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# One-Pot SELEX: Identification of Specific Aptamers against Diverse Steroid Targets in One Selection

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Supporting Information

**ABSTRACT:** Aptamers are well-established biorecognition molecules used in a wide variety of applications for the detection of their respective targets. However, individual SELEX processes typically performed for the identification of aptamers for each target can be quite time-consuming, labor-intensive, and costly. An alternative strategy is proposed herein for the simultaneous identification of different aptamers binding distinct but structurally similar targets in one single selection. This one-pot SELEX approach, using the steroids estradiol, progesterone, and testosterone as model targets, was achieved by combining the benefits of counter-SELEX with the power of next-generation sequencing and bioinformatics analysis. The pools from the last stage of the selection were compared in order to discover sequences with preferential abundance in only one of the pools. This led to the identification of aptamer candidates with potential specificity to a single steroid target. Binding studies demonstrated the high affinity of each selected aptamer for its respective target, and low nanomolar range dissociation constants calculated were similar to those previously reported for steroid-binding aptamers selected using traditional SELEX approaches. Finally, the selected aptamers were exploited in microtiter plate assays, achieving nanomolar limits of detection, while the specificity of these aptamers was also demonstrated. Overall, the one-pot SELEX strategy led to the discovery of aptamers for three different steroid targets in one single selection without compromising their affinity or specificity, demonstrating the power of this approach of aptamer discovery for the simultaneous selection of aptamers against multiple targets.


## 1. INTRODUCTION

In almost 30 years since their initial report,[1,2] aptamers selected through the SELEX process have been established as reliable recognition molecules for therapeutic and diagnostic applications. They have been proposed as viable antibody replacements because of their numerous attractive properties,[3,4] including (i) the possibility to bind virtually any kind of target, (ii) high affinity and specificity, (iii) reproducible chemical synthesis, (iv) stability at a variety of environmental conditions, (v) reversible denaturation, (vi) in vitro selection, (vii) possibility of site-directed modification, and (viii) potential tuning of kinetic parameters.

The classic SELEX process exploited for aptamer selection involves the initial use of a highly diverse oligonucleotide library ($10^{14}$ to $10^{16}$ variants) for performing iterative rounds of target binding, partitioning of bound sequences, and subsequent amplification for following rounds. Different variants of this process have been proposed over the years to accommodate specific targets and applications, as has been extensively reviewed.[5−7] Negative SELEX was introduced to eliminate sequences with affinity to the matrix used for target immobilization,[2] while counter-SELEX was incorporated to remove sequences with affinity to structurally similar or potentially interfering molecules.[8] Other variants have been developed to shorten the selection process (e.g., FluMag-SELEX,[9] capillary electrophoresis,[10] and microfluidic SELEX[11]) or to enable the selection of aptamers to different types of targets (e.g., cell SELEX,[12] capture-SELEX,[13] and graphene oxide SELEX[14]) or with desired properties (e.g., Photo SELEX[15]). The idea of using next-generation sequencing (NGS) to identify aptamer candidates instead of the classic cloning-sequencing approach was first introduced by Hoon et al.[16] Since then NGS has not only enabled the analysis of thousands of sequences instead of just a hundred at best but it

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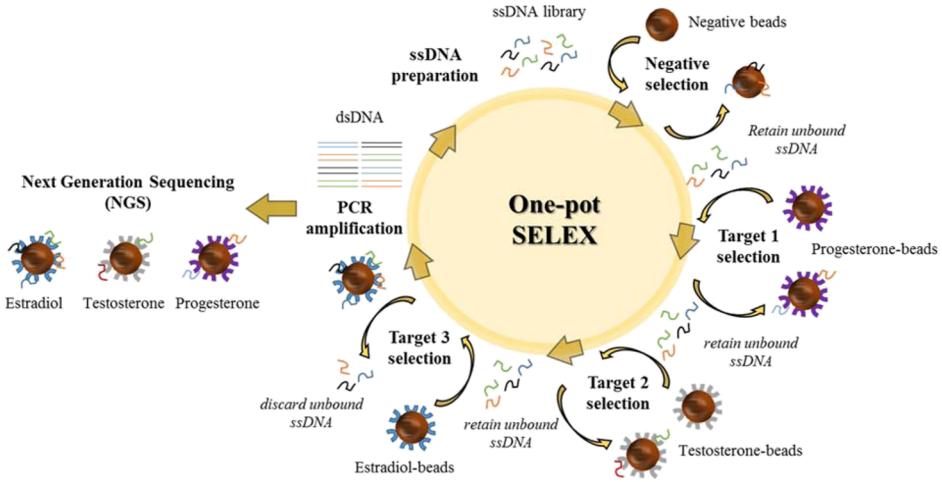


**Figure 1.** One-pot SELEX for the simultaneous selection of aptamers to different targets.

has also paved the way to gain further insights into the selection process. The realization of massive parallel sequencing of multiple rounds has facilitated the monitoring of the abundance and persistence of certain sequences throughout SELEX, and new bioinformatics approaches have consequently been developed to enable the analysis of the vast data sets obtained from NGS.[17,18]

