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Optimizing Cross-reactivity with Evolutionary Search for Sensors

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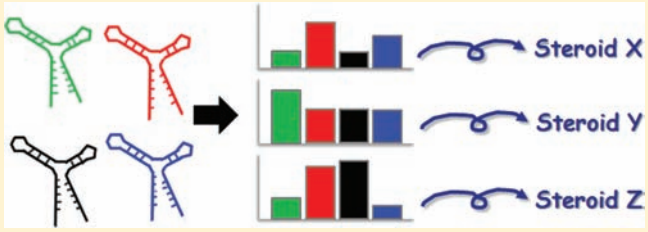
Supporting Information

ABSTRACT: We report a straightforward evolutionary procedure to build an optimal sensor array from a pool of DNA sequences oriented toward three-way junctions. The individual sensors were mined from this pool under separate selection pressures to interact with four steroids, while allowing cross-reactivity, in a manner designed to achieve perfect classification of individual steroids. The resulting sensor array had three sensors and displayed discriminatory capacity between steroid classes over full ranges of concentrations. We propose that similar protocols can be used whenever we have two or more classes of samples, with individual classes being defined through gross differences in ratios of dominant families of responsive components.



INTRODUCTION

Inspiration for cross-reactive sensor arrays[1−5] (chemical or electronic noses) comes from the ability of the mammalian olfactory system to differentiate a large number of odorants over a wide range of concentrations and against various backgrounds. In mammalian olfaction, the relative sparseness of the receptor space is made up for by a complex computing process in the neural system.[6−8] Analogously, in many artificial electronic noses, advanced machine learning techniques are used to optimize classification.[9] If applied to molecular sensing problems, the arrays may lead to immediate classification of samples (e.g., urine) into various classes, shortening the time required for differential diagnosis.[10]

We previously described[11] nonspecific hydrophobic receptors and their derivative sensors—inspired by nucleic acid-based three-way junctions (TWJ)[12]—suitable for the construction of minimal cross-reactive arrays. In more recent work, we focused on methods for constructing minimal high-resolution cross-reactive arrays.[13] For example, we demonstrated a set of only three sensors that perfectly classifies ten hydrophobic analytes over a range of concentrations.[13c] A drawback of our earlier approaches was that we had to manually screen a large number of individually synthesized receptors, containing modified bases, in order to identify optimal sensors. We now ask: What is the optimal way to search the space of interactions between analytes and sensors for suitable sets of sensors acting as highly effective cross-reactive arrays? And also: Is the natural space of hydrophobic receptors based on nucleic acids with the four standard bases sufficiently rich to obtain good classification results, or do we need to use modified bases? The latter question is of more general significance as well, because it directly addresses doubts as to whether nucleic acids can efficiently form hydrophobic recognition regions.

Taking advantage of the nucleic acid ancestry of our receptors, we now report an *in vitro* selection and amplification procedure resulting directly in optimal cross-reacting arrays[14,15] while favoring the fittest receptors isolated from large pools on the basis of interactions with what is common to a class of samples. In the specific demonstration, we target samples of pure steroids, as model compounds for broad families of urinary steroids.

RESULTS AND DISCUSSION

General Principles. With smaller oligonucleotide libraries (less than 4^{22} members), we have the ability to exhaustively screen the entire receptor space spanned by the sequences, and we can do so each time we perform a selection (i.e., we assume that our initial conditions are the same in each selection). Suppose that (i) we have two sets of samples (**A** and **B**) that will be used in two separate selections, each set with a different dominant family of analytes that is responsive to receptors (in our case families of related steroids are modeled by characteristic steroids); (ii) in our library of receptors there are members that are more sensitive (differentially cross-reactive) to the major components of **A** and **B**; and (iii) we perform the selection so the frequency of receptors identified in the final pool (defined as the number of times we observe each receptor after cloning and sequencing of, e.g., 100 pool members) is proportional to the ability of receptors to interact with responsive components in samples.

