Supplemental Information

RE-SELEX: Restriction Enzyme-Based Evolution of Structure-Switching Aptamer Biosensors

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MATERIALS AND METHODS

Design of restriction enzyme libraries

EcoRI Library or BamHI library was hybridized using slow cooling from 95 °C to 25 °C over 30 minutes in a thermal cycler to varying lengths of capture sequences (8-14 nt) in a 1:1 ratio in 1X Cut Smart Buffer (50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 100 µg/mL bovine serum albumin, pH 7.9, New England Biolabs) to a final concentration of 1 µM library and 1 µM capture sequence. The hybridized complex was then divided into 10 µL portions and incubated with either 1.5 U of EcoRI-HF or BamHI-HF (New England Biolabs). The solutions were incubated for 2 h at 37 °C, followed by 20 min at 65 °C. Samples were analyzed by denaturing 10% PAGE to monitor digestion of the biosensor. The gels were imaged using a GE Amersham Typhoon RGB scanner and a 488 nm excitation laser and the Cy2 525BP20 emission filter. Digestion efficiency was determined by the percent cleaved (fluorescence band volume for the cleaved product/total lane volume) using ImageJ.

Bead assisted restriction enzyme SELEX

For the bead assisted restriction enzyme SELEX, capture strand provided directly on the oligo-affinity polymeric support (26-4001-01, Glen Research) as PS Bead-spacer18-spacer18-photocleavable linker (10-4913, Glen Research) -3' AGTCTTAAGTAA-5' from the University of Utah DNA/Peptide Synthesis Core Facility. 500 µL of 1 mg/mL of beads were washed 2X by vortexing and centrifugation (16 xg, 2 minutes) with 500 µL of the selection buffer (50 % 1X Cut Smart Buffer (New England Biolabs) 50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 100 µg/mL bovine serum albumin (pH 7.9); 1X artificial cerebrospinal fluid buffer, 0.05 % Tween-20). The beads were resuspended in with 1 nmol of library in a total of 500 µL selection buffer. After round 1, 100 picomole were used with 100 µL beads in a total of 100 µL selection buffer. The library was hybridized to the beads for 1 h at 25 °C. The beads were then washed 3X with 500 µL selection buffer as described above. Biosensor was collected following UV irradiation (365 nm) for 45 minutes and resuspended in 10 mM target in selection buffer and incubated for 1 hr at 25 °C.

Bulk biosensor test

Round 10 and 11 Library and 3'-BHQ-1 (12 nt) and capture strand were diluted in selection buffer (50 % 100 mM sodium chloride, 20 mM tris-hydrochloride, 2 mM magnesium chloride, 5 mM potassium chloride, and 1 mM calcium chloride (pH 7.6); 50 % 1X Cut Smart Buffer (New England Biolabs) 50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 100 µg/mL bovine serum albumin (pH 7.9)). Biosensors were constructed by combining library (1 µM) with capture strand (2 µM) in selection buffer and heating to 95 °C and slow-cooling to 25 °C over 30 mins in a thermal cycler. Biosensor was then added to a Corning Costar 384-well black plate. Buffer or kanamycin A (1 mM) was then added and re-incubated for 1 h at 25 °C, covered in foil. Samples were normalized to a well with library in selection buffer alone. Percent displacement was calculated using equation 1.

Kanamycin immobilization on beads and binding test

Pierce™ NHS-activated magnetic beads (300 µL) were washed with 1 mL of ice-cold 1 mM hydrochloric acid. After discarding the supernatant, 300 µL of 10 mg/mL kanamycin A was added to the beads and incubated with gentle shaking for 18 h at 25 °C. The beads were then collected using a magnetic stand and washed twice with 1 mL 50 mM borate buffer, pH 8.5 followed by ultrapure water once. Quenching buffer (1 mL) was then added and incubated for 2 h at 25 °C. The beads were then collected, washed twice with ultrapure water and stored in 300 µL selection buffer. For bead binding, 20 µL beads were diluted in 100 µL selection buffer containing 100-200 pmol of each aptamer candidate (n=1). The samples were incubated at 25 °C for 1 h, then washed twice using selection buffer before eluting twice for 5 min at 95 °C. Absorbance was measured using a BioTek Take3 plate set to ssDNA mode. Percent bound was calculated from quantifying elution compared to input. We included #3-19 to benchmark positive hits. We also included a random ssDNA as a negative control to set a baseline for non-specific binding.
RESULTS