Despite the huge advances made in the aptamer selection process, a new selection still has to be performed for each new target. In an effort to bypass this limitation, a one-pot SELEX strategy is proposed in this work, as depicted in Figure 1. It relies on the use of counter-SELEX, NGS, and bioinformatics analysis in order to simultaneously identify aptamers for three distinct but structurally similar targets in one single selection. The selection was performed by successive incubations of the ssDNA sub-libraries with each of the targets, followed by NGS of the pools from the last stage of the selection and monitoring of the sequence variability and abundance in each pool to identify unique persistent sequences. It was hypothesized that the preferential abundance of a sequence in only one pool originating from a single target would be related to its specificity for the desired target and absence of cross-reactivity with the other two targets used in the same selection process. Even though counter-SELEX is a common approach for removing sequences binding to undesired molecules and NGS has been used before for aptamer discovery, the combination of the two with the bioinformatics analysis proposed herein has not been reported before. The model targets used to validate this hypothesis were the steroids estradiol, progesterone, and testosterone. Steroids belong to a family of hormones involved in many physiological processes and are used as biomarkers in many fields, including reproductive health,[19,20] cancer diagnostics[21] as well as in doping control,[22] and the detection of environmental pollutants.[23,24] Steroid-binding aptamers have already been reported, with the majority of these reports targeting estradiol, and their binding properties were evaluated in a previous work.[25] Of the various reported estradiol aptamers, those developed by Kim et al.[26] and Alsager et al.[27] have been widely adopted in various applications.[28−31] In the case of progesterone, only one aptamer has been reported to date,[32] which has been exploited[33,34] despite the fact that it cross reacts with other steroids.[33−35] Finally, testosterone-binding aptamers have been reported[36] but to date, no application of this aptamer has been reported. The structural similarities between steroids are quite high thus obtaining aptamers with high specificity for these small molecules is a challenging task. In this work, we establish the suitability of the proposed one-pot SELEX approach to identify multiple specific aptamers in one selection process. The binding properties of the identified aptamers were characterized, and the selected aptamers were exploited in microtiter plate assays for the individual detection of each of estradiol, progesterone, and testosterone. Finally, their performance was compared to previously reported aptamers to further highlight the benefits of the proposed strategy.

## 2. RESULTS AND DISCUSSION

**2.1. Selection Strategy.** The objectives of the selection process employed were twofold: (1) perform one single selection and identify multiple aptamers binding to distinct but structurally similar targets and (2) achieve specificity for each desired target with no cross-reactivity with the other non-target molecules. To demonstrate the proposed one-pot SELEX approach for selection of aptamers against several targets, the steroids estradiol, progesterone, and testosterone were chosen as models because of their high structural similarities (see structures in Figure S1, Supporting Information) and their simultaneous occurrence in biological samples, where any cross-reactivity would impede their reliable quantification. To promote specific aptamer selection, counter-SELEX is typically employed using potentially interfering molecules, separately or in combination. In this work, up to four separate incubations were performed sequentially per round, each utilizing different types of modified magnetic beads: (a) control beads (with their reactive groups blocked with sulfo-NHS acetate) to exclude sequences with affinity for the matrix of the beads alone, (b) progesterone, (c) testosterone, and (d) estradiol-modified beads to select sequences with affinity for each of these steroids. The beads used in each round are detailed in Table S1 (Supporting Information), and a schematic representation of the selection is illustrated in Figure 1. The selection was performed at different stages, starting with the estradiol beads and gradually incorporating the other steroid beads in the selection according to Figure S3 (Supporting Information). During the last stage (rounds 11−15), the ssDNA library was initially incubated with the control beads and the supernatant from this incubation, containing unbound sequences, was then sequentially incubated with the progester-



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one, testosterone, and estradiol beads in three separate incubation steps, using the specific conditions for each round (Table S1 and Figure S3, Supporting Information). To simplify the selection, minimize the duration and also lower the costs, only the estradiol-binding pool from each round was ultimately used for the preparation of ssDNA for the following round. Because of the high structural similarities between the three targets, cross-reactive sequences in all pools were expected. A preliminary evaluation of the selection process up to round 11 indeed confirmed this. The ssDNA pool prepared from the estradiol beads (last incubation step in each round) appeared to bind to all three steroid-bead types (Figure S4, Supporting Information).

**2.2. Identification of Aptamer Candidates.** To identify sequences from the last round of selection with the desired specificity to each target, several points were considered: (i) the three targets share very high structural similarities (Figure S1, Supporting Information); therefore, complete elimination of cross-reacting sequences from the pools was considered unlikely; (ii) each sub-library was prepared from the DNA bound on the estradiol beads at the end of each round; therefore, it was expected that the majority of binders to the other two targets progesterone and testosterone were removed from the selection; (iii) comparison of the composition of the data sets obtained from the last round for each target (E2−R15, P4−R15, and T−R15) was expected to be useful in identifying sequences with preferential abundance in only one of the target pools; (iv) the pools from round 15 binding to the progesterone and testosterone beads would presumably contain persistent, generic steroid binders as well as some unique sequences binding specifically to each steroid target.

