SUPPORTING INFORMATION

One-pot SELEX: identification of specific aptamers against diverse steroid targets in one selection

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**S1. Materials and reagents**

17β-Estradiol (estradiol), estradiol-6-one 6-(O-carboxymethyl)oxime (estradiol-CMO), progesterone, progesterone-3-(O-carboxymethyl)oxime (progesterone-CMO), testosterone, testosterone-3-(O-carboxymethyl)oxime (testosterone-CMO), 11-amino-1-undecanethiol hydrochloride (MUAM), cysteamine, TMB liquid substrate system for ELISA, rabbit anti-mouse-HRP conjugate and maleimide-activated microplate strip wells were supplied by Sigma (Spain). The Dynabeads M-270 Amine (2.8 µm diameter) magnetic beads, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), sulfo-NHS-acetate, DreamTaq DNA polymerase and lambda exonuclease were from Fisher Scientific (Spain) whereas the SiMAG-Amine (1 µm diameter) magnetic beads from Chemicell (Germany). The murine monoclonal antibodies to estradiol (clone 9F9), progesterone (clone 9F44) and testosterone (clone 5E801) were obtained from USBiological Life Sciences (provided by VWR, Spain) and the DNA purification kits (Oligo Clean & Concentrator kit and DNA Clean & Concentrator kit) from Zymo Research (supplied by Ecogen, Spain). Streptavidin-polyHRP80 was purchased from SDT-Reagents (supplied by Bionova, Spain). The random 86 nt-long library (5’-TAGGGAAGAGAACATATGAT-N_{40}-TTGACTATGACCATGACCTTTGA-3’) was from TriLink Biotechnologies (USA) and all other oligonucleotides were synthesized by Biomers.net (Germany).

![Figure S1. Structures of the target steroids and their CMO derivatives used for immobilization.](image-url)
S2. Selection

(a) Steroid immobilisation on magnetic beads

Each of the CMO-steroid (estradiol, progesterone and testosterone) derivatives were separately immobilised on amine-functionalized magnetic beads as previously described,¹ using sulfo-NHS acetate to block any unreacted amine groups. Approximately 700-800 pmol of each CMO-steroid was used for the immobilization on 3 mg of amine-magnetic beads. The selection was performed using Dynabeads M-270 Amine beads whereas SiMAG-Amine magnetic beads were used for the characterization of the aptamer candidates by APAA. The immobilisation level of each steroid on the corresponding beads was estimated by a semi-quantitative competitive ELISA assay using monoclonal antibodies against each steroid¹ as shown in Figure S2. Sulfo-NHS acetate-blocked beads were also prepared (control-beads) for performing the negative selection and also for control experiments.

Figure S2. Quantification of steroids immobilised on magnetic beads used during the selection and characterization of aptamer candidates. The upper panel shows the calibration curves obtained from the detection of each steroid (as BSA conjugates) using monoclonal anti-steroid antibodies. The lower panel corresponds to the quantification of the steroid-modified beads after extrapolation from the calibration curves.
(b) Design of the selection process

The simultaneous selection of steroid-binding aptamers with different specificities was completed after 15 rounds. The specific conditions are shown in Table S1 and a detailed schematic representation of the different stages in Figure S3. The duration of the incubation step of the ssDNA with each bead type (30 min) as well as the washing steps (3 x 200 µl of selection buffer) between steps were maintained constant during the entire selection.

