Evelin Florencia Cardozo

Simultaneous detection of progesterone and estradiol using aptamer-based assays: problems and prospects.

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supervised by Dr. Markéta Svobodová & Prof. Ciara K. O'Sullivan

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Simultaneous detection of progesterone and estradiol using aptamer-based assays: problems and prospects

Evelin F. Cardozo[1], Markéta Svobodová[1] & Ciara K. O'Sullivan[1,2]

[1]INTERFIBIO Consolidated Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, 43007 Tarragona, Spain.

[2]Institució Catalana de Recerca i Estudis Avancats (ICREA), Passeig Lluís Companys 23, 08010 Barcelona, Spain.

Abstract

The simultaneous detection of steroids is of increasing importance due to their implications in the early diagnosis, treatment and monitoring of different pathologies and diseases. There is a need for the rapid and reliable detection of sex hormones such as progesterone (P4) and estradiol (17-β estradiol) in a wide range of environmental samples, foods, medical samples, and cosmetics. The overall objective of this work was the simultaneous detection of P4 and 17-β estradiol using a three-step aptamer-based detection method. Different surfaces were analyzed for optimal target immobilization and detection based on a competitive assay. In parallel, the amplification and detection of E28 and P5 aptamers via the polymerase chain reaction, or isothermal amplification in combination with an enzyme-linked oligonucleotide assay was pursued. Future work will focus on the combination of the two assays.

1. Introduction

Steroids, particularly progesterone and estradiol as part of endocrine disruptors (EDC´s), are hormones that have a crucial role in the proper development, preservation and control of the sexual reproductive system and tissues in animals and humans[1,2]. The analysis of these steroids has been a key tool for the diagnosis and monitoring of several endocrinal disorders and other pathologies[3]. For example, an estradiol:progesterone ratio out of the normal range is related to different types of cancer[1,4–7], menstrual period alterations, irregular libido[1], difficulties in conceiving, and miscarriages[8,9]. Progesterone and estradiol are also present as pollutants in aquatic environments, representing an emerging risk to animal and human health as well as affecting wildlife[7]. In fish populations, there is evidence of reproductive issues leading to an unbalanced sex ratio and a resulting decrease in fish numbers[10]. The identification of progesterone and estradiol in a large variety of environmental samples, foods, medical samples or cosmetics has been facilitated by an increasing number of assays dedicated to the detection of these molecules[11]. For several decades, single steroid detection methods have been used for specific steroid-related endocrine diseases. However, there has been a big interest in expanding this limited approach to a larger group of steroids[3].

Currently, the techniques employed for multiplexed detection of steroids involves the combination of techniques such as mass spectroscopy (MS) and liquid or gas chromatography[12] (LC or GC). Those techniques have the great advantage of achieving high accuracy and sensitivity,[12,13] but they require the use of high-end equipment, pre-treatment of the sample and highly specialized personnel, thus resulting in a high cost. Electrochemical sensors are another alternative due to their low cost, ease of use, sensitivity, and applicability to use at the point-of-need[14]. Diverse sensors exploiting nanomaterials such as molecularly imprinted polymers[15], single-walled carbon nanotubes[16] and C60[17], have been reported, but many of these suffer from non-specific interferences.

Immunoassays offer an alternative to existing LC/GC-MS methods, as they are easy-to-perform techniques and of relatively low cost. However,

immunoassays used for steroids detection can suffer from problems with reproducibility, sensitivity and selectivity[18–20]. These problems became from the difficulty to generate highly specific antibodies against steroids, due to their small size and highly similar structures (Figure 1).

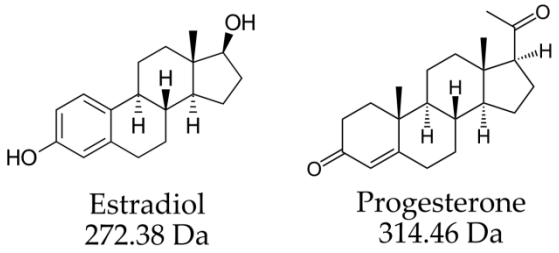

Figure 1.- Schematic representation of estradiol and progesterone chemical structures.

Aptamers have emerged as a substitute for antibodies and have garnered increasing interest since they were first reported[21–23], especially for the recognition of small molecules[24–27]. Aptamers are synthetic oligonucleotides selected by a process called Systematic Evolution of Ligands by Exponential enrichment (SELEX)[28]. They can be selected against a wide variety of molecules, such as proteins[29], toxins[30,31], pesticides[32,33], and hormones[24,34] among others. In addition to that, their post-SELEX synthesis is much less expensive than antibodies, they can be amplified, are more stable, can be easily modified and can present a higher specificity due to the inclusion of a counter selection step against potential interferents in the SELEX process[35,36].

Looking for a cost-effective solution for use at the point-of-need, a three-step aptamer-based method for the simultaneous detection of progesterone and estradiol was proposed in this work. The process starts with the detection of estradiol and progesterone using a competitive Enzyme-Linked Aptamer Assay (ELAA). Previously published aptamers selected against progesterone (P5) and estradiol (E28)[24] are then amplified by Polymerase Chain Reaction (PCR) or Recombinase Polymerase Amplification (RPA) and the amplified product could be quantitatively detected using an Enzyme-Linked Oligonucleotide Assay (ELONA).

In order to reach the final objective, extensive optimization of each step was carried out. Different types of target immobilization were evaluated to find the best system with the lowest limit of detection and reproducibility. Optimization of the competitive assay, as well as the design and use of specific primers for use in thermal cycling/ isothermal amplification, were also studied.

