PD1-9                                       |                     |  |                                     |            |
| PD1-10                                      |                     |  |                                     |            |
| PD1-11/12                                   |                     |  |                                     |            |
| PD1-2/3/13/14/15/16/17/18/19/20             |                     |  |                                     |            |
| PD1-21<br>-22                               |                     |  |                                     |            |
| NAD1  | TRLENIMWKQISNELNHII | LENDMKFTVVVG <mark>DVS</mark> GIL <mark>4</mark> | AQGKKMIRPQPMEHKYSWKS                | WGK 120    |
| PD1-1/4/5/6                                 | π                   |  | N                                   |            |
| PD1-7                                       | -<br>T              | DVT  |                                     |            |
| PD1-8                                       | -<br>T              | DVAZ   |                                     |            |
| PD1-9                                       | -<br>T              | DVA  |                                     |            |
| PD1-10                                      | -<br>T              | NVA  | A                                   |            |
| PD1-11/12                                   | -<br>T              | DAN  | r                                   |            |
| PD1-2/3/13/14/15/16/17/18/19/20             | -<br>T              | DAN  | -<br><b>-</b>                       |            |
| PD1-21                                      | -<br>A              | DAN  | -<br>                               |            |
| PD1-22                                      | т                   | DAN  | -<br>r                              |            |
| NAD1  | AKIIGADVONTTFIIDGP  | TPECPDNORAWNIWEVED                               | YGFGIFTTNIWLKLRDSYTC                | VCD 180    |
| PD1-1/4/5/6                                 | DVN                 | D  | <b>I</b>                            | <b>v</b>   |
| PD1-7                                       | DVN                 | IDD  | <mark>I</mark>                      | - <b>v</b> |
| PD1-8                                       | DVN                 | ID   | <b>I</b>                            | - <b>v</b> |
| PD1-9                                       | DVN                 | ID   | <b>I</b>                            | <b>v</b>   |
| PD1-10                                      | DVN                 | ID   | <b>I</b>                            | - <b>v</b> |
| PD1-11/12                                   | <b>DTL</b>          | )D   | <b>v</b> v                          | - <b>v</b> |
| PD1-2/3/13/14/15/16/17/18/19/20             | DTI                 | )DD  | <b>v</b> v                          | -M         |
| PD1-21                                      | DT                  | )DD  | <b>v</b>                            | -M         |
| PD1-22                                      | ETE                 | )DD  | <mark>v</mark>                      | - <u>M</u> |
| NAD1  | HRLMSAAIKDSKAVHADMO | GYWIESEKNETWKLARASF1                             | IEVKTCIWP <mark>K</mark> SHTLWSNGVL | ESE 240    |
| PD1-1/4/5/6                                 |                     |  | <b>K</b>                            |            |
| PD1-7                                       |                     |  | <mark>k</mark>                      |            |
| PD1-8                                       |                     |  | <mark>k</mark>                      |            |
| PD1-9                                       |                     |  | K                                   |            |
| PDI-IU<br>11/12                             |                     |  | K                                   |            |
| PDI-II/IZ<br>D1-2/2/12/14/15/16/17/19/10/20 |                     |  | K<br>P                              |            |
| PD1-2/3/13/14/13/10/17/10/13/20             |                     |  | <u>k</u>                            |            |
| PD1-21<br>1-22                              |                     |  | <u>R</u>                            |            |
| NAD1  | MTTPKTYGGPTSOHNYRPG | YFTOTAGPWHIGKLELDF                               | LCEGTTVVVDEHCGNRGPS                 | T.RT 300   |
| PD1-1/4/5/6                                 | -I                  |  |                                     |            |
| PD1-7                                       | - <b>I</b>          | <b>r</b>   | D                                   |            |
| PD1-8                                       | - <b>v</b>          | <b>I</b>   | D                                   |            |
| PD1-9                                       | -I                  | <b>N</b>   | N                                   |            |
| PD1-10                                      | - <b>I</b>          | <mark>I</mark>                                   | D                                   |            |
| PD1-11/12                                   | - <b>I</b>          | <b>N</b>   | N                                   |            |
| PD1-2/3/13/14/15/16/17/18/19/20             | -I                  | ·  | N                                   |            |
| PD1-22                                      | - <b>T</b>          |  | N                                   |            |
| NAD1  | TTVTGKTIHEWCCRSCTLE | PLRFKGEDGCWYGMEIRP                               | VKEKEENLVKSMVSA 352                 |            |
| PD1-1/4/5/6                                 | I                   | FR   | K                                   |            |
| PD1-7                                       | I                   | <b>F</b> R                                       | <b>K</b>                            |            |
| PD1-8                                       | I                   | FR   | <b>K</b>                            |            |
| PD1-9                                       | I                   | <b>F</b> R                                       | <b>K</b>                            |            |
| PD1-10                                      | I                   | YR   | <b>K</b>                            |            |
| PD1-11/12                                   | I                   | <b>F</b> R                                       | <b>R</b>                            |            |
| PD1-2/3/13/14/15/16/17/18/19/20             | II                  | FR   | <b>R</b>                            |            |
| PD1-21                                      | I                   | <b>F</b> R                                       | <b>R</b>                            |            |
| PD1-22                                      | I                   | <b>F</b> R                                       | <b>R</b>                            |            |

Supplementary Fig. S15: Differences in the amino acid sequences of DEN1-NS1 proteins in the clinical samples, PD1-1 through PD1-22. Alignment of the amino acid sequences of DEN1-NS1 proteins in clinical samples and the original DEN1-NS1 protein (NAD1) used in AptD1 aptamer generation as the target. For AptD1b generation, we used the prepared recombinant DEN1-NS1 from PD1-2 and the clinical sample PD1-13. The common amino acids in the sequences are represented by hyphens.



Supplementary Fig. S16: NS1 sequence variations of dengue serotype 1 clinical samples. The amino acids that differed from those in each target dengue NS1 protein from The Native Antigen Company were mapped onto the tertiary structure of the dengue NS1 dimer (PDB: 4O6B), with one subunit in gray and the other colored by domains (cyan,  $\beta$  roll; yellow/orange, wing/connector subdomain; pink,  $\beta$  ladder). The amino acid variations found only in variant 1 (the sequences of The Native Antigen Company and PD1-1 to PD1-10) or variant 2 (PD1-11 to PD1-22) are indicated in blue, while those in PD1-1 to PD1-10 and PD1-11 to PD1-22, which might include critical amino acids for our AptD1 and AptD1b aptamers' binding, are indicated in red. Since the amino acid residue at position 162 would be located within the NS1 hexamer, this residue would be not critical for the specificity of the AptD1 and AptD1b aptamers.



**Supplementary Fig. S17: Possible topologies of the G-quadruplex of AptD2-1.** The G-to-A scanning experiments indicate the importance of G15 and G48, as well as G25, G32, G37, and G43 in the G-tracts (Fig. 4).