Table S1. Selection conditions. Each bead type was used according to the order shown below.

| Selection round | ssDNA input (pmol) | Magnetic beads (15 mg/ml) |
|-----------------|--------------------|--------------------------|
|                 |                    | Negative | Progesterone | Testosterone | Estradiol |
| R1              | 300                | -        | -            | -            | 3 µl      |
| R2              | 4                  | 2 µl     | -            | -            | 2 µl      |
| R3-R10          | 5 - 10             | 2 µl     | 2 µl         | -            | 2 µl      |
| R11 – R15       | 20                 | 2 µl     | 2 µl         | 2 µl         | 2 µl      |
Figure S3. Graphical representation of the different stages of the selection process.
S3. Evolution of the selection process

To evaluate the progress of the selection process, equal amounts of ssDNA from different rounds (in binding buffer) was separately incubated with the four types of steroid-magnetic beads (control/negative, progesterone, testosterone and estradiol) for 30 min at 22°C under tilt rotation. After thorough washing, the re-suspended beads were used for amplification and determination of relative binding according to the APAA assay. The results are shown in Figure S4.

Figure S4. Evolution of the selection process by APAA. (a) Gel electrophoresis and (b) corresponding band intensity. c: control beads; P4: progesterone-beads; T: testosterone-beads; E2: estradiol-beads; nc: PCR negative control (no template).
S4. High-throughput analysis of the selection process by Ion Torrent Next Generation Sequencing

Five different DNA pools were sequenced: rounds 11, 13 and 15 for estradiol (E2), and round 15 for progesterone (P4) and testosterone (T) binding sequences. The general statistics of the analysis performed using the Galaxy server and the FASTQC tool are shown in Table S2.

Table S2. High-throughput sequencing of the different DNA pools. R11, R13, R15 denote the selection round whereas E2, P4, T the steroid target estradiol, progesterone and testosterone, respectively.

| Selection round | E2-R11 | E2-R13 | E2-R15 | P4-R15 | T-R15 |
|-----------------|--------|--------|--------|--------|-------|
| **Total sequences** | 268 | 97,867 | 97,650 | 108,169 | 116,286 |
| **Sequence length** | 34 – 164 | 25 – 169 | 25 – 228 | 25 – 252 | 25 – 199 |
| **Sequences 80-100 bp** | 254 | 70,772 | 82,815 | 85,356 | 90,782 |
| **Unique 80-100 bp sequences %** | 127 | 9,832 | 11,264 | 11,947 | 13,168 |
| **Top100 %** | 50.0 | 13.9 | 13.6 | 14.0 | 14.5 |
| **Top10 %** | 227 | 50,144 | 58,336 | 59,456 | 62,487 |
| **%** | 89.4 | 70.9 | 70.4 | 69.7 | 68.8 |

Initially, the most over-represented sequences (most copy numbers based on 100 % sequence identity) found in the estradiol pools from the sequencing of rounds 11 and round 13 were evaluated. These were designated as Seq.1 – Seq.7. Their ranking and number of copies in all the pools sequenced are shown in Table S3.

Table S3. Ranking and abundance of the most over-represented sequences from the estradiol E2-R11 and E2-R13 pools in all the pools sequenced. The first number denotes the ranking and the second one the number of copies.

| Sequence ID | E2-R11 | E2-R13 | E2-R15 | P4-R15 | T-R15 |
|------------|--------|--------|--------|--------|-------|
| **Seq.1** | 3-17 | 2-9380 | 2-3979 | 1-8176 | 2-9261 |
| **Seq.2** | 1-35 | 1-7845 | 16-519 | 7-2606 | 6-2063 |
| **Seq.3** | 4-16 | 5-4206 | 8-1862 | 3-5240 | 3-6781 |
| **Seq.4** | 2-22 | 4-3963 | 9-1105 | 2-7151 | 4-5605 |
| **Seq.5** | 7-3 | 7-3117 | 54-113 | 23-473 | 24-449 |
| **Seq.6** | 5-13 | 3-2838 | 19-370 | 10-1538 | 7-1468 |
| **Seq.7** | 6-4 | 6-587 | 139-45 | 45-272 | 43-193 |
The specificity of these sequences was evaluated by APAA. Equal concentration of each sequence (in binding buffer) was separately incubated with the four types of steroid-magnetic beads (control/negative, progesterone, testosterone and estradiol) used during the selection for 30 min at 22°C under tilt rotation. After thorough washing, the resuspended beads were amplified and the relative binding of each sequence to each steroid was evaluated using APAA. The results are shown in Figure S5.