2. Materials and methods

2.1. Materials and reagents

Progesterone-P4, Progesterone3-(O-carboxymethyloxime), β-Estradiol, β-Estradiol-6-one 6-(O-carboxymethyloxime), skimmed milk powder, DNA single-stranded from salmon testes, and 6-Mercapto-1-hexanol were purchased from Sigma Aldrich (Spain). All oligonucleotides' sequences were synthesized by Biomers (Germany). Dynabeads™ M-270 Amine, Pierce™ Maleimide Activated Plates, Pierce™ NeutrAvidin™ Coated Plates, Corning™ PureCoat™ amine microplate, horseradish peroxidase (HRP), substrate formulation tetramethylbenzidine (TMB), dNTPs, sulfo-N-Hydroxysuccinimide (Sulfo-NHS), Pierce™ Sulfo-NHS-Acetate, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), Poly-HRP Streptavidin (SA-PolyHRP), Phosphate-Buffered Saline (PBS) Tablets, Tween20 and UltraPure™ DEPC-Treated water were purchased from Fischer Scientific (Spain). 11-Amino-1-undecanethiol (MUAM) was purchased from Tebu-bio (Spain). SiMAG Amine beads were purchased from Chemicell (Germany). Agarose gel powder was purchased from Bio-Rad Laboratories S.A. (Spain), GelRed™ Nucleic Acid Gel Stain from Biotium (Spain) and TwistAmp Basic kit from TwistDX (United Kingdom). The Binding Buffer (BB) used in the different assays consisted of 20 mM Tris Base, 2 mM $MgCl_2$ and 100 mM NaCl (pH 7.5).

2.2. Affinity dissociation constants (K_D) determination by Bead-ELAA

Dynabeads™ M-270 Amine were rinsed 3 times with 100 mM MES buffer and then coated with the corresponding CMO-steroid using EDC/NHS (15 mg/ml each) in 100 mM MES buffer.

Subsequently, 10 mM Sulfo-NHS-Acetate in PBS was used to block any remaining free-amine groups. Biotinylated aptamers E28 and P5 were incubated at room temperature for 30 min in BB under agitation. Streptavidin-polyHRP80 (0.05 µg/ml in PBS with Tween20 (PBST)) was then added and incubated for 30 min at room temperature, before finally adding TMB for 5 min followed by the addition of 1 M H_2SO_4 to stop the reaction. Prior to absorbance reading at 450 nm, the supernatant was separated from the beads using a magnet and transferred to a microtiter plate. Each incubation step was carried out using 50 µl/well except for the blocking and washing steps with PBS where 200 µl/well were used. Each washing step involved 3 repetitions. The absorbance was read at 450 nm using a Bio-Nova SpectraMax® 340PC microplate reader.

SiMAG™ amine beads were rinsed 3 times with 100 mM MES buffer and then coated with the corresponding CMO-steroid using EDC/NHS (50 mg/ml and 10 mg/ml respectively) in 50 mM MES buffer. Sulfo-NHS-Acetate (1mM) in PBS was then used to block any remaining free-amine groups followed by blocking with 200 µl/well of 2 %w/v skimmed milk in PBST for 1 h at room temperature. Biotinylated aptamers, streptavidin polyHRP80, TMB and H_2SO_4 were used as described above.

2.3. Affinity dissociation constants (K_D) determination by Plate-ELAA

The same protocol employed for the Dynabeads™ M-270 Amine was used for the Corning™ PureCoat™ microtiter plate, with some minor modifications. Following Sulfo-NHS-Acetate blocking, a second blocking with 1 %w/v of skimmed milk in PBST was carried out for 1 h at room temperature. Biotinylated aptamers (P5 and E28) with 0.5 mg/ml of salmon sperm DNA were added to the wells of the plate and incubated at room temperature for 30 min in BB under agitation. Streptavidin polyHRP80, TMB and H_2SO_4 were used as described above. Each incubation step was carried out using 50 µl/well except the blocking and PBST washing steps where 200 µl/well were used. Each washing step involved 3 repetitions.

In the case of the Pierce™ Maleimide Activated Plates, following 3 times washing with PBST, MUAM (300µM) was added and the plate was incubated overnight at 4ºC. The corresponding CMO-steroid was then coupled by a carbodiimide reaction using EDC/NHS (50 mg/ml and 10 mg/ml respectively) in 50 mM MES buffer. Sulfo-NHS-Acetate (1 mM) in PBS was incubated for 1 h at room temperature followed by a second blocking with 10 mM 6-Mercapto-1-hexanol in PBS. Biotinylated aptamers, streptavidin polyHRP80, TMB and H_2SO_4 were used as described above.

2.4. Detection of progesterone and estradiol by competitive-ELAA

For competitive-ELAA, the same protocol was used for steroid immobilization as that for K_D determination. The corresponding biotinylated aptamers were pre-incubated for 30 min at room temperature with the steroid to be measured. This was followed by the addition of the biotinylated aptamer-steroid mixture to the beads or microplates. For all surfaces studied, a range of aptamer concentrations ranging from 0.312 nM to 10 nM and steroid concentrations of 0 µM to 100 µM were used. In the case of the amine plates, aptamer concentrations of 20 nM and 100 nM were also analyzed. Following incubation, and thorough washing the SA-polyHRP, TMB and H_2SO_4 were added, as described above.

2.5. Amplification techniques

2.5.1. Synthetic oligonucleotides sequences

In order to achieve the simultaneous detection of both steroids, specific primers for each aptamer were designed and combined with single-stranded sequences (tails). All sequences used in this study can be found in Table 1.