As already mentioned above, the pool from round 11 contained sequences binding to all three targets, even though preferential binding to estradiol was observed (Figure S4, Supporting Information). This was expected considering that only the estradiol-binding pool was used for the preparation of the sub-library for each succeeding selection round. Also, the ssDNA pool used for the last selection round (round 15) contained very few remaining sequences binding to the progesterone and testosterone beads compared to the estradiol beads [apta-PCR affinity assay (APAA) assay, data not shown].

The bioinformatics analysis aiming at discovering specific binders to each steroid target was initiated by performing a general statistics analysis of the raw data. This can be found in the Supporting Information (Table S2). With the exception of round 11 of the estradiol selection (E2−R11) that gave a very low read number (268), which can be attributed to sample preparation issues, all the other pools sequenced gave an average of 100 000 reads thus enabling in-depth analysis of sequence variability and representation. The seven sequences with highest number of copies (most over-represented sequences based on 100% sequence identity) in pools E2−R11 and E2−R13 were designated as Seq.1−Seq.7 and were initially analyzed. Their ranking and high abundance in each of the pools sequenced suggested possible binding to all three steroid targets (Table S3, Supporting Information). This was confirmed experimentally with an APAA which verified their cross-reactivity with all three steroids tested (Figure S5, Supporting Information). This finding reinforces the widespread belief that the most abundant sequences are not necessarily the best binders, in terms of binding affinity and/or specificity. In search of specific sequences in silico, the pools from round 15 for the three steroids were compared in order to identify sequences with preferential abundance in only one of them as detailed in the Supporting Information. This analysis led to the identification of the sequences E1−E30, P1−P18, and T1−T7 for estradiol, progesterone, and testosterone, respectively. Their sequences can be found in Tables S4−S6 (Supporting Information) and information regarding ranking and abundance in Table S7 (Supporting Information). Multiple sequence alignment of all these sequences let to the identification of sequence families as shown in Figure S6 (Supporting Information). Based on the information of abundance and sequence families, a total of six aptamer sequences were chosen for further characterization. These were E11, E26, and E28 for estradiol, P5 and P6 for progesterone, and T6 for testosterone. Their abundance in the different target pools from round 15 can be seen in Figure 2, while their sequences and predicted structures are found in Table S8 and Figure S7 (Supporting Information). These sequences were ultimately chosen for experimental evaluation.

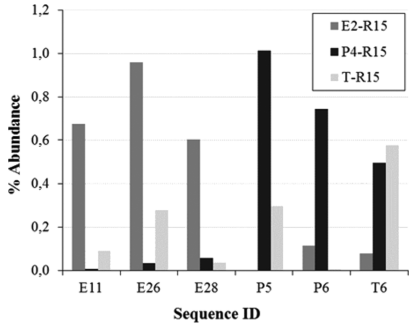


**Figure 2.** Abundance of the selected aptamer sequences in each of the target pools sequenced. E2, P4, and T correspond to the steroids estradiol, progesterone, and testosterone, respectively, whereas *R* indicates the selection round sequenced.

**2.3. Screening of the Aptamer Candidates.** The characterization of the individual aptamer candidates was performed with the aim of determining their affinity for their respective target and ultimately choosing the most appropriate candidate(s) for assay development and cross-reactivity studies. It is essential to study the binding properties of small molecule binding aptamers with multiple techniques in order to confirm their affinity and also exclude the effect and/or participation of the matrix used for target immobilization to the binding event. It is also critical to corroborate their performance in application-specific conditions because their performance in one application does not guarantee their functionality in another one. To this end, the affinity dissociation constants ($K_D$) of the selected aptamer candidates were evaluated using three independent methods. Initially, the APAA was used. The utility of this assay for monitoring aptamer-small molecule interactions has already been demonstrated.[25,36] The conditions employed for this assay mimic the ones used during the selection process, where the steroid targets are immobilized on magnetic beads and the binding of unmodified aptamers is detected after PCR amplification and gel electrophoresis. The beads used for target immobilization were different compared to the ones used during the selection, in size and binding capacity. This was done deliberately to demonstrate the absence of matrix participation in the binding event. As can be seen in Table 1 and Figure S8a (Supporting Information), all aptamers exhibited very high affinity for their


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respective target with the calculated $K_D$s in the low nanomolar range (1.55−27.15 nM).