**Figure S5.** Specificity of the most abundant sequences (Seq.1 – Seq.7) in the E2-R11 and E2-R13 pools evaluated by APAA. P4, T and E2 refer to progesterone, testosterone and estradiol steroids, respectively immobilized on magnetic beads.

In order to select highly specific sequences for each target, sequences with preferential abundance in only one of the target pools were identified. To achieve this, identical sequences in each dataset from the last selection round (E2-R15, P4-R15 and T-R15) were collapsed into single sequences. Then, the top 100 sequences from each dataset with most copies were combined and collapsed again. Sequences with only one copy, corresponding to sequences with presence in only one of the target pools, were chosen for further analysis. These selected sequences were named E1-E30, P1-P18 and T1-T7 and their sequences are shown in Tables S4–S6, respectively. Then, the number of copies of each of these sequences within the first megabyte of the raw data from the last selection round for each target (E2-R15, P4-R15 and T-R15) was manually counted. The objective was to find sequences with preferential abundance in the desired target pool compared to the other two non-target pools. The ranking and number of copies of the selected sequences can be found in Table S7. Multiple sequence alignment using the external tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was then performed to identify sequence families and facilitate the selection of aptamer candidates for characterization. This clustering into sequence families is shown in Figure S6 whereas the sequences of the final selected aptamer can be found in Table S8 and their predicted structures in Figure S7.
| ID  | Sequence (5' to 3')                                                                 |
|-----|-----------------------------------------------------------------------------------|
| E1  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E2  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E3  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E4  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E5  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E6  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E7  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E8  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E9  | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E10 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E11 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E12 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E13 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E14 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E15 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E16 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E17 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E18 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E19 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E20 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E21 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E22 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E23 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E24 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E25 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E26 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E27 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E28 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E29 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| E30 | TAGGGAAAGGAGAGCGGAGTACATGTAAGCGGAGGCTGAGCTCCGAGGAGCTGAGCTCAGGAGTGCAGCAGCAGGAGT |
| ID | Sequence (5' to 3') |
|----|-------------------|
| P1 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P2 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P3 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P4 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P5 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P6 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P7 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P8 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P9 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P10 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P11 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P12 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P13 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P14 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P15 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P16 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P17 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
| P18 | TACGAGAAGAAAGGACATATGACATATCTCTGCGAAGCTATGTCATGCCAACATTCATCAGGTCTGTTCTCAGGCTCTGACTAGTACATGACCACTTGAGG |
Table S7. Ranking and abundance of the candidate sequences with preferential abundance in each target pool. The first number denotes the ranking and the second one the number of copies of each sequence.