**Supplementary Figure 1.** DNA sequences used in restriction enzyme library optimization. FAM = fluorescein

| Name                        | Sequence (5'-3')                                                                 |
|-----------------------------|---------------------------------------------------------------------------------|
| EcoRI-library               | /FAM/CGCATACCCAGCTTAGTTCAGAGAATTTCATTTN40-AGATAGTAAAGTGCAATCTCGGCG              |
| EcoRI-8 nt CS               | TGAATCTC                                                                         |
| EcoRI-10 nt CS              | ATGAATCTCG                                                                      |
| EcoRI-12 nt CS              | AATGAATCTCGA                                                                     |
| BamHI-library               | /FAM/CGCATACCCAGCTTAGTTCAGAGAATTTCATTTN40-AGATAGTAAAGTGCAATCTCGGCG              |
| BamHI-8 nt CS               | GGATTC                                                                            |
| BamHI-10 nt CS              | TGGATCTCGA                                                                       |
| BamHI-12 nt CS              | ATGGATCTCGA                                                                     |
| BamHI-14 nt CS              | AATGGATCTCGA                                                                     |

**Supplementary Figure 2.** DNA sequences used for SELEX and biosensor characterization. FAM = fluorescein; Sp9 = spacer 9; BHQ1 = black hole quencher 1

| Name                        | Sequence (5'-3')                                                                 |
|-----------------------------|---------------------------------------------------------------------------------|
| EcoRI-library               | /FAM/CGCATACCCAGCTTAGTTCAGAGAATTTCATTTN40-AGATAGTAAAGTGCAATCTCGGCG              |
| forward primer              | /FAM/CGCATACCCAGCTTAGTTCAGAGAATTTCATTTN40-AGATAGTAAAGTGCAATCTCGGCG              |
| unlabeled forward primer    | CGCATACCCAGCTTAGTTCAGAGAATTTCATTTN40-AGATAGTAAAGTGCAATCTCGGCG                  |
| reverse primer              | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Supplementary Figure 3. Maximizing digestion efficiency of restriction enzyme-based libraries. Restriction enzyme mediated digestion of library hybridized to capture strands of varying length for EcoRI and BamHI. Digestion of the 5'-fluorescein labeled libraries was carried out for 2 h at 37 °C and cleavage products were analyzed by 10 % PAGE. Band intensity was used to quantify percent cleaved.
Supplementary Figure 4. Bead assisted EcoRI SELEX to generate structure-switching aptamers. (a) Progression of bead assisted EcoRI SELEX to generate structure-switching aptamers. (b) Following digestion, cleavage products were monitored by 10% PAGE. Band intensity was used to quantify percent uncleaved. (c) Potential structure-switching aptamer candidates and their corresponding enrichment values from round 4 to round 7. The EcoRI restriction site is underlined. The N40 restriction site is underlined with a single mismatch (bold, red).
Supplementary Figure 5. Optimization of EcoRI-based SELEX steps. (a) Scheme overview of EcoRI-based SELEX. Steps b, c, and d were optimized to reduce background enrichment. (b) The initial N40 5'-fluorescein labelled ssDNA library was hybridized to capture strand in a 1:2 ratio in selection buffer. This complex was digested with EcoRI-HF. The 70 nt cleaved product was gel excised and purified following 10% PAGE, 1X SYBR Gold staining, and visualization on a UV-transilluminator. (c) Full length product was recovered through ligation by T4 DNA ligase and visualized using 10% PAGE. Percent ligated was calculated by the band intensity using ImageJ and was used to calculate final library concentration in the biosensor. (d) 10% PAGE analysis of EcoRI digestion of purified ligation from (b) after hybridization to free capture strand in a 1:2 ratio. Percent cleaved was calculated from band intensity using ImageJ.