Recall our assumption that there exist receptors with differential response to samples **A** and **B**; that is, there is at least

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one such pair. As long as these receptors are also sensitive enough, they will be selected in the two selections (i.e., they will have nonzero frequency). To identify the best such pair, we rank our receptors postcloning according to their frequencies, separately for the two selections. We discard those highest-ranked receptors that have the same rank in both selections. Let **A**′ and **B**′ be the next highest-ranked receptors in each selection; they then exhibit differential response to samples **A** and **B**. **A**′ and **B**′ will form a small cross-reactive array with perfect classification of classes used in selections (as training sets), because at all responsive concentrations of analytes one receptor will be more responsive to one class than the other and *vice versa*. This situation by definition will lead to two classes being separated easily by a simple method (if **A**′ < **B**′, then [CLASS **A**] or *vice versa*) over the full range of concentrations.

Selection Design. Setting up a selection requires defining the initial library of three-way junctions and the selection conditions for interactions between the library and the steroid samples. We strictly focus on screening libraries of unmodified oligonucleotides (Figure 1b) in order to explore the potential

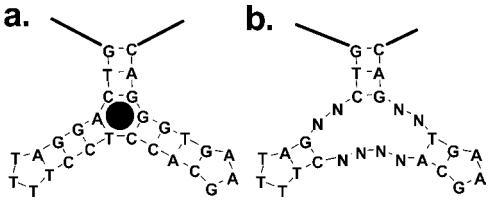

Figure 1. (a) Fully matched three-way junction with an analyte (black circle) binding to the hydrophobic pocket at the intersection of three helices. (b) Template for a randomized library of hydrophobic pockets based on three-way junctions; each N represents any of the four bases (A, T, C, G).

of unmodified nucleic acids to form hydrophobic pockets. We decided to use a highly focused library of three-way junctions with eight randomized positions (Figure 1b), i.e., with 4^8 members. This library is significantly smaller than those used in conventional SELEX procedures, which typically sample around a 4^{40} receptor space (i.e., with a 40-mer randomized region). But, we considered that in the next stage of our work we may be interested in monitoring the evolutionary process and assessing changes in clonal frequencies during selection. Therefore, we preferred to have a limited 65,536-member (4^8) library that can always be fully reproduced in all selections, monitored via NextGen sequencing,[16] or even mimicked on microarrays; that is, we prefer a library in which differences in results could not be attributed to differences in the outcome of a random initial choice of sequences, but to actual selection conditions. As we shall see later, our results did not fully conform to these expectations, in part because any synthetic library has further sources of diversity, e.g., errors in synthesis and fidelity failures during PCR amplification.

Because in the future our selections will be done on urine samples, even in the present work we eschew the standard procedure, in which affinity material is in the solid phase.[14a,b] The groups of Ellington and Li reported a direct selection procedure for structure-switching sensors[15] that used solution-phase affinity for small molecules. Inspired by these reports, we settled on a somewhat simpler procedure using one "competitor" oligonucleotide, complementary to the primer region (**C** in Figure 2a) and binding next to the junction (reflecting our previous results[13c] with structure-switching sensors). This complement is used to capture the library to a column, coupling the ability of library members to survive cycles of selection (released from column) to their ability to interact with steroids in a way that competes with **C**.

This selection allows us to immediately transform receptors into sensors and characterize binding *via* fluorescence sensing. Taking advantage of this benefit dictated that one of the stems must not be randomized, because we needed to preserve the potential for the full binding to the competitor oligonucleotide in the vicinity of the binding site. Otherwise, our selection conditions might lead to the preferred release from the column, strictly owing to shorter complementarity (weaker binding) with the competitor oligonucleotide.

After some initial experimentation, we chose the selection buffer with very high salt concentrations, in contrast with our previous work.[13c] This choice both favors the retention of the library on the column and reflects the need to overcome the variability in salt concentrations of urines. From the outset, we also made the choice not to run counter-selections and, in particular, not to seek sensors specific to single analytes. Hydrophobic receptors are never fully specific, and even if we achieved selectivity over one steroid, there would be cross-reactivity with others. Also, a general argument in favor of cross-reactive arrays is that sets of two or three specific sensors would be unable to detect more than two or three analytes, respectively. Finally, without standardization against creatinine, specific sensors would be useless in urine, while cross-reactive sets would still provide us with a characteristic pattern.