| Sequence ID | Estradiol (E2-R15) | Progesterone (P4-R15) | Testosterone (T-R15) |
|-------------|-------------------|-----------------------|-------------------|
| E1          | 46-132            | 235-27                | 130-53            |
| E2          | 41-153            | 419-14                | 157-43            |
| E3          | 69-90             | 761-7                 | 202-31            |
| E4          | 71-88             | 340-17                | 207-30            |
| E5          | 43-142            | 434-14                | 139-50            |
| E6          | 75-80             | 344-17                | 186-36            |
| E7          | 100-60            | 298-20                | 206-31            |
| E8          | 44-135            | 204-31                | 127-55            |
| E9          | 22-304            | 181-36                | 110-66            |
| E10         | 80-74             | 1519-4                | 583-11            |
| E11         | 79-76             | 11213-1               | 501-12            |
| E12         | 101-60            | 734-8                 | 165-40            |
| E13         | 55-109            | 512-11                | 179-37            |
| E14         | 58-108            | 308-19                | 240-26            |
| E15         | 94-65             | 1138-5                | 437-14            |
| E16         | 74-82             | 1735-3                | 239-26            |
| E17         | 40-128            | 336-18                | 169-39            |
| E18         | 48-123            | 186-34                | 150-46            |
| E19         | 73-85             | 946-6                 | 359-17            |
| E20         | 95-63             | 491-12                | 159-43            |
| E21         | 50-108            | 342-17                | 131-53            |
| E22         | 68-100            | 284-21                | 210-30            |
| E23         | 99-60             | 1163-5                | 387-16            |
| E24         | 64-102            | 576-10                | 163-42            |
| E25         | 84-71             | 2266-3                | 389-16            |
| E26         | 60-108            | 1563-4                | 181-37            |
| E27         | 88-69             | 737-8                 | 525-12            |
| E28         | 90-68             | 792-7                 | 1295-5            |
| E29         | 38-165            | 1064-5                | 373-16            |
| E30         | 56-109            | 239-27                | 189-36            |
| P1          | 684-7             | 68-142                | 171-38            |
| P2          | 318-18            | 94-91                 | 102-76            |
| P3          | 467-12            | 78-123                | 160-43            |
| P4          | 461-12            | 98-87                 | 164-40            |
| P5          | -                 | 80-121                | 168-39            |
| P6          | 421-13            | 96-89                 | 7997-1            |
| P7          | 281-19            | 72-137                | 112-65            |
| P8          | 604-8             | 95-89                 | 148-46            |
| P9          | 318-17            | 87-102                | 167-39            |
| P10         | 246-23            | 64-150                | 108-67            |
| P11         | 430-13            | 65-147                | 113-65            |
| P12         | 2663-2            | 99-83                 | 248-23            |
| P13         | 1254-4            | 71-138                | 138-50            |
| P14         | 319-17            | 93-91                 | 122-56            |
| P15         | 524-10            | 60-161                | 123-56            |
| P16         | 501-11            | 89-100                | 134-72            |
| P17         | 365-15            | 76-124                | 115-61            |
| P18         | 228-25            | 84-109                | 103-76            |
| T1          | 206-28            | 125-66                | 90-84             |
| T2          | 213-27            | 120-68                | 77-101            |
| T3          | 256-21            | 112-72                | 89-84             |
| T4          | 249-22            | 140-54                | 95-79             |
| T5          | 164-34            | 107-76                | 75-103            |
| T6          | 575-9             | 133-59                | 101-76            |
| T7          | 208-27            | 124-66                | 84-91             |
Figure S6. Multiple sequence alignment of the candidate sequences with preferential abundance in each target pool. The sequence families are boxed and the selected aptamers are highlighted.
Table S8. Sequences of the selected aptamers.

| Target     | Aptamer | Sequence (5’ – 3’)                                                                 | Length (nt) |
|------------|---------|----------------------------------------------------------------------------------|-------------|
| Estradiol  | E11     | TAGGGAAGAGAAGGACATATGATAATCATGTCTGACC GGAGGCTGACCCGAAATGAGGAAATTCGTACCATTGACT AGTACATGACCACCTTA |
|            | E26     | TAGGGAAGAGAAGGACATATGATCTCTGACC GGAGGCTGACCGAAGTGAGGAATTCGTACCTATTGACTAGTACAT GACCACCTTA |
|            | E28     | TAGGGAAGAGAAGGACATATGATACATATCCGAAGGGTCCTGACC GGAGGCTGACCGAAGTGAGGAATTCGTACCTATTGACTAGTACAT GACCACCTTA |
| Progesterone| P5      | TAGGGAAGAGAAGGACATATGATACCTCCGAAATGATCATGAGCAGTGACC GGAGGCTGACCGAAGTGAGGAATTCGTACCTATTGACTAGTACAT GACCACCTTA |
|            | P6      | TAGGGAAGAGAAGGACATATGATCGAGGTACTCCTTCACTACGTACGTTCCCTGACC GGAGGCTGACCGAAGTGAGGAATTCGTACCTATTGACTAGTACAT GACCACCTTA |
| Testosterone| T6     | TAGGGAAGAGAAGGACATATGATGCGTGAATACAGGCCGTCCGCTCCGCTTTGACTAGTACATGACCACCTTA | 86          |
Figure S7. Predicted structures of the selected steroid aptamers using the mfold server. Calculations were made at 25°C, 100 mM NaCl and 2 mM MgCl₂.
S5. Determination of the binding affinity ($K_D$) of the aptamer candidates by different approaches.