Supplementary Figure 6. 10% PAGE analysis of SELEX rounds. Digestion of the 5'-fluorescein labelled libraries was carried out for 2 h at 37 °C. Cleavage products for buffer (-) and 10 mM kanamycin (+) were analyzed by 10% PAGE. Percent cleaved was quantified using band intensity in ImageJ using library as a positive control lane. Rounds increase from left to right.
Supplementary Figure 7. Bulk biosensor activity of SELEX rounds 10 and 11. PCR amplified library (1 µM) from rounds 10 (n=1) and 11 (n=2) were hybridized to the 12 nt BHQ1 capture strand (2 µM). 1 mM kanamycin A or selection buffer was added to the biosensors and incubated for 1 h at 25 °C. Displacement was quantified by measuring the fluorescence intensity on a Cytation 5 multi-mode plate reader (BioTek) using excitation at 490 nm and emission at 520 nm (bandwidth 9, read height 6.5 mm). All samples were normalized to wells with aptamer alone. Percent displacement was calculated using equation 1 and plotted using GraphPad Prism.
Supplementary Figure 8. Bioinformatics analysis of SELEX rounds 9, 10, and 11. (a) Percent unique sequences over SELEX rounds calculated from the total sequences and number of unique sequences populated using the FASTAptamer-Count function. The count files were then further analyzed using the FASTAptamer-Compare function to show scatter plots of read distribution from (b) replicate reads for round 11 (c) rounds 9 and 10 and (d) rounds 10 and 11.
Free energy of secondary structure: -13.60 kcal/mol

Free energy of secondary structure: -14.20 kcal/mol

Free energy of secondary structure: -12.54 kcal/mol

Free energy of secondary structure: -15.31 kcal/mol

Free energy of secondary structure: -16.48 kcal/mol

Free energy of secondary structure: -23.05 kcal/mol
Supplementary Figure 9. Secondary structure analysis of aptamer sequences using NUPACK. Structures were predicted at 25 °C using standard conditions.
Supplementary Figure 10. Initial bead binding screen for top aptamer candidates. Candidates were incubated with 10 mg/mL kanamycin A magnetic beads for 1 h at 25 °C, followed by two washes. Bound sequences were eluted at 95 °C for 5 min, repeated once. The eluted ssDNA was quantified using absorbance at 260 nm using a Biotek Take3 plate on the plate reader. Percent bound was calculated from (eluted ssDNA/input) *100 %. Sequences that had higher % bound than the negative control (random) were carried forward for biosensor testing. (n=1)

Supplementary Figure 11. Increased ratio of 9 nt capture strand does not increase K16-1 stability. Displacement as a function of kanamycin A concentration using increasing K16-1:capture strand ratios using (a) 9 nt and (b)10 nt capture strands. Samples resulting in negative displacement were denoted 0 % displacement. Error bars denote standard error (n=3).
Supplementary Figure 12. Biosensor activity of #3-19. (a) Structure of #3-19, a top binding candidate from Capture SELEX hybridized to the 12 nt capture strand. The docking site is in highlighted in red. Structure estimated using NUPACK at 25°C using standard conditions. (b) Biosensor concentration impacts signal quenching from hybridization of #3-19 to 12 nt capture strand. (c) Normalized RFU to show there was no displacement as a function of kanamycin A concentration using capture stand lengths. Error bars denote standard error (n=3).

Supplementary Figure 13. Normalized RFU of K16-1 with aminoglycosides. Normalized RFU to show there was a concentration dependent drop in fluorescence as a function of aminoglycoside concentration. Error bars denote standard error (n=3).
Supplementary Figure 14. Binding isotherm for K16-1 and #3-19 determined by MST. Graphs are displayed as fraction bound versus kanamycin A concentration. Errors represent the standard error of three independent trials. The data was graphed using GraphPad Prism and fit according to the law of mass action to determine $K_D$.

Supplementary Figure 15. Tabular data for K16-1 hybridization with 12 nt capture strand in percent quenched and raw fluorescence values. Errors represent the standard error of three independent trials.

| Biosensor concentration (nM) | % Quenched  | Aptamer (RFU) | Biosensor (RFU) |
|------------------------------|-------------|---------------|-----------------|
| 10                           | 12.6 ± 7.2  | 1066 ± 105    | 989 ± 65        |
| 50                           | 11.9 ± 9.2  | 6528 ± 446    | 5828 ± 671      |
| 100                          | 34.7 ± 6.5  | 11067 ± 1023  | 7097 ± 205      |
| 250                          | 49.5 ± 5.6  | 27913 ± 674   | 14009 ± 1994    |
| 500                          | 51.2 ± 4.7  | 55966 ± 2971  | 27107 ± 1623    |
| 1000                         | 50.4 ± 5.8  | 107056 ± 4931 | 52498 ± 3568    |