**Table 1. Determination of the Affinity Dissociation Constants ($K_D$s) of the Aptamers Using APAA**

| Aptamer candidate | | $K_D$ (nM) | $R^2$ |
|---|---|---|---|
| Estradiol | E11 | 4.74 ± 6.28 | 0.8845 |
| | E26 | 27.15 ± 64.83 | 0.9645 |
| | E28 | 3.10 ± 1.93 | 0.9590 |
| Progesterone | P5 | 7.37 ± 4.20 | 0.9722 |
| | P6 | 1.55 ± 0.19 | 0.9251 |
| Testosterone | T6 | 2.61 ± 0.52 | 0.9790 |

The second assay used for $K_D$ determination was bead-enzyme linked aptamer assay (bead-ELAA). The same steroid-magnetic beads used for APAA were employed for this assay, while the aptamers were modified with a 5′-biotin label to allow direct colorimetric detection using a streptavidin-polyHRP reporter. The calculated $K_D$s are shown in Table 2,

**Table 2. Determination of the Affinity Dissociation Constants ($K_D$s) of the Aptamers Using Bead-ELAA**

| Aptamer candidate | | $K_D$ (nM) | $R^2$ |
|---|---|---|---|
| Estradiol | E11 | ambiguous | 0.9450 |
| | E26 | no binding | |
| | (T15)−E26 | 9.12 ± 9.43 | 0.8477 |
| | E28 | 15.21 ± 3.75 | 0.9462 |
| Progesterone | P5 | 0.44 ± 0.14 | 0.9596 |
| | P6 | 39.09 ± 7.09 | 0.9825 |
| Testosterone | T6 | 1.26 ± 0.16 | 0.9936 |

and the binding curves are shown in Figure S8b (Supporting Information). Aptamers E28 for estradiol, P5 and P6 for progesterone, and T6 for testosterone performed equally well in bead-ELAA as in APAA, with the $K_D$s calculated again to be in the low nanomolar range (0.44−39.09 nM). On the other hand, even though the binding of the E11 aptamer to estradiol was clearly detected, the calculation of the $K_D$ was ambiguous; thus, its affinity could not be determined. Surprisingly, no binding of the E26 aptamer to the estradiol beads was observed, and it was hypothesized that this could be attributed to the modification of the sequence with the 5′-biotin label. Indeed, after the introduction of a T15 spacer between the biotin label and the 5′-end of the aptamer, the binding properties of the aptamer were restored. The $K_D$ of the (T15)−E26 aptamer was determined to be 9.12 nM, which was in the same range as the other aptamers. Because of the loss of binding of the 5′-modified E26 aptamer to its target, it can be assumed that its 5′-end is involved in the binding event.

Finally, a microtiter plate-based assay was developed for the facile, quantitative measurement of the steroids. This plate-enzyme-linked aptamer assay (plate-ELAA) was based on the immobilization of the CMO-steroid derivatives on maleimide-activated plate wells through an amino-thiol compound. Two different amino-thiols were evaluated (Figure S10, Supporting Information), and 11-amino-1-undecanethiol containing a long C11 spacer arm (15 Å) resulted in the specific detection of the immobilized steroid using a biotin-modified aptamer. Cysteamine was not suitable because it resulted in high background signals in the absence of the aptamer and steroid, maybe because of insufficient blocking of the surface by the layer formed by the short chain (C2, 4 Å). Following immobilization of the steroids on the plate, the binding affinity of the biotin-modified aptamers to their respective targets was determined. The calculated $K_D$s are shown in Table 3, and the binding

**Table 3. Determination of the Affinity Dissociation Constants ($K_D$s) of the Aptamers Using Plate-ELAA**

| Aptamer candidate | | $K_D$ (nM) | $R^2$ |
|---|---|---|---|
| Estradiol | E11 | 0.87 ± 0.04 | 0.9979 |
| | (T15)−E26 | 0.95 ± 0.30 | 0.9639 |
| | E28 | 2.22 ± 0.17 | 0.9959 |
| Progesterone | P5 | 2.03 ± 3.24 | 0.9418 |
| | P6 | 0.57 ± 0.84 | 0.9132 |
| Testosterone | T6 | 0.80 ± 0.18 | 0.9738 |

curves are shown in Figure S8c (Supporting Information). The high affinity of all aptamers demonstrated with the previous two assays was also successfully corroborated with plate-ELAA, with $K_D$s in the low/sub-nanomolar range (0.57−2.22 nM). The small variations observed in the $K_D$s obtained with the different assays could be partially attributed to the different immobilization levels and spatial distribution of each steroid on the respective surfaces used (magnetic bead or microplate well). In addition, the modification of the aptamers at their 5′-end could potentially affect their binding properties, as was observed with the E26 aptamer. A further determining factor could be the steric accessibility, facilitating aptamer-small molecule target binding, which to some extent is determined by the spacer used to immobilize the target molecule. The binding of aptamers to small molecules is often an intercalation event within stems, loops, or three-way junctions, and it has been proposed that the hydrophobic cavities formed in the central branching points of three-way junctions can accommodate low polarity compounds such as steroids[37] and cocaine.[38] This kind of DNA folding can also be observed in the predicted structures of the selected steroid-binding aptamers (Figure S7, Supporting Information). It is therefore plausible that the long C11 spacer used to tether the steroid to the microplate surface provided more steric freedom to facilitate binding, as well as a favorable spatial distribution. On the other hand, the magnetic beads contain a short C3 spacer arm which might not facilitate equally well the recognition or stabilization of the complex after binding. However, the aptamers were expected to perform well in the APAA and bead-ELAA assays because magnetic beads were actually used for target immobilization during the selection process.