Table 1.- Synthetic oligonucleotide sequences. E28-F_t and E28-R_t, as well as P5-F_t and P5-R_t, refer to forward and reverse tailed primers employed in the amplification step. E28_C1, P5_C1 and E28/P5_R refer to the capture and reporter probes used in ELONA assays.

	Sequence
E28	5'-tagggaagagaaggacatatgatacatatccgaag ggtcctgaccggaggctgaccggagtgggaattg actagtacatgaccacttgagg-3
P5	5'-tagggaagagaaggacatatgatacctccgaagta tcatgcggagcatgtcccgaatttcattcgttctcgtgact tgactagtacatgaccacttgagg-3
E28-F_t	5'-ctagtagccgaattcctag-3′-5′-acatatccgaagggtcctgac-3'
E28-R_t	5'-tgtaaaacgacggccagt-3′-5′-aattcccactccggtcagc-3'
P5-F_t	5'-ataggctggttcgtaatcgg-3′-5′-ctccgaagtatcatgcggag-3'
P5-R_t	5'-tgtaaaacgacggccagt-3′-5′-aagtcacgaga acgaatgaaat-3'
E28_C1	5'-ctaggaattcggctacttagttttttttttttttt-3'
P5_C1	5'-ccgattacgaaccagcctatttttttttttttttt-3'
E28/P5_R	5'-actggccgtcgttttaca-3'

2.5.2.PCR Amplification

Polymerase Chain Reaction (PCR) protocol was optimized for the specific set of primers used in this study, containing primers annealing temperature and concentration, cycles number and cycling protocol. Optimized conditions include: 1X Buffer, 200 µM dNTPs, 200 nM tailed primers, 1U Taq Dream DNA Polymerase, H_2O (nuclease-free, DECP-treated) as needed. All reagents except the template were mixed in a single master mix and distributed in 0.2 ml tubes and placed in a T100 BioRad iCycler® cycler with the following program: 2 min at 95°C (only the first cycle), 30 sec at 95°C, 30 sec at 58°C, 30 sec at 72°C for 25cycles. The final extension was performed at 72°C for 5 min.

2.5.3. RPA Amplification

The Recombinase Polymerase Amplification (RPA) protocol recommended by the TwistDX was used with some minor modifications. Reagent concentrations (dNTPs, template, and tailed primers) and amplification times (5 min-30 min) were optimized for each template. Optimized conditions were found to be 14 mM magnesium acetate, 1x E-mix, 1x Core Reaction, 200 µM dNTPs, 200 nM tailed primers using a range of concentrations of the template DNA. All reagents except the template and magnesium acetate were mixed in a single master mix and distributed in 0.2 ml tubes. Finally, the template was added, followed by magnesium acetate to initiate the reaction and placed at 37ºC for 20 min in a T100 BioRad iCycler®. All steps were carried out using a frozen rack to avoid reaction initialization due to room temperature.

2.6. Detection of the amplified product by ELONA

Pierce™ NeutrAvidin™ Coated Plates were rinsed 3 times with PBST washing buffer and coated with 100 nM capture probe solution in PBST for 30 min at room temperature, under shaking conditions. The resulting RPA/PCR amplified product was diluted (1/50) and added to the functionalized neutravidin plates for a further 30 min, followed by a washing step with PBST, and subsequent addition of 10 nM reporter probe labelled with HRP. After a final washing step, the presence of HRP was measured by adding TMB substrate and stopping the enzymatic reaction with 1 M H_2SO_4 after 5 min. Each incubation step was carried out using 50 µl/well except the washing steps where 200 µl/well of PBST were used 3 times.

2.7. Raw data processing

Data analysis was performed using GraphPad Prism® software (v. 9.0.2). Determination of the aptamer affinity constants was carried out by applying the "One Site – Specific Binding with Hill Slope" model. For the calibration curves, the raw data was fitted using "Sigmoidal, 4PL, x is log(concentration)" model, were the limits of detection (LODs) for the competition assay were defined as the highest value obtained (Top best-fit value) minus three times its standard deviation (3x Top Std. Error). In the case of the ELONA assays, the limits of detection (LODs) were defined as the

lowest value obtained (Bottom best-fit value) plus three times its standard deviation (3x Bottom Std. Error). All other data were analyzed using Microsoft Excel®. The OligoAnalyzer™ Tool from Integrated DNA Technology (IDT) was used for the analysis of interactions between primers, tails, and tailed primers.

3. Results and discussion

3.1. Detection of progesterone and estradiol by competitive-ELAA

The work reported here aimed at achieving a three-step process comprising target detection by competitive ELAA, aptamer amplification by PCR/RPA and quantification using ELONA.

The characterization of the E28 and P5 aptamers was performed to (i) determine their affinity for their respective targets and (ii) choose the most appropriate surface for target immobilization for the competitive ELAA assay. To this end, the affinity dissociation constant (K_D) of aptamers were evaluated using 4 different surfaces, including maleimide and amine activated plates, as well as two different-sized, amine-functionalized beads purchased from different companies. Because the steroids do not directly have any functional group for the immobilization, their CMO-modified versions were used. In the case of the maleimide plate, CMO steroid derivates were immobilized through 11-amino-1-undecanethiol (MUAM), which contains a long C11 spacer arm. In the rest of the cases, CMO steroid derivates were immobilized directly using carbodiimide chemistry between the amine group on the surface and the carboxyl group of CMO steroid derivates. For all studied surfaces, the concentration of targets, pH of the buffers solutions as well as blocking reagents used for the immobilization were optimized. As can be seen in Figure 2, both aptamers have almost the same behavior when using Dynabeads for steroid immobilization, with comparable sensitivity to the maleimide microplates. In the case of the amine microplates and SiMAG beads, the best results are observed using SiMAG beads, but in both cases, the aptamer P5 shows more sensitivity and specificity as compared to the aptamer E28. The summary of the K_D values for E28 and P5 aptamer using different surfaces can be observed in Table 2.