Supplementary Figure 16. Tabular data for K16-1 dose-displacement with 9, 10, and 12 nt capture strand in percent displacement. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt     | 10 nt    | 12 nt    |
|------------------|----------|----------|----------|
| 0                | 0.0 ± 0.0| 0.0 ± 0.0| 0.0 ± 0.0|
| 10               | 3.0 ± 1.9| 0.0 ± 0.0| 0.1 ± 0.1|
| 100              | 19.9 ± 4.8| 5.9 ± 5.0| 14.7 ± 7.9|
| 1000             | 28.5 ± 10.0| 15.7 ± 4.9| 11.9 ± 4.9|
| 10000            | 56.8 ± 10.9| 46.8 ± 5.7| 21.7 ± 5.0|
Supplementary Figure 17. Tabular data for K16-1 dose-displacement with 9, 10, and 12 nt capture strand in raw fluorescence values. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt       | 10 nt       | 12 nt       |
|------------------|------------|-------------|-------------|
| 0                | 9966 ± 132 | 16927 ± 294 | 14681 ± 311 |
| 10               | 9801 ± 379 | 16535 ± 523 | 14372 ± 217 |
| 100              | 10545 ± 103| 16948 ± 158 | 15596 ± 192 |
| 1000             | 10804 ± 306| 17531 ± 170 | 15420 ± 37  |
| 10000            | 11608 ± 231| 18718 ± 72  | 16006 ± 101 |

Supplementary Figure 18. Tabular data for K1-1 dose-displacement with 9, 10, and 12 nt capture strand in percent displacement. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt       | 10 nt       | 12 nt       |
|------------------|------------|-------------|-------------|
| 0                | 0.0 ± 0.0  | 0.0 ± 0.0   | 0.0 ± 0.0   |
| 10               | 0.1 ± 0.1  | 2.5 ± 2.5   | 0.0 ± 0.0   |
| 100              | 1.8 ± 0.9  | 6.4 ± 6.4   | 0.0 ± 0.0   |
| 1000             | 22.8 ± 3.2 | 14.9 ± 4.5  | 0.2 ± 0.2   |
| 10000            | 39.7 ± 1.5 | 15.4 ± 3.9  | 0.5 ± 0.5   |

Supplementary Figure 19. Tabular data for K1-1 dose-displacement with 9, 10, and 12 nt capture strand in raw fluorescence values. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt       | 10 nt       | 12 nt       |
|------------------|------------|-------------|-------------|
| 0                | 4945 ± 100 | 4308 ± 104  | 2718 ± 81   |
| 10               | 4874 ± 140 | 3882 ± 491  | 2437 ± 33   |
| 100              | 4943 ± 104 | 4418 ± 154  | 2479 ± 42   |
| 1000             | 5355 ± 47  | 4677 ± 30   | 2659 ± 62   |
| 10000            | 5651 ± 50  | 4682 ± 10   | 2696 ± 30   |

Supplementary Figure 20. Tabular data for K1-2 dose-displacement with 9, 10, and 12 nt capture strand in percent displacement. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt       | 10 nt       | 12 nt       |
|------------------|------------|-------------|-------------|
| 0                | 0.0 ± 0.0  | 0.0 ± 0.0   | 0.0 ± 0.0   |
| 10               | 5.0 ± 5.0  | 8.3 ± 6.7   | 0.0 ± 0.0   |
| 100              | 6.7 ± 5.3  | 16.8 ± 5.0  | 2.7 ± 1.2   |
| 1000             | 12.7 ± 3.3 | 28.6 ± 4.9  | 0.0 ± 0.0   |
| 10000            | 34.4 ± 7.6 | 19.3 ± 9.0  | 0.1 ± 0.1   |
**Supplementary Figure 21.** Tabular data for K1-2 dose-displacement with 9, 10, and 12 nt capture strand in raw fluorescence values. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt   | 10 nt   | 12 nt   |
|------------------|--------|---------|---------|
| 0                | 5553 ± 33 | 4281 ± 13 | 2775 ± 25 |
| 10               | 5469 ± 185 | 4296 ± 103 | 2645 ± 19 |
| 100              | 5660 ± 129 | 4411 ± 14  | 2841 ± 23 |
| 1000             | 5790 ± 81  | 4513 ± 33  | 2742 ± 30 |
| 10000            | 6200 ± 159 | 4413 ± 54  | 2729 ± 47 |