Selection Process. We performed selections with four different steroids (Figure 2b), representing large families. Three were of actual diagnostic interest[17] (DOG, DIS, and DCA). The fourth (β-estradiol, BE) was primarily of structural interest—it has an aromatic A ring, and we had previously not identified a single sensor that would prefer it over other steroids. Further, the perfect classification of DOG and DIS over a range of concentrations with unmodified junctions was traditionally the most challenging task for small arrays; thus, isolation of a pair of sensors that can do this effectively would be of practical

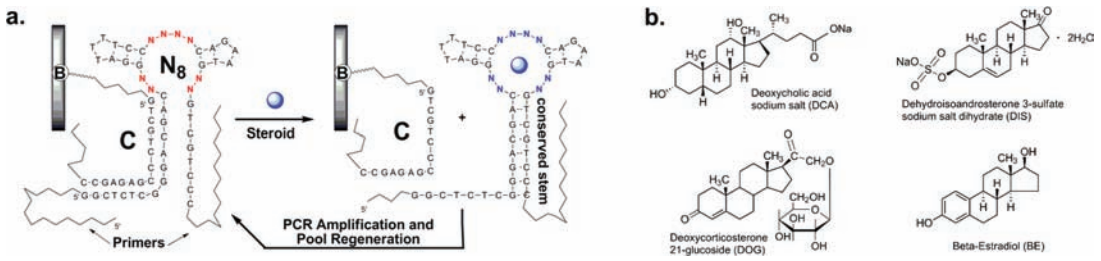

Figure 2. (a) Selection design: A library N_8 is attached to a column via a "competitor" oligonucleotide; those members that are specifically washed away from the columns by addition of steroids are amplified and used in the next round of selection. (b) Four steroids used in selections.

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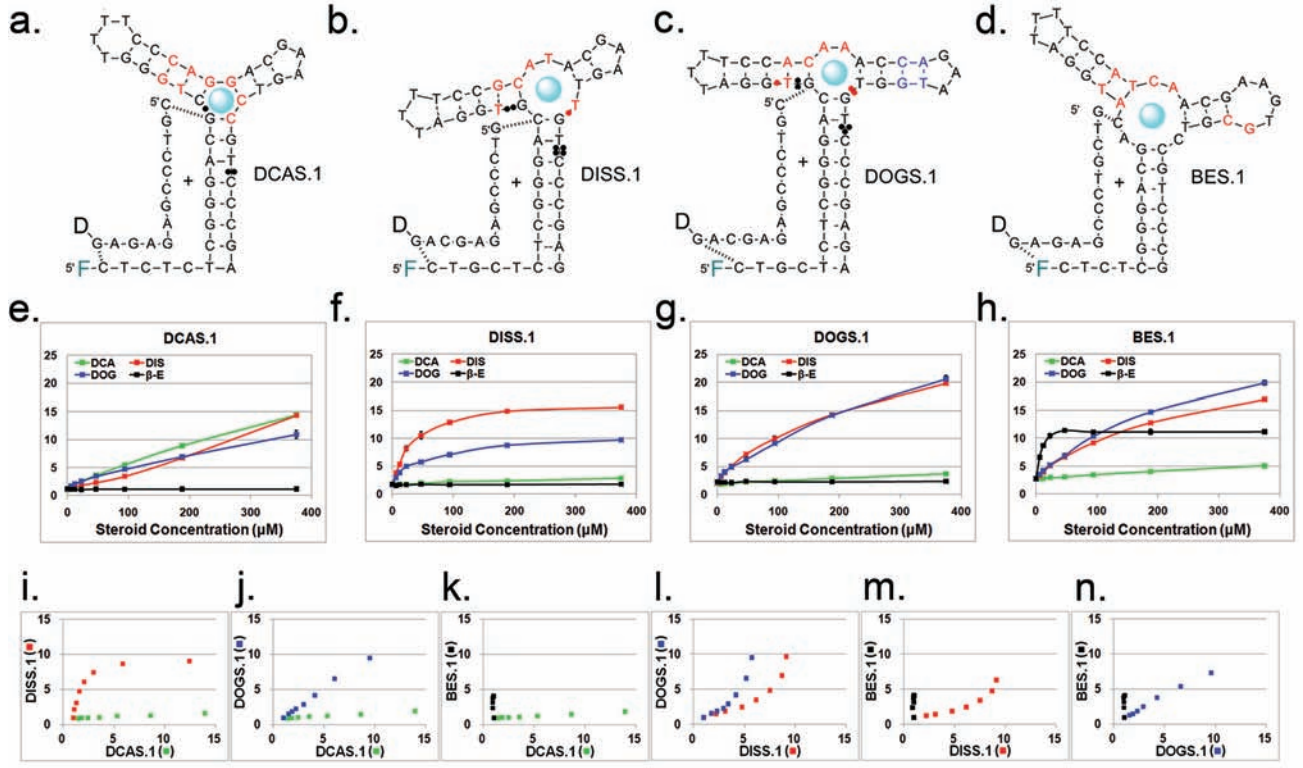

Figure 3. Structures (a–d) and reactivity (e–h) of the steroid sensors used in the final array, with black dots representing changes from the original library (missing bases). These sensors come from the most frequent sequences from individual SELEX runs. Graphs show fluorescence intensity vs all four steroid concentrations (μM), with standard deviations of triplicate measurements shown. (i–n) 2D plots with data (-fold increase) from pairwise two sensors, showing (i) the potential for perfect classification and (ii) that our initial design of a procedure yielding perfect classification based on decision trees is correct; i.e., all sensor pairs (DCAS.1 vs DISS.1; DCAS.1 vs DOGS.1; DCAS.1 vs BES.1; DISS.1 vs DOGS.1; DISS.1 vs BES.1; DOGS.1 vs BES.1) can be used to classify pairwise the steroids used to isolate them.

significance. Concentrations of steroids in selections were set significantly below concentrations typically present in the urines of patients (20 vs 100s μM). Thus, we focused selection on sensors that should be diagnostically useful for urines diluted with buffer.

First selections and optimizations of conditions were implemented in parallel with DIS and DCA, with final cloning performed after 11 rounds. In two other selections, cloning was performed after 9 rounds for estradiol and 13 rounds for DOG. The decision when to sequence was made on the basis of the strength of the elution from the column, according to a signal obtained via semiquantitative PCR. After each cloning, we obtained snapshots of the pool, by sequencing and analyzing up to 24 clones.

Analysis of Receptors/Sensors Isolated in the Selections. High-frequency sequences from four cloned pools from the individual selections were individually tested in their sensor form (Table S2–S3 of the Supporting Information). The analysis of results indicated that the actual selection pressure was the result of the balance between interactions with steroids, as expected, and reduced binding to the complementary oligonucleotide on the affinity column (Figure 3a–c). For example, only one of the four most abundant structures from the four selections could be completely mapped to the initial library as ordered (Figure 3d), while many other sequences (Figure 3a–c) had missing bases (e.g., up to seven, Figure 3c). Further, none of the four most abundant sequences folded according to our initial proposal (including the fully matched structure from DCA SELEX), although all of them found a way to form some variation of the three-way junction that should have existed in the initial library as well (where folding is that of the minimum free energy structure as reported by Mfold[18]). Some sequences also had unexpected mutations, presumably as a result of errors during PCR amplification, while others even had extra bases.

After further optimization of structures, in particular their stems and complementary oligonucleotides, to improve sensing (cf. Supporting Information), we settled on four sensors actually representing the most abundant aptamers from each selection, as shown in Figure 3. These four sensors did not fit the "best-case scenario"—with complete inversions of selectivity in each case—that we outlined above, but they were still strikingly differentially cross-reactive. Nevertheless, as predicted in our initial design considerations, we could take any two analytes pairwise and separate them over a range of concentrations using the two sensors from the corresponding selections (Figure 3i–n). Furthermore, three sensors were sufficient for perfect separation of these four analytes in the 3D space (Figure 4) formed (without any coordinate transformations) by the sensors from the BES, DIS, and DCA selections. This result confirms our previous results[13c] that the number of analytes that can be perfectly classified with cross-reactive arrays can exceed the number of sensors.

Overall, although the results were not exactly what we expected on the basis of the input library (owing to the presence of mutations and errors), and our receptor space did not in all cases provide receptors with inverse selectivity (this may require some form of counter-selection), the evolutionary procedure resulted in a very satisfactory array, one that perfectly classified

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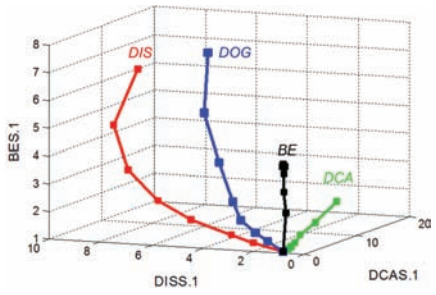

Figure 4. 3D plot representation of steroid classification. Data (-fold increase) from three sensors (DCAS.1, DISS.1, and BES.1) were plotted for four steroid analytes.

steroids over a range of concentrations that matched the concentrations of similar analytes present in urines even after dilutions.

Comparison of Sensors and Receptors with Previously Known Hydrophobic Receptors. Next, we compared the structures of the receptors that were isolated in our evolutionary procedure with those isolated in manual screenings. Of note, none of these sensors had any modifications in their binding sites; that is, the sensors were based on pure DNA. Thus, even though our library was very small (for typical oligonucleotide-based libraries), we obtained a clear indication of the richness in hydrophobic receptors available for any future evolutionary selections.

Three of the steroids yielded new types of junctions: none of the most abundant sequences had been tested in our previous manual screening. The BES.1 sensor, with the highest selectivity for estradiol, is a representative of a completely new motif with extended linkers connecting hydrophobic surfaces; nothing similar had even been considered for manual testing, as there was no rationale to propose testing such a design. Historically, random individual sampling of similar expanded junctions resulted in presumably "collapsed" hydrophobic pockets, that is, junctions with no response to steroids.

Junctions used in DISS.1 and DOGS.1 are, at first glance, similar to some receptors that had been previously tested,[13a,b] but these tests had resulted in no satisfactory results; for example, we had tested nonresponsive variants with two T's in a linker vs two A's in DOGS.1, and a variant with T instead of A in DISS.1. These results again showcase the principal advantage of evolutionary search—exhaustive access to sensors.

Selection for binding to deoxycholic acid served in a fashion as a positive control. Kato and co-workers previously demonstrated[12c,d] that three-way junctions are optimal receptors for this class of steroids, and we confirmed their results with our sensors as well; selection with DCA resulted exclusively in the fully matched three-way junction type receptors. Thus, moving away from this type of receptor would require different SELEX conditions for bile acids.

Comparison with Previous Methods for Construction of Aptameric Cross-Reactive Arrays. We[13] and others[10b] have used receptors either directly isolated from SELEX or subsequently modified to construct various cross-reactive arrays, including those with perfect classification.[13c] In contrast to previous methods, we now obtain direct support for the hypothesis that, within finite pools of naturally cross-reactive receptors, two nonidentical highest ranking (most abundant) receptors isolated during separate selections for binding to two analytes can constitute a minimal cross-reactive array with perfect classification over a continuous range of concentrations for these two analytes. Of course, it still remains to be demonstrated that our approach would work in the same manner with real sets of samples reflecting shifts in dominant responsive families of components.

■ CONCLUSIONS

In this report we offer the following advances: (i) From the perspective of hydrophobic receptors and sensors, we demonstrated the unexpected richness of motifs in the junction space, in contrast to our previous work, in which we had been able to achieve similar classification results only by using modified junctions and nonjunction sensors.[13] The preference of BES.1 for estradiol provides the outstanding example of new motifs, from both structural and functional points of view. (ii) From the perspective of practical cross-reactive arrays, we established a straightforward evolutionary search. The procedure is performed with analytes in the solution phase, so it is suitable to be expanded to different classes of hydrophobic compounds, including those in actual patients' urine samples. (iii) From the perspective of the basic science of cross-reactive arrays with high classification power, we confirmed that minimal sets capable of perfect classification could be built "bottom-up", combining differential sensors isolated through interactions with individual classes. In contrast, we previously used "top-down" methods, starting from larger collections of junctions, using manual screening followed by machine-learning techniques to trim down initial sets of sensors by studying the classification power of individual subsets. (iv) Furthermore, in our approach we used the partially randomized libraries with preexisting structural motifs in order to focus our selection on variations of known steroid-binding motifs; this approach is akin to a knowledge-based prefiltering, a method used to reduce the search space in the data mining field. The opportunity cost of this approach is that it misses potential receptors that interact more strongly with steroids but are not close to the known steroid-binding motifs.

We also note the conceptual closeness of our approach to the procedures relying on combinatorial chemistry[19] to collect receptors with differential activity. There are three important differences: (i) In our procedure there is a direct connection between genotype (sequence or primary structure) and phenotype (binding structure to steroids); (ii) in theory, at least, our procedure guarantees perfect classification due to the mathematical properties of SELEX, the procedure we use, and the properties of our libraries and samples (cf. *General Design*); and (iii) we can cover up to 4^{22} sequences, while (in principle) guaranteeing nearly identical starting points for each selection.

In conclusion, the key for our successful demonstration was the direct connection between genotype (sequence) and phenotype (hydrophobic interactions) in our receptors. This enables the application of evolutionary methods to mine the rich junction space for receptors responsive to particular steroids, leading to a straightforward approach to cross-reactive arrays.

■ EXPERIMENTAL SECTION

General. All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). The oligonucleotides for the library and primers were used without further purification; for the modified oligonucleotide types (e.g., biotinylation, fluorophore conjugation), HPLC-purified grade oligonucleotides were used. All compounds were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise noted. Nuclease-free water was used for all purposes, e.g., oligonucleotide dissolution and buffer preparation.

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Preparation of Buffer and Steroid Solution. Three buffers were prepared: (1) reaction buffer (20 mM Tris, pH 7.4, 1 M NaCl, 10 mM $MgCl_2$); (2) no $MgCl_2$ washing buffer (20 mM Tris, pH 7.4, 1 M NaCl), and (3) 2X SELEX reaction buffer (40 mM Tris, pH 7.4, 2 M NaCl, 20 mM $MgCl_2$).

The target molecules are four different steroids: deoxycholic acid sodium salt (DCA), dehydroisoandro sterone-3-sulfate (DIS), deoxycorticosterone-21 glucoside (DOG), and β-estradiol (BE). 100 mM steroid stock solutions for DCA, DIS, DOG, and 50 mM β-estradiol were prepared according to the manufacturer's recommendation. Steroid concentrations from 375 μM to 6 μM were prepared through serial dilution in SELEX buffer of the 100 mM stock solutions.

***In Vitro* Selection Process.** The oligonucleotides for steroids SELEX are as follow: (1) Random (N_8) library (78-mer), 5′-GGTATTGAGGAGGCTCTC-GGGACGAC(N_2)GGATTTTCC(N_4)ACGAAGT(N_2)GTCGTCCCGATCCTCCTAACGTACGACT-3′; (2) forward-primer (24 mer), 5′- GGTATTGAGGAGGCTCTCGGGACG-3′; (3) reverse-primer (20 mer), 5′-AGTCGTACGTTAGGAGGATC-3′; (4) biotinylated reverse-primer (20 mer), 5′-biotin-AGTCGTACGTTAGGAGGATC-3′; (5) biotinylated column immobilizing sequence (I-DNA; 25 mer), 5′-GGTCCGTCGTCCCGAGAGCCGGACC-biotin-3′.

For the first round of SELEX, the oligonucleotide mixture containing 0.5 nmol I-DNA and 0.1 nmol random library was prepared in 250 μL of SELEX reaction buffer and incubated at 95 °C for 5 min. The mixture was cooled down to room temperature (>10 min) and then added to a streptavidin agarose column. The streptavidin agarose column was prepared with 250 μL of the streptavidin agarose resin (1−3 mg Biotinylated BSA/ml resin; Thermo Scientific, IL, USA) in a micro biospin chromatography column (Biorad, CA, USA). To equilibrate the column, streptavidin agarose was washed five times with the same volume of SELEX reaction buffer, and then the oligonucleotide mixture was flowed through the column. The mixture was collected and applied to the column again two more times. The column was then washed ten times with SELEX buffer. Eluent from each of these washes was collected into separate tubes. After washing, we eluted the DNA−steroid complex using 3 × 250 μL of 20 μM steroid solution (collecting each 250 μL fraction separately). The eluted samples were used as a template for PCR after concentrating to 50 μL by Amicon Ultra centrifugal filter (Millipore, Cork, Ireland).

PCR protocol: 1 cycle of 95 °C, 2 min, 13 cycles of [95 °C, 15 s; 57.5 °C, 30 s; 72 °C, 45 s], and 1 cycle of 72 °C, 2 min. The PCR amplicons were concentrated by centrifugal filter, and the original library size of product was analyzed using 4% E-gel (Invitrogen, CA, US) and purified with a QIAEX II gel purification kit (QIAGEN, Maryland, USA).

Following purification, the double strand PCR amplicons were captured on a streptavidin agarose column via the biotinylated antisense strand; 0.2 mL of streptavidin agarose resin was used and washed three times with strand separation buffer (20 mM Tris, pH 7.4, 1 M NaCl). After loading the sample into the column, the resin was washed five times with the strand separation buffer and incubated with 0.20 mL of 0.2 M NaOH. The flow-out drops were collected, neutralized, and concentrated using a centrifugal filter for the next round of SELEX as the library.

Cloning and Sequencing. During the *in vitro* selection step, the elution profile of the SELEX was monitored by PCR amplification of the flow outs collected during the washes and the target addition. After obtaining a clear elution profile (marked by a large increase in the PCR band intensity where the target was added), cloning was performed. To prepare the insert DNA for cloning, PCR amplification was carried out under the same PCR conditions as those aforementioned, except for the final extension step, where it was increased to 15 min at 72 °C to add A-tail sufficiently for the T/A clone system. The amplicons were purified by the same method mentioned above, and the purified products were directly incorporated to plasmid vectors using a TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. The plasmids were subsequently transformed into OneShot TOP10 competent cells (Invitrogen), and positive clones containing recombinant plasmid were DNA screened via blue/white screening. Some 20−30 colonies were picked in each cloning, and the plasmids were isolated using a PureLink Quick Plasmid Miniprep Kit (Invitrogen). Positive clones were confirmed by PCR, and sequencing was performed by the in-house DNA sequencing service facilities (https://www.dnasequencing.hs.columbia.edu/). The analysis of electropherograms files (AB1files) and verification of each error were completed by Chromas Lite (http://www.technelysium.com.au). The multiple sequence alignments were carried out using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/c lustalw2/). A total of 149 sequences were identified after cloning, out of which 101 sequences showed 3-way junction folding (Table S2 of the Supporting Information) on secondary structure analysis (mfold, http://mfold.rna.albany.edu/).

Sensor Selection and Measurement of Steroid Reactivity. Among the predicted three-way junction sensors based on folding analysis (mfold), we selected four steroid sensors: DCAS.1, DISS.1, DOGS.1, and BES.1. The composition of the sensor sequence and the optimized capture sequence is in Table S3 of the Supporting Information.

All measurements were performed in SELEX reaction buffer with the indicated steroid concentration. A mixture of sensors and Q-capture strand was incubated for 5 min at room temperature, and then a series of standard dilutions of all compounds (the stock solution of each compound was adjusted to pH 7.4) were added to the mixture solutions to final concentrations of 50 nM for sensors and 150 nM for Q-capture. The mixtures were incubated for 30 min (after 15 min signal was constant) and transferred into a 384-well nonbinding surface, flat bottom, black polystyrene assay plate (Corning, NY, USA). The fluorescence in each well was measured with a 485-nm excitation filter and a 535-nm emission filter on a Perkin-Elmer Victor II microplate reader (Shelton, CT, USA). All measurements were done in triplicate. To calculate the response fold increase of the fluorescence response, the average fluorescence intensity value F in each well was divided by the background fluorescence F_0 (solution containing the sensor and buffer solution only), and the latter was assigned the value of 1.0. 2D plots were drawn on Microsoft Excel, and 3D-plot analysis was carried out in MatLab (MathWorks, MA, USA), respectively.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Sequencing and sequence frequency and analysis data (Table S1−S3), the steroid reactivity of sensors (Table S4), original aptamer sequence before optimization (Figure S1), and total 3D plot classification (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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