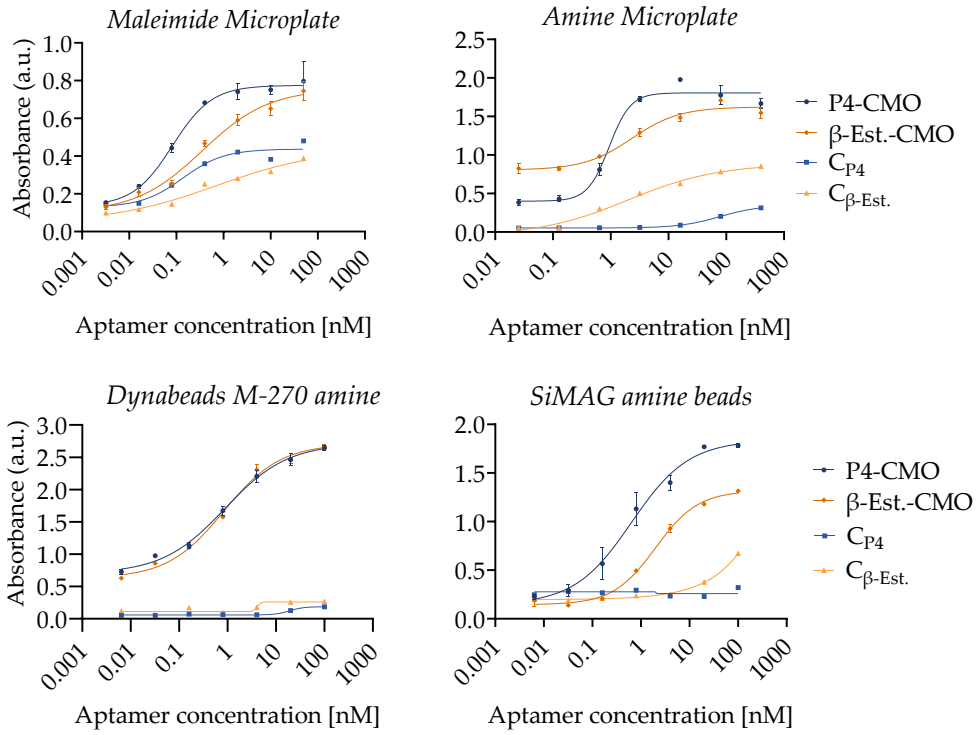

Figure 2.- Direct assays results using different surfaces for steroid's immobilization. P5 and E28 aptamers were analyzed in presence of progesterone-CMO (P4-CMO) and estradiol-CMO (β Est.-CMO), respectively, as well as non-target controls (CP4 and Cβ-Est.).

The lowest K_D values as well as the best coefficients of determination (R^2) were reached using maleimide microplates followed by amine microplates, Dynabeads and SiMAG amine beads. All the K_Ds were in the nM range (0.22 nM to 1.95 nM) except for the P5 aptamer that reached an improved dissociation affinity constant with a value of 40 pM when using the maleimide microplates.

Table 2.-Dissociation affinity constants determination for the studied surfaces for E28 and P5 aptamers.

Surface	Aptamer	K_D [nM]	R^2
Dynabeads M270 amine	E28	0.27 ± 0.12	0.987
	P5	0.62 ± 0.24	0.985
SiMAG amine beads	E28	1.95 ± 0.60	0.969
	P5	0.53 ± 0.20	0.979
Maleimide Microplates	E28	0.30 ± 0.11	0.988
	P5	0.04 ± 0.01	0.991
Amine Microplates	E28	0.22 ± 0.09	0.975
	P5	0.52 ± 0.20	0.985

The competitive-ELAA was based on the competition of the immobilized CMO-steroid derivates and unmodified steroids in solution to bind to the aptamer. The aptamer that binds to the immobilized steroid is the one that gives signal, leading to a signal inversely proportional with respect to the concentration of the steroid in solution.

Several critical parameters of the competition assay, such as the concentration of aptamers and steroids were analyzed to find the optimal conditions.

For maleimide microplates, in the case of progesterone, competition worked with aptamer concentrations below 5 nM, reaching the best result at 2.5 nM. Using this concentration, a calibration curve was performed (Figure 3) and a limit of detection (LOD) of 8.572 µM was obtained. This LOD is considerably higher than the one required for current applications. Furthermore, the assay, surprisingly, was not reproducible, which could be attributable to a new batch of the MUAM that was purchased from a different company.

In the case of estradiol, competition was not observed at any of the aptamer concentrations studied. Results obtained from different experiments performed during optimization can be found in Figures S1 and S2.

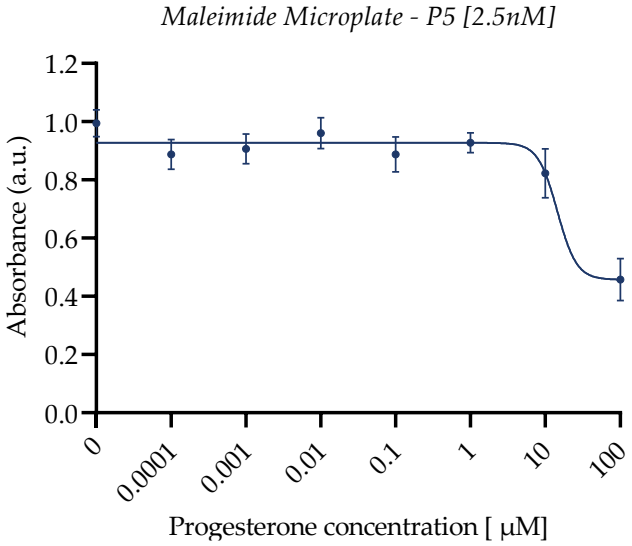

Figure 3. Competition ELAA assay for progesterone (P5) using maleimide microplate for steroid immobilization. Aptamer's concentration employed was 2.5 nM.

Because of the drawbacks found with maleimide microplates, the use of amine plates was subsequently studied. This surface facilitates a direct immobilization of the steroid on the surface via carbodiimide coupling reaction without the need for a linker molecule. Unfortunately, using these plates, neither of the planned competition assays for progesterone and estradiol worked. With increasing steroid concentration, behavior opposite to that expected was obtained (Figure 4).

Results of the different experiments performed during optimization can be found in Figure S3.

Dynabeads offered another possibility to carry out the competitive-ELAA. In the case of estradiol, competition worked at aptamer concentrations below 2.5 nM, with the best result observed at 1.25 nM. A calibration curve was performed using 1.25 nM of E28 aptamer (Figure 5) and a LOD of 3.899 µM was achieved. However, again it was not possible to reproduce these results. New reagents were purchased, and new optimizations were

performed by modifying E28 concentrations and trying different combinations of pre-incubation and incubation times (5 min / 30 min and 30 min / 30min) at different temperatures (room temperature and 4°C) without any improvement in the results obtained.

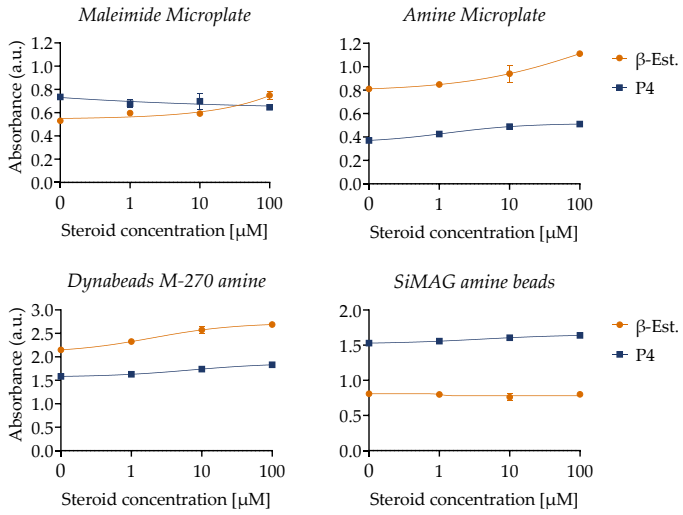

Figure 4.-Summary of results obtained for competitive ELAA assay using different surfaces for steroid's immobilization. P5 and E28 aptamers (2.5 nM) were analyzed in presence of progesterone (P4) and estradiol (β Est.), respectively.

On the other hand, in the case of progesterone, competition was not observed at any of the aptamer concentrations used. Results of the different experiments performed during optimization can be found in Figures S4 and S5.

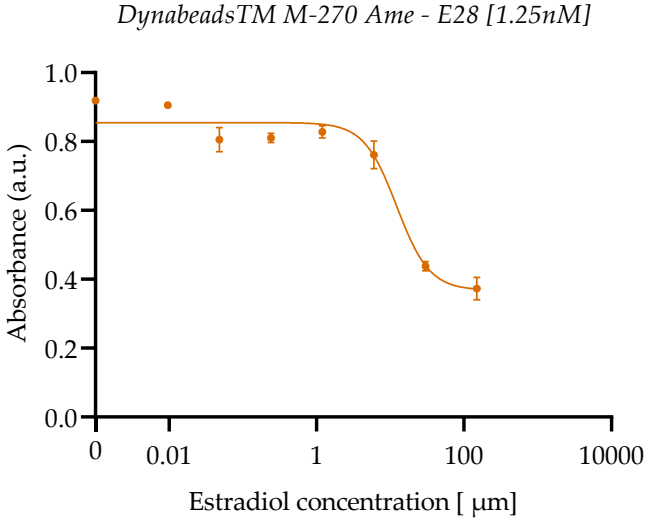

Figure 5.- Competition ELAA assay for detection of β-Estradiol using Dynabeads™ M-270 amine for steroid immobilization. Aptamer's concentration employed was 1.25 nM.

Finally, SiMAG amine beads were tested. SiMAG beads are almost 3 times smaller than Dynabeads leading to a considerable increase in their surface/volume ratio, which could affect the way the aptamers interact with the corresponding steroid. Unfortunately, also in this case no competition was observed, independent of the aptamer concentration employed. Results of the different experiments performed during optimization can be found in Figure S6.

All results obtained for the competition assays using different surfaces suggest the preference of the aptamers to bind to the immobilized target instead of the steroid in solution. Work will continue to identify the conditions required to achieve a functioning competitive assay, but due to time constraints, the focus of the work switched to the amplification part of the assay.

3.2. Amplification and detection of amplified product by ELONA.

RPA is an isothermal amplification technique ideal for application at the point-of-need because it has a wide operating temperature range (22-45ºC), with reliable results and a robust character[37]. In addition, it is faster and more sensitive as compared with PCR. Regarding the detection of amplified products, whilst there have been some reports detailing the use of ELONA for the multiplexed detection of oligonucleotide sequences[38–40], to date this method has not been employed for the simultaneous detection of aptamers against estradiol and progesterone amplified using tailed primers.

Several experiments for the simultaneous PCR and RPA amplification of P5 and E28 aptamers were performed. First, PCR amplification was performed using untailed primers specifically designed for each aptamer to confirm their specificity. As can be observed in Figure 6, electrophoresis results showed two well-defined bands indicating successful dual amplification.

Following the design of the tailed primers, PCR was performed again but amplification was not observed to be reproducible. A deep analysis of potential interactions between tailed primers was

performed using OligoAnalyzer™ software from IDT. Complementarity of up to 5 bases between tailed primers was found, which could explain the problems in reproducible amplification. New tails were thus designed, a range of primer concentrations was used, and an alternative engineered DNA polymerase, Kapa2G Robust was used, but still dual simultaneous amplification could not be achieved. In parallel, simultaneous RPA amplification using tailed primers was also performed and similar issues to PCR were observed. In this case, a sequential experiment where the primers were added with a lag-time was designed to try to avoid the interactions between the tailed primers, but only one aptamer was amplified. Results of the different experiments performed during optimization can be found in Figures S7-S9.

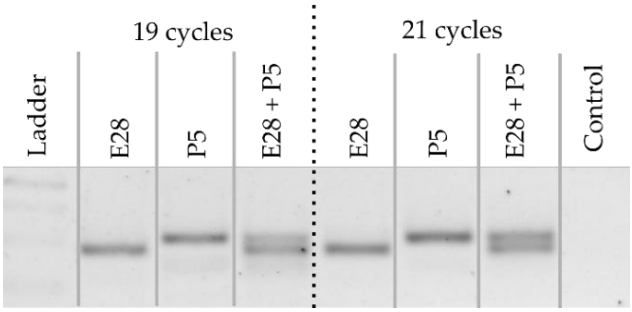

Figure 6.- Electrophoresis result obtained after 19 and 21 cycles of simultaneous PCR amplification of E28 and P5 aptamers.

Finally, single aptamer PCR and RPA amplification followed by detection using ELONA was performed. Calibrations curves were carried out for each aptamer using 50-fold dilutions of P5 and E28 amplified aptamers by PCR and RPA achieving limits of detection of 2.82 fM and 1.75 fM for PCR, and 298 aM and 585 aM for RPA, respectively. As can be seen in Figure 7, the best results are obtained with RPA due to its wider linear range and better sensitivity.

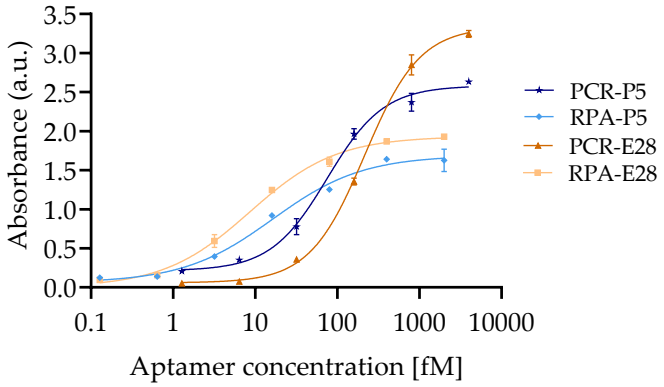

Figure 7.- Calibration curves for detection of PCR/RPA amplified aptamers by ELONA.

4. Conclusions

A three-step aptamer-based method for the simultaneous detection of progesterone and estradiol was proposed. Four different surfaces were studied to find the most suitable system to carry out the competition assay; maleimide and amine activated plates, Dynabeads M-270 amine and SiMAG amine beads. For all the studied surfaces a deep optimization of the concentration and pH of the reagents, incubation temperatures, times, and blockings required was performed. Subsequently, the affinity dissociation constant for each surface was determined to be in the low nanomolar range except for the aptamer P5 that was observed to have a K_D of 40 pM.

Unfortunately, competitive-ELISA results suggest a preference for the aptamer to bind to the immobilized target instead of the steroid in solution, which may be attributable to the SELEX process employed in the selection of these aptamers. Under specific conditions, this preference seems to be decreased and a working competitive-ELAA for progesterone using maleimide activated plates and for estradiol using Dynabeads was obtained, but possibly due to new batches of reagents, these assays could not be repeated, and work is ongoing to elucidate the optimum results.

Whilst simultaneous amplification of the P5 and E28 aptamers using non-tailed primers was successfully achieved, when the tails were introduced, this dual amplification could no longer

be achieved. Even following a redesign of the tails, the problem persisted, and it is postulated that the tails are provoking some type of three-dimensional structure that causes interactions to form between tails, even though there should be no base complementarity. Individual amplification could however be achieved using these tailed primers. PCR and RPA calibrations curves for detection of each of the amplified aptamers using ELONA were obtained, and the LODs obtained for P5 and E28 aptamers were in the fM range (1.75 fM and 2.82 fM, respectively) for PCR and in the aM range (298 aM and 585 aM, respectively) for RPA.

Work is ongoing to elucidate the correct conditions for the competitive assays, or to use a displacement type assay such as that recently reported by Du et al., 2021[41]. In parallel, another set of tails are being designed to facilitate simultaneous amplification, with the final goal being the realization of a lateral flow assay for the ultrasensitive simultaneous detection of estradiol and progesterone in urine samples.

5. Acknowledgements

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Simultaneous detection of progesterone and estradiol using aptamer-based assays: problems and prospects

Evelin F. Cardozo[1], Markéta Svobodová[1] & Ciara K. O'Sullivan[1,2]

[1]INTERFIBIO Consolidated Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, 43007 Tarragona, Spain.

[2]Institució Catalana de Recerca i Estudis Avancats (ICREA), Passeig Lluís Companys 23, 08010 Barcelona, Spain.

Supplementary Information

Contents

Results and discussion

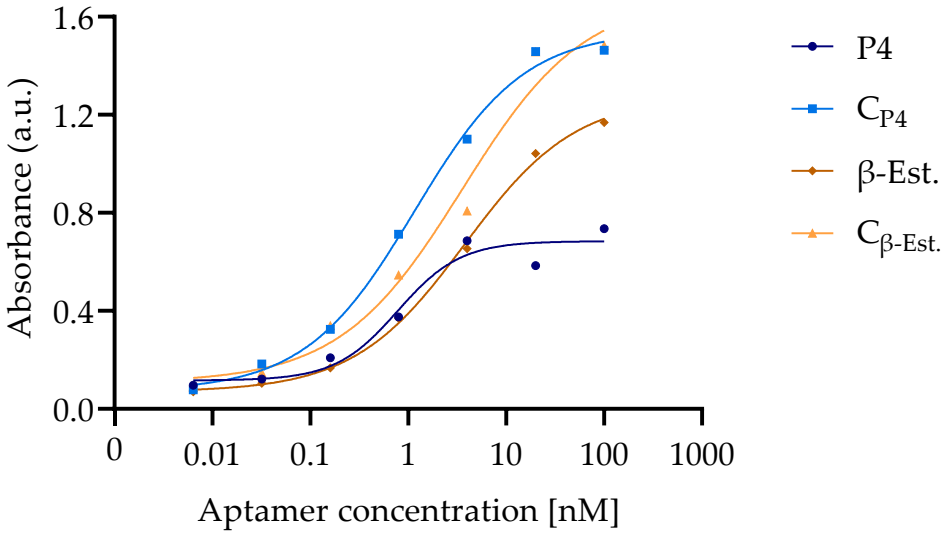

Figure S1.- Direct assay result for progesterone and estradiol when using maleimide microplates after the acquisition of new reagents. Controls signals are higher than positive signals, suggesting unspecific binding of the aptamer.

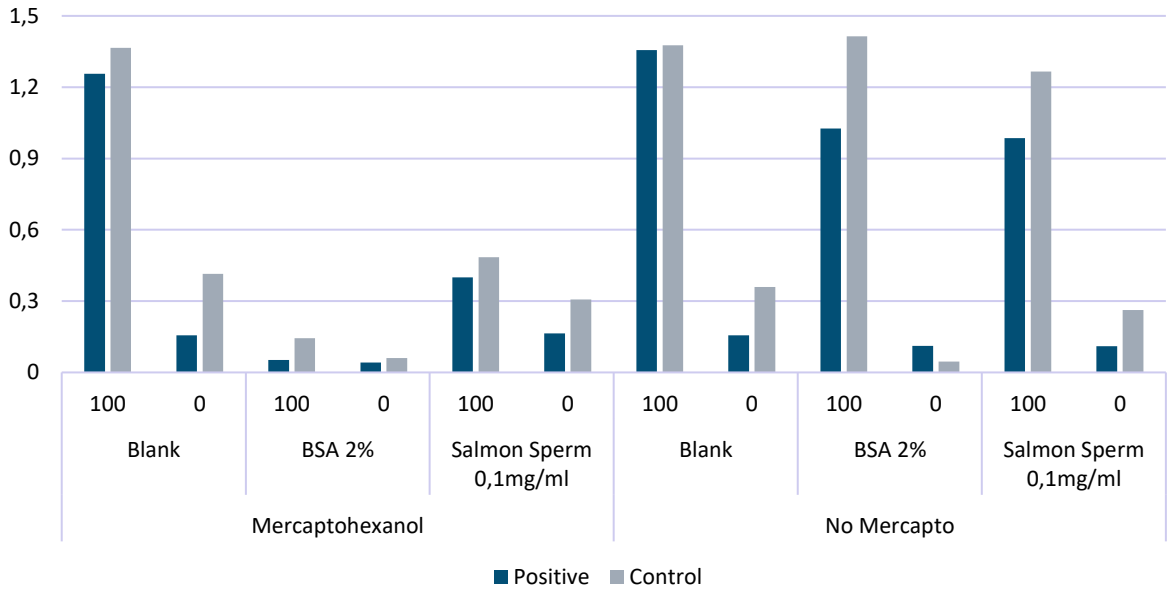

Figure S2.- Direct assay results when analyzing multiples blockings combinations to avoid the unspecific binding of the aptamer and decrease the control signal. The concentrations of aptamer employed were 100nM and 0nM. All the combinations decrease the signals for both, positive and control but none of them achieved the requirements.

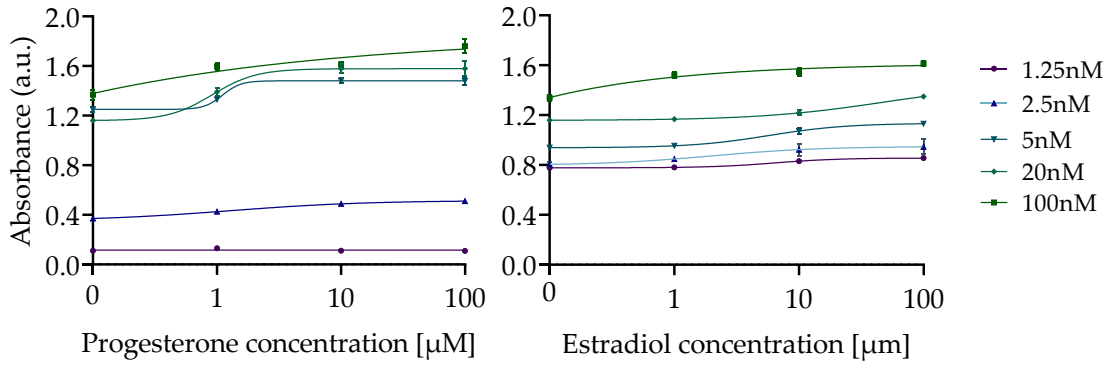

Figure S3.- Competition assay results for progesterone and estradiol detection using amine plate for its immobilization. The graph illustrates the system response using P5 and E28 different aptamers concentrations (100nM, 20nM, 5nM, 2.5nM, 1.25nM) as a function of the corresponding steroid concentration.

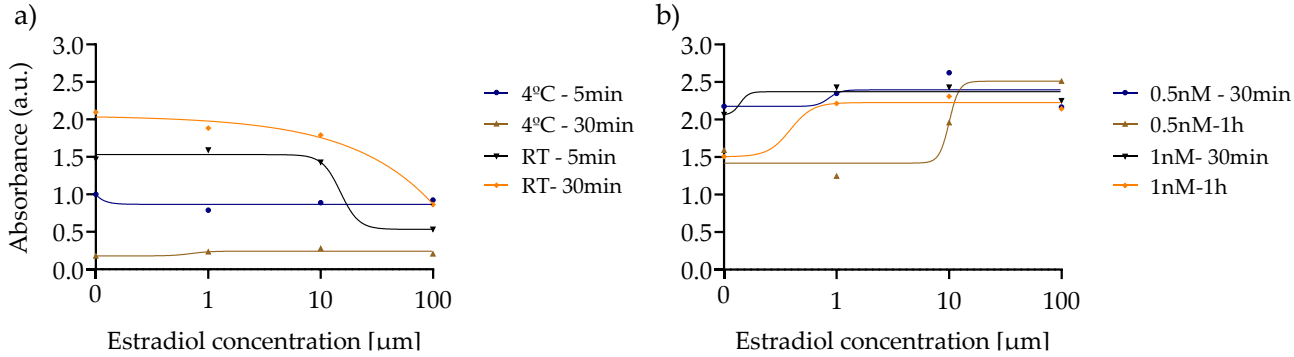

Figure S4.-Competition assay results for estradiol. a) Analysis of the effect of the pre-incubation time and temperature of the incubation step using DynabeadsTM M-270 Amine as surface and 0.5nM of the aptamer. The same analysis was done for 2.5nM and 0.25nM, b) Repetition of the best result obtained in a), using an additional aptamer concentration, and increasing pre-incubation time.

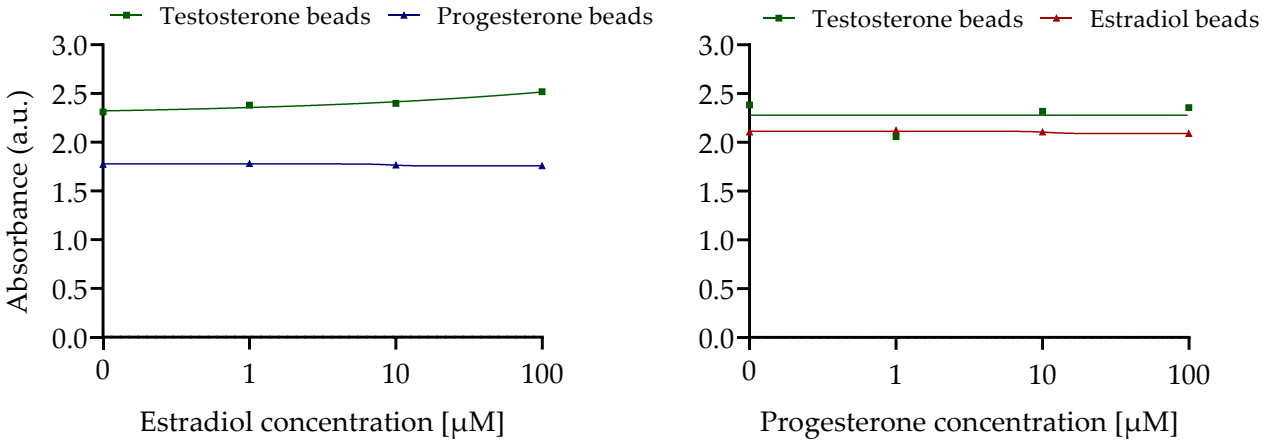

Figure S5.- Competition assay results for estradiol and progesterone. a) Analysis of the effect of the pre-incubation time and temperature of the incubation step using DynabeadsTM M-270 Amine as surface and 0.5nM of the aptamer. The same analysis was done for 2.5nM and 0.25nM, b) Repetition of the best result obtained in a), using an additional aptamer concentration and increasing pre-incubation time.