**Supplementary Figure 22.** Tabular data for K2-1 dose-displacement with 9, 10, and 12 nt capture strand in percent displacement. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt | 10 nt | 12 nt |
|------------------|------|-------|------|
| 0                | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 10               | 1.1 ± 1.1 | 0.2 ± 0.2 | 0.4 ± 0.4 |
| 100              | 2.3 ± 1.4 | 11.0 ± 2.4 | 2.5 ± 2.2 |
| 1000             | 9.9 ± 6.0 | 4.1 ± 2.1 | 0.8 ± 0.8 |
| 10000            | 26.6 ± 5.4 | 1.3 ± 0.9 | 1.1 ± 1.1 |

**Supplementary Figure 23.** Tabular data for K2-1 dose-displacement with 9, 10, and 12 nt capture strand in raw fluorescence values. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt | 10 nt | 12 nt |
|------------------|------|-------|------|
| 0                | 7248 ± 71 | 5681 ± 101 | 3730 ± 104 |
| 10               | 6876 ± 159 | 5635 ± 77  | 3521 ± 87  |
| 100              | 7305 ± 41  | 6130 ± 98  | 3885 ± 44  |
| 1000             | 7384 ± 203 | 5794 ± 48  | 3618 ± 38  |
| 10000            | 7858 ± 77  | 5721 ± 140 | 3701 ± 42  |

**Supplementary Figure 24.** Tabular data for K16-1 dose-displacement with 9 nt capture strand in percent displacement and raw fluorescence values. Errors represent the standard error of nine independent trials.

| Kanamycin A (µM) | % Displacement | RFU     |
|------------------|----------------|---------|
| 0                | 0.0 ± 0.0 | 7600 ± 453 |
| 90               | 3.8 ± 2.2 | 7461 ± 591 |
| 180              | 5.1 ± 3.3 | 7674 ± 493 |
| 300              | 15.8 ± 5.4 | 8090 ± 595 |
| 600              | 22.2 ± 6.7 | 8287 ± 606 |
| 900              | 26.6 ± 7.1 | 8509 ± 657 |
| 1800             | 26.6 ± 7.3 | 8543 ± 601 |
| Biosensor concentration (nM) | % Quenched   | Aptamer (RFU) | Biosensor (RFU) |
|-----------------------------|--------------|---------------|-----------------|
| 10                          | 46.4 ± 3.1   | 271 ± 9       | 146 ± 13        |
| 50                          | 54.6 ± 1.4   | 2891 ± 139    | 1308 ± 28       |
| 100                         | 68.3 ± 2.1   | 8374 ± 320    | 2643 ± 89       |
| 250                         | 66.8 ± 0.3   | 22932 ± 546   | 7613 ± 234      |
| 500                         | 71.0 ± 0.7   | 51430 ± 914   | 14896 ± 101     |
| 1000                        | 71.7 ± 0.4   | 99221 ± 2224  | 28080 ± 365     |

**Supplementary Figure 25.** Tabular data for #3-19 hybridization with 12 nt capture strand in percent quenched and raw fluorescence values. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 9 nt       | 10 nt      | 12 nt      |
|------------------|------------|------------|------------|
| 0                | 5457 ± 139 | 4956 ± 130 | 2785 ± 68  |
| 10               | 5349 ± 135 | 4829 ± 275 | 2883 ± 65  |
| 100              | 5809 ± 65  | 5163 ± 185 | 2971 ± 29  |
| 1000             | 5434 ± 63  | 4853 ± 41  | 2831 ± 57  |
| 10000            | 5370 ± 115 | 4593 ± 52  | 2656 ± 68  |

**Supplementary Figure 26.** Tabular data for #3-19 dose-displacement with 9, 10, and 12 nt capture strand in raw fluorescence values. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 1:1         | 1:2         | 1:3         |
|------------------|-------------|-------------|-------------|
| 0                | 0.0 ± 0.0   | 0.0 ± 0.0   | 0.0 ± 0.0   |
| 10               | 3.0 ± 1.9   | 7.6 ± 7.6   | 15.8 ± 13.5 |
| 100              | 19.9 ± 4.8  | 16.5 ± 16.5 | 22.6 ± 12.6 |
| 1000             | 28.5 ± 10.0 | 28.1 ± 15.4 | 30.7 ± 9.8  |
| 10000            | 56.8 ± 10.9 | 81.4 ± 8.2  | 43.8 ± 9.6  |