The affinity of the different aptamer candidates was evaluated three different approaches: Apta-PCR Affinity Assay (APAA), bead-Enzyme Linked Aptamer Assay (bead-ELAA) and plate-Enzyme Linked Aptamer Assay (plate-ELAA). The format of each assay and the binding curves are shown in Figure S8.

**Figure S8.** Binding curves used for the calculation of the binding affinity ($K_D$) of the candidate aptamers using (a) APAA, (b) bead-ELAA and (c) plate-ELAA. The format of each assay is also shown.
S6. Competitive plate-ELAA for steroid detection

A competitive plate-ELAA assay was designed for the detection of steroids using the selected aptamers and the format is shown in Figure S9.

Figure S9. Competitive plate-ELAA for steroid detection.

(a) Optimisation of conditions for the immobilisation of the steroids on maleimide-activated plates

Different amino-thiol compounds and concentrations of the CMO-steroid derivatives were evaluated in order to optimise the immobilisation of the steroids on maleimide-activated microtitre plate wells. The objective was the optimal detection of the steroids using a biotin-modified aptamer. The compounds evaluated were cysteamine (C2 spacer arm) and 11-amino-1-undecanethiol (C11 spacer arm) which were prepared at 100 µM in PBS and they were incubated in the maleimide-activated wells overnight at 4°C. Unreacted maleimide groups were blocked with sulfo-NHS-acetate (1 mM in 1 M sodium carbonate) whereas 100 - 1000 µM of the CMO-steroid, pre-activated with EDC/NHS, was added to the plate and incubated for 1 h. Detection of the steroid was performed with the biotin-modified aptamer (0 – 100 nM) in combination with streptavidin-polyHRP (80 HRP molecules per streptavidin molecule) and the results are shown in Figure S10. The incubation was performed at 22°C under mild agitation. 11-Amino-1-undecanethiol was finally chosen for the assays in combination with 100 µM of CMO-steroid derivative.
Figure S10. Steroid detection of steroid immobilised on microtitre plates using a biotin-modified aptamer. The amino-thiols (a) cysteamine and (b) 11-amino-1-undecanethiol were used for the immobilisation of the CMO-steroid derivative (100 or 1000 µM) on maleimide-activated plate.

(b) Duration of the incubation steps

Using the optimal conditions for steroid immobilisation, the competitive plate-ELAA assay was then optimised in terms of duration of the incubation steps. Both the pre-incubation of the aptamer with the free steroid (”pre-incubation step”) and the subsequent incubation of the aptamer-steroid mixture on the plate containing immobilised steroid (”incubation step”) were simultaneously varied from 5 min to 30 min. All the incubation steps were performed at 22ºC under tilt rotation or mild agitation. The calibration curves are shown in Figure S11 and the sensitivity achieved (LODs) in Table S9.
**Figure S11.** Optimisation of the duration of the incubation steps for optimal detection of steroids with a competitive plate-ELAA using a biotin-aptamer. The times on the graphs refer to pre-incubation time – incubation time. The optimisation was performed for estradiol detection using the biotin-E28 aptamer.