**2.4. Competitive Plate-ELAA for the Analytical Determination of Steroids.** The detection of small molecular weight targets is typically achieved using competitive assays. The design of the competitive ELAA developed in this work for aptamer-based steroid detection is illustrated in Figure S9 (Supporting Information). The assay was based on the ELAA described above, which was used for the determination of the binding affinity of the selected aptamers. Several factors were optimized in order to enhance the performance of the assay. The amino-thiol compound used for the immobilization of the steroids on the maleimide-activated microplate wells was 11-amino-1-undecanethiol, as detailed above and in the Supporting Information (Figure S10). The concentration of the CMO-steroid used for immobilization was 100 $\mu$M as the use of higher concentrations was not



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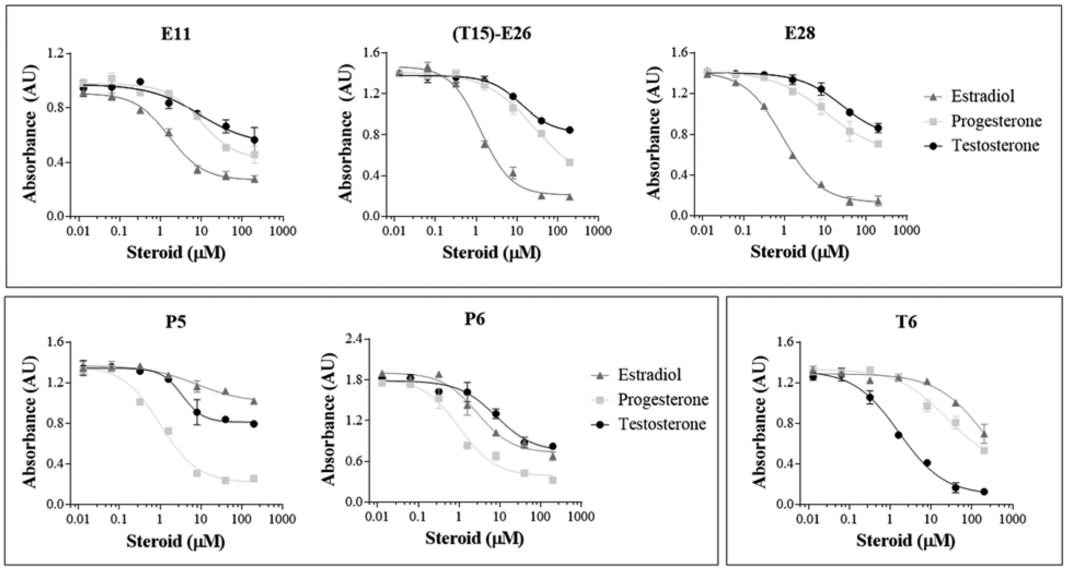


| Immobilized steroid on plate | Aptamer | Steroid in solution | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Estradiol | | | Progesterone | | | Testosterone | | |
| | | LOD (nM) | EC50 (nM) | $R^2$ | LOD (nM) | EC50 (nM) | $R^2$ | LOD (nM) | EC50 (nM) | $R^2$ |
| Estradiol | E11 | 181 | 1,667 | 0.9898 | 1,185 | 9,805 | 0.9740 | 2,272 | 9,146 | 0.8933 |
| | E26 | 175 | 1,300 | 0.9902 | 554 | 28,060 | 0.9899 | 1,366 | 12,710 | 0.9821 |
| | E28 | 46 | 925 | 0.9971 | 462 | 11,120 | 0.9813 | 1,261 | 25,610 | 0.9819 |
| Progesterone | P5 | 1,373 | 7,731 | 0.9290 | 112 | 994 | 0.9890 | 1,060 | 3,460 | 0.9702 |
| | P6 | 432 | 2,767 | 0.9742 | 183 | 1,006 | 0.9776 | 1,096 | 8,066 | 0.9650 |
| Testosterone | T6 | 4,725 | 287,900 | 0.9626 | 1,090 | 23,280 | 0.9756 | 103 | 1,650 | 0.9913 |

**Figure 3.** Sensitivity and specificity of the competitive plate-ELAA assays for steroid detection. The upper panel shows the binding curves of all aptamers, whereas the LOD and EC50 values calculated for each steroid are shown below.

observed to significantly increase the signals. In order to achieve the lowest possible detection limits, the duration of the preincubation and incubation steps needs to be very carefully optimized. The preincubation step is performed off-plate and consists of the incubation of the biotinylated aptamer with the sample containing the target molecule. The sample−aptamer mixture is then added to the wells containing the immobilized target, and any aptamer not bound to the target in solution is thus available to bind the immobilized target. Finally, the aptamer−target complexes in solution are washed away, while the aptamer bound to the plate-immobilized target is detected using via addition of streptavidin-polyHRP. Several combinations of preincubation/incubation times were tested varying from 5 to 30 min for each step (Figure S11, Supporting Information), achieving limits of detection (LOD) in the range of 14.5−565.4 nM, with shorter incubation times enhancing sensitivity (Table S9, Supporting Information). To facilitate sample handling within a reasonable period of time, a combination of 10 min for each of the preincubation and incubation steps was selected to be optimal. The effect of temperature on assay performance was then evaluated. The LOD achieved at 22 °C was lower (80 nM estradiol) compared to that obtained at 37 °C (1079 nM), and 22 °C was thus chosen for all further experiments (Figure S12, Supporting Information). These conditions were in fact observed to be optimal for all aptamers (data not shown). The concentration of each aptamer was also optimized as detailed in the Supporting Information (Figure S13) and the concentrations used for the final assays (0.5−12.5 nM) are shown in Table S10 (Supporting Information).

Using these optimized parameters, competition assays were carried out with steroid concentrations in solution ranging from 13 pM to 200 $\mu$M. To establish the selectivity of the aptamers for their cognate targets, the assays were also performed for the other two non-target steroids. Each aptamer was therefore studied in combination with its designated target immobilized on the plate, whereas different concentrations of the non-target steroids where used in solution for the competition. The LOD of each aptamer for its respective target was calculated in the range of 46−183 nM (Figure 3).

Aptamer E28 was found to be the most specific aptamer for estradiol among those tested, with an LOD for the target steroid at least 10-fold lower than those obtained for the non-target steroids (LOD of 46 nM for estradiol, compared to 462 nM for progesterone, and 1261 nM for testosterone). Additionally, the EC50 values for progesterone and testosterone were >10-fold higher. The P5 progesterone aptamer also performed well, achieving an LOD of 112 nM for progesterone and significantly higher LODs of 1373 and 1060 nM for estradiol and testosterone, respectively. Finally, the testosterone aptamer also performed well, achieving an LOD of 103 nM for testosterone, and again having markedly higher detection limits of 1090 and 4725 nM for progesterone and estradiol, respectively. The excellent binding properties of this aptamer are also reflected by the EC50 values, which were more than 14-fold and 170-fold higher for progesterone and


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estradiol, respectively, as compared to the EC50 value for testosterone.

The binding properties of the aptamers developed in this work were compared to other steroid-binding aptamers previously reported in the literature. In the case of estradiol, various aptamers have been reported, but the most exploited ones for assay development exhibited a $K_D$ of 130[26] and 50 nM.[27] Only one progesterone aptamer has been reported with a $K_D$ of 17−112 nM, depending on the method used by the authors to monitor the binding event.[32] There is also only one report in the literature describing testosterone aptamers and the $K_D$ of the various candidates ranged from 0.5 to 50 nM.[36] All these aptamers were identified using classic SELEX approaches where each selection was dedicated to a single target, and their binding properties were determined using different approaches. To enable the direct comparison of all the reported aptamers, an ELAA was used for $K_D$ determination. As can be seen in Figure S14a,b (Supporting Information), the estradiol-binding aptamers reported by Kim et al.[26] and Alsager et al.[27] exhibited very high affinity for estradiol with $K_D$s in the low nanomolar range, 7.29 and 5.12 nM, respectively. The binding affinity of these aptamers is thus comparable with the affinity exhibited for the estradiol-binding aptamers reported in this work ($K_D$s of 0.87−2.22 nM). The high affinity of the P4G13 progesterone aptamer reported by Contreras Jiménez et al.[32] was also corroborated ($K_D$ = 1.65 nM, Figure S14c, Supporting Information), which was within the same range as the affinity displayed by the progesterone aptamers identified by the one-pot SELEX detailed here ($K_D$s of 0.57−2.03 nM). Finally, the T5 testosterone aptamer developed by Skouridou et al.[36] also demonstrated extremely high affinity ($K_D$ = 0.5 nM, Figure S14d, Supporting Information), very similar to the affinity exhibited by the T6 candidate selected in this work ($K_D$ = 0.80 nM). Overall, it can be concluded that the devised SELEX strategy for one-pot selection using multiple but structurally similar targets can lead to the identification of specific aptamers with high affinity, comparable or even superior to that of aptamers selected via classic SELEX approaches.

## 3. CONCLUSIONS

The selection of aptamers for various targets employing different SELEX strategies has been widely reported. However, typically each SELEX is dedicated to the selection of aptamers for a single target. We demonstrate that the combination of counter-SELEX selection steps, NGS, and bioinformatics analysis can lead to the identification of aptamers binding distinct targets. The steroids estradiol, progesterone, and testosterone were used as the model target molecules. The estradiol-binding pool at the end of each round was used as a sub-library for all succeeding rounds. Progesterone and testosterone were gradually incorporated in the selection which consisted of sequential incubations of the ssDNA sub-library with each target. Upon completion of the one-pot SELEX, NGS was performed to monitor the abundance of sequences within each pool from the last stage of the selection. This aimed at identifying potential aptamer candidates with preferential abundance in only one of the pools which was hypothesized to be related to their specificity. The binding properties of the selected aptamer candidates were evaluated using three independent methods. All aptamers exhibited very high affinity for their corresponding target with $K_D$s in the range of 0.44−27.15 nM, depending on the method used. Finally, competitive microtiter plate assays were developed using the selected aptamers and the detection of all steroids was achieved with LODs in the range of 46−183 nM. In terms of specificity, most of the aptamers evaluated performed remarkably well because the obtained LODs for each target steroid were at least 10-fold lower compared to the ones for the non-target steroids. The best performing aptamers, E28 for estradiol and T6 for testosterone, also demonstrated more than 10-fold higher EC50 values for the non-target steroids compared to the values for their cognate target. These aptamers will be exploited in lateral flow assays for use at the point-of-care/need, with several potential applications including monitoring of fertility, where it is important to specifically detect estradiol and progesterone, while testosterone levels, which are innately low in females, should be of low or no significance. An alternative application is the combined detection of estradiol and testosterone to define maturity of sturgeon eggs for caviar collection, where the levels of progesterone are irrelevant. It is crucial to demonstrate the specificity of the aptamers in the context of the final application assay, and we have demonstrated that the presence of up to ca. 1.6 $\mu$M of the non-target steroids did not significantly affect the signals obtained with the competitive plate-ELAA assays; therefore, they are not expected to interfere with the detection of the target steroid. The strategy presented in this work could be further expanded to other targets and allow the simultaneous identification of aptamers binding structurally similar targets with appropriate specificity depending on the assay requirements, thus shortening the overall duration and reducing costs related to aptamer discovery.

## 4. EXPERIMENTAL SECTION

**4.1. Selection Process.** For the simultaneous selection of aptamers binding independently to estradiol, progesterone or testosterone, a SELEX process was designed, comprising of successive steps of negative and positive selections using control beads and three different types of steroid-functionalized magnetic beads (Figures 1 and S2, Supporting Information). The preparation of the steroid beads is described in the Supporting Information (Figure S1) and the selection strategy is summarized in Table S1. Each steroid was incorporated at different stages of the selection as it is summarized in Figure S3 (Supporting Information). In the first round, the random library was incubated with the estradiol-functionalized beads in binding buffer (20 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$, pH 7.5), followed by washing with the same buffer and recovering the bound ssDNA for use in the next round. Negative selection using control magnetic beads (blocked with sulfo-NHS acetate) was incorporated in the second round to remove sequences with affinity for the bead matrix. The supernatant obtained following this incubation step, which contained unbound ssDNA sequences, was then transferred to a tube containing the estradiol-beads for positive selection. For rounds 3−10, progesterone-beads were introduced to the selection process for incubation with the unbound ssDNA pool after incubation with the control beads. Unbound ssDNA from the progesterone-beads (supernatant from the progesterone-beads) was then incubated with the estradiol-beads. For the final rounds 11−15, the testosterone-beads were incorporated. The ssDNA input pool was incubated first with the control beads, and unbound ssDNA from each step was successively incubated with the progesterone-beads, testosterone-beads, and finally the estra-



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diol-beads. At the end of each selection round, the estradiol-beads were resuspended in 20 $\mu$L of dd$H_2O$ and were used directly for PCR amplification with library-specific forward and phosphorylated reverse primers. For this, 2 $\mu$L of the resuspended beads were added to 100 $\mu$L of PCR master mix, and a three-step program was performed as follows: 2 min at 95 °C, 8−16 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C, and 5 min at 72 °C. The PCR reaction was then used as the template for asymmetric PCR (20 $\mu$L per 100 $\mu$L of the master mix) which was done under the same conditions as the first PCR with the exception of the exclusion of the reverse primer from the master mix and the performance of only eight cycles during the second step. Finally, lambda exonuclease was added to the asymmetric PCR reaction for digestion (1.5 h at 37 °C) and the generated ssDNA was isolated by column purification. Before each selection round, the ssDNA input pool was diluted in binding buffer to a final volume of 100 $\mu$L, heated at 95 °C for 3 min, and then slowly cooled down to 4 °C before use. Each incubation step was performed for 30 min at 22 °C under tilt rotation using 2 $\mu$L of each bead type. The progress of the selection process is detailed in the Supporting Information (Figure S2).

**4.2. NGS and Bioinformatics Analysis.** DNA bound to the estradiol-beads (selection rounds 11, 13, and 15), progesterone-beads (selection round 15), and testosterone-beads (selection round 15) was analyzed using NGS. Each sample containing the respective beads with bound DNA was individually amplified using PCR with different forward primers, containing distinct barcode sequences, a common reverse primer, and the resulting dsDNA was column-purified. The pools were sequenced using Ion Torrent NGS (Centre for Omic Sciences, Eurecat Technology Centre, Reus, Spain) and the fastq raw data was imported into the Galaxy web server for analysis. An overall evaluation of the data and general statistics were obtained using the "FASTQC" tool. The format of the data was then converted to FASTA using the "FASTQ to FASTA converter". The length of the sequences was constrained to library length (80−100 bp) using the "Filter sequences by length" tool in order to remove amplification and sequencing artifacts from the data sets. Unique sequences were then identified using the "Collapse" tool. The most abundant sequences from the estradiol rounds 11 and round 13 were identified after collapsing, analyzed, and characterized as detailed in the Supporting Information. The first 100 sequences in each pool from the last selection round with the most number of copies were then searched for unique sequences present in only one of the pools. The number of copies of each of the selected sequences within the first megabyte of raw data of each pool from the last selection round was then registered. Finally, selected candidates with preferential abundance in only one of the pools were identified as potential binders with specificity to each target (see the Supporting Information for more details). Multiple sequence alignment of these sequences using the external tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was finally performed in order to identify sequence families. Based on this information and the number of copies of each sequence in each of the target pools, the final aptamer candidates were chosen for characterization.

**4.3. Affinity Dissociation Constants ($K_D$) Determination by APAA.** APAA was performed as previously described.[36] Each unmodified aptamer (156 pM to 10 nM) was incubated with each of the four types of magnetic beads (control, progesterone, testosterone, and estradiol) in binding buffer for 30 min at 22 °C under tilt rotation. After thorough washing, steroid beads with bound sequences were amplified using PCR using library-specific primers and the intensity of the gel electrophoresis bands was estimated using the ImageJ software. The $K_D$s of the aptamers were calculated using the GraphPad Prism 6 software and the "One site—Specific binding with the Hill slope" model. Triplicate measurements were carried out for each aptamer concentration tested.

**4.4. $K_D$ Determination by Bead-ELAA.** For bead-ELAA, the aptamer candidates were modified with a 5′ biotin label, with or without a T15 spacer. Each modified aptamer (1.56−200 nM) was separately incubated with the four different types of magnetic beads (control, progesterone, testosterone, and estradiol) for 30 min at 22 °C under tilt rotation in binding buffer. The beads with bound aptamers were then washed and streptavidin-polyHRP80 (0.05 $\mu$g/mL in binding buffer) was added, followed by another 30 min incubation step. After a final washing step, the beads were resuspended, TMB substrate was added, and following a brief incubation at room temperature, an equal volume of 1 M $H_2SO_4$ was added to stop color development. The supernatants were separated from the beads using a magnet, transferred to a microtiter plate, and the absorbance was measured at 450 nm. The $K_D$s of the aptamers were calculated using the GraphPad Prism 6 software and the "One site—Specific binding with Hill slope" model. Triplicate measurements were carried out for each aptamer concentration tested.

**4.5. $K_D$ Determination by Plate-ELAA.** Plate-ELAA was performed using maleimide-activated plate wells functionalized with 11-amino-1-undecanethiol hydrochloride (MUAM, 100 $\mu$M in PBS) after overnight incubation at 4 °C. The wells were washed with PBS followed by the addition of a mixture of the CMO-steroid derivatives with EDC/NHS and incubation for 2 h at 22 °C for steroid immobilization. After washing with PBS, the wells were blocked with sulfo-NHS acetate (1 mM in PBS) for 1 h at 22 °C followed by a final washing step with PBS. Different concentrations of each modified aptamer (with a 5′ biotin label, with or without a T15 spacer, in binding buffer) were then added to each of the wells and incubated for 30 min at 22 °C, followed by washing, the addition of streptavidin-polyHRP80 (0.05 $\mu$g/mL in PBST), and another 30 min incubation. Finally, the wells were washed with PBST, the TMB substrate was added, and color development was terminated by the addition of an equal volume of 1 M $H_2SO_4$ followed by measurement of absorbance at 450 nm. The $K_D$s of the aptamers were calculated using the GraphPad Prism 6 software and the "One site—Specific binding with Hill slope" model. Triplicate measurements were carried out for each aptamer concentration tested.

**4.6. Detection of Steroids by Competitive Plate-ELAA.** The selected aptamers were used for the development of competitive microplate-based assays for the detection of each steroid. The optimization of various assay parameters is detailed in the Supporting Information. MUAM-functionalized microplate wells were initially prepared followed by the immobilization of the CMO-steroid derivatives as detailed in the "Plate-ELAA" section. Each aptamer (0.5−12.5 nM) was pre-incubated with free steroid solutions (13 pM to 200 $\mu$M in binding buffer containing 5% v/v ethanol) for 10 min at 22 °C ("pre-incubation step"). The aptamer-steroid mixtures were then transferred to the steroid-immobilized microplate wells, followed by another 10 min incubation at 22 °C ("incubation



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step"). After thorough washing with PBST, streptavidin-polyHRP80 (0.05 $\mu$g/mL in PBST) was added and incubated for further 30 min at 22 °C, followed by a final washing step and addition of the TMB substrate. The signal at 450 nm was recorded following the addition of an equal volume of 1 M $H_2SO_4$. The signals obtained as a function of the concentration of the free steroid were plotted with GraphPad Prism and fitted to a sigmoidal model (four parameter logistic model where $X$ is the log concentration). The LODs were defined as the highest signal obtained (top of the fitted curve) minus three times its standard deviation (Top − 3SDTop), and they were interpolated from the fitted curves. The EC50 values were calculated directly by the software. Triplicate measurements were performed for each target concentration.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02412.

Materials and reagents, selection, evolution of the selection process, high-throughput analysis of the selection process by Ion Torrent NGS, determination of the binding affinity ($K_D$) of the aptamer candidates by different approaches, competitive plate-ELAA for steroid detection, evaluation of previously reported steroid-binding aptamers with the plate-ELAA, and references (PDF)

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### Notes

The authors declare no competing financial interest.

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