**Supplementary Figure 27.** Tabular data for K16-1 dose-displacement at 1:1, 1:2, and 1:3 ratio aptamer: 9 nt capture strand in percent displacement. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 1:1         | 1:2         | 1:3         |
|------------------|-------------|-------------|-------------|
| 0                | 9966 ± 132  | 9356 ± 326  | 8828 ± 415  |
| 10               | 9801 ± 379  | 9272 ± 45   | 9220 ± 51   |
| 100              | 10545 ± 103 | 9335 ± 282  | 9461 ± 148  |
| 1000             | 10804 ± 306 | 9806 ± 160  | 9662 ± 99   |
| 10000            | 11608 ± 231 | 10826 ± 289 | 9997 ± 52   |

**Supplementary Figure 28.** Tabular data for K16-1 dose-displacement at 1:1, 1:2, and 1:3 ratio aptamer: 9 nt capture strand in raw fluorescence values. Errors represent the standard error of three independent trials.
**Supplementary Figure 29.** Tabular data for K16-1 dose-displacement at 1:1, 1:2, and 1:3 ratio aptamer: 10 nt capture strand in percent displacement. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 1:1                  | 1:2                  | 1:3                  |
|------------------|----------------------|----------------------|----------------------|
| 0                | 0.0 ± 0.0            | 0.0 ± 0.0            | 0.0 ± 0.0            |
| 10               | 0.0 ± 0.0            | 4.3 ± 2.7            | 1.1 ± 1.1            |
| 100              | 5.9 ± 5.0            | 25.0 ± 5.0           | 15.1 ± 9.9           |
| 1000             | 15.7 ± 4.9           | 34.5 ± 6.3           | 19.2 ± 4.6           |
| 10000            | 46.8 ± 5.7           | 56.7 ± 9.5           | 36.5 ± 0.8           |

**Supplementary Figure 30.** Tabular data for K16-1 dose-displacement at 1:1, 1:2, and 1:3 ratio aptamer: 10 nt capture strand in raw fluorescence values. Errors represent the standard error of three independent trials.

| Kanamycin A (µM) | 1:1                  | 10 nt                | 12 nt                |
|------------------|----------------------|----------------------|----------------------|
| 0                | 16927 ± 294          | 13957 ± 273          | 9223 ± 73            |
| 10               | 16535 ± 523          | 13962 ± 217          | 9159 ± 43            |
| 100              | 16948 ± 158          | 14871 ± 76           | 9498 ± 223           |
| 1000             | 17531 ± 170          | 15205 ± 228          | 9637 ± 141           |
| 10000            | 18718 ± 125          | 16011 ± 387          | 10015 ± 49           |

**Supplementary Figure 31.** Tabular data for K16-1 raw fluorescence in the presence of aminoglycosides. Errors represent the standard error of three independent trials.

| Target concentration (µM) | Kanamycin A | Kanamycin B | Streptomycin | Tobramycin |
|---------------------------|-------------|-------------|--------------|------------|
| 0                         | 13829 ± 44  | 8174 ± 74   | 13829 ± 44   | 13829 ± 44 |
| 10                        | 13094 ± 162 | 8005 ± 21   | 13016 ± 84   | 13381 ± 54 |
| 100                       | 13241 ± 130 | 8210 ± 444  | 13066 ± 100  | 12919 ± 74 |
| 1000                      | 12925 ± 660 | 7379 ± 40   | 13566 ± 101  | 12142 ± 254|
| 10000                     | 14830 ± 130 | 1767 ± 11   | 11831 ± 236  | 6619 ± 20  |

**Supplementary Figure 32.** Tabular data for K16-1 displacement with 9 nt capture strand in percent displacement for aminoglycosides at 1 mM in percent displacement and normalized fluorescence. Errors represent the standard error of three independent trials.

| Target                  | % Displacement |
|-------------------------|----------------|
| Biosensor alone         | 0.0 ± 0.0      |
| Kanamycin A             | 41.9 ± 7.7     |
| Kanamycin B             | 0.0 ± 0.0      |
| Tobramycin              | 2.8 ± 3.5      |
| Streptomycin            | 2.8 ± 1.7      |
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