**Table S9.** Effect of pre-incubation and incubation time on the sensitivity of estradiol detection with the competitive plate-ELAA using the biotin-E28 aptamer. ND: not determined.

| Pre-incubation (min) | Incubation (min) | LOD (R²)       |
|----------------------|------------------|----------------|
|                      | 5                | 14.5 nM (0.9977) | 62.4 nM (0.9920) | 174.0 nM (0.9928) | 565.4 nM (0.9901) |
|                      | 10               | 198.3 nM (0.9833) | 80.3 nM (0.9863) | ND                | ND               |
|                      | 20               | 277.9 nM (0.9817) | ND               | 358.3 nM (0.9758) | ND               |
|                      | 30               | 179.2 nM (0.9887) | ND               | ND                | 322.4 nM (0.9813) |
(c) Assay temperature

The effect of the temperature, used to perform all the incubation steps, on the performance of the competitive assays for steroid detection was evaluated. The temperatures tested were 22°C and 37°C using optimised times for the pre-incubation and incubation steps (10 min each) and the results are shown in Figure S12.

![Graph showing the effect of temperature on assay performance](image)

**Figure S12.** Effect of the temperature on the performance of the competitive plate-ELAA for estradiol detection using the biotin-E28 aptamer.

| Assay temperature | LOD (µM) | R² |
|-------------------|----------|----|
| 22°C              | 0.080    | 0.9863 |
| 37°C              | 1.079    | 0.9887 |

(d) Biotin-aptamers concentration

The concentration of each aptamer used in the final competitive plate-ELAA assays was optimised as follows: a direct assay was initially performed using varying concentrations of the biotin-aptamer for the detection of the steroid immobilised on a microtitre plate. After a 10-min incubation step at 22°C (1st incubation), the supernatant from each well containing unbound aptamer was transferred to a fresh microtitre plate with immobilised steroid and the incubation step was repeated (2nd incubation). Both plates were finally incubated with the streptavidin-polyHRP and signal was obtained after the addition of TMB substrate. The concentrations of aptamer for which the signal difference between the first and the second incubation was largest (2 – 4 distinct concentrations for each aptamer) were then used and the same 2-step assay was repeated in order to choose the final concentration of aptamer for the competitive assays. The assay was performed for all aptamers: E11, E26 and E28 for estradiol, P5 and P6 for progesterone and T6 for testosterone. As an example, Figure S13 shows the results obtained for the estradiol E28 aptamer, whereas the concentrations of all aptamers used for the final assays are shown in Table S10.
Figure S13. Optimisation of aptamer concentration used for the competitive plate-ELAA assays for steroid detection. Aptamer E28 is shown as an example for estradiol detection using (A) a wide range of aptamer concentrations and (B) selected aptamer concentrations for which the signal difference between the two incubations was greatest.

Table S10. Concentrations of the aptamers used for the competitive plate-ELAA for steroid detection.

| Steroid target | Aptamer candidate | Biotin-Aptamer concentration (nM) |
|----------------|-------------------|----------------------------------|
| Estradiol      | E11               | 3.0                              |
|                | (T15)-E26         | 2.0                              |
|                | E28               | 12.5                             |
| Progesterone   | P5                | 0.5                              |
|                | P6                | 0.8                              |
| Testosterone   | T6                | 2.0                              |
S7. Evaluation of previously reported steroid-binding aptamers with the plate-ELAA.

Previously reported steroid-binding aptamers were evaluated with the plate-ELAA developed in this work. The aim was to compare their binding affinity with the newly developed aptamers. The aptamers evaluated were: the estradiol-binding aptamers reported by Kim et al.\textsuperscript{3} and Alsager et al.\textsuperscript{4}, the progesterone aptamer reported by Contreras Jiménez et al.\textsuperscript{5} and the testosterone T5 aptamer reported by Skouridou et al.\textsuperscript{1} The binding curves and the calculated affinity ($K_D$) of these aptamers for their respective target are shown in Figure S14.

![Binding curves of previously reported aptamers with their cognate steroid target](image)

Figure S14. Binding curves of previously reported aptamers with their cognate steroid target: (a) Kim estradiol aptamer; (b) Alsager estradiol aptamer; (c) P4G13 progesterone aptamer; (d) T5 testosterone aptamer.
S8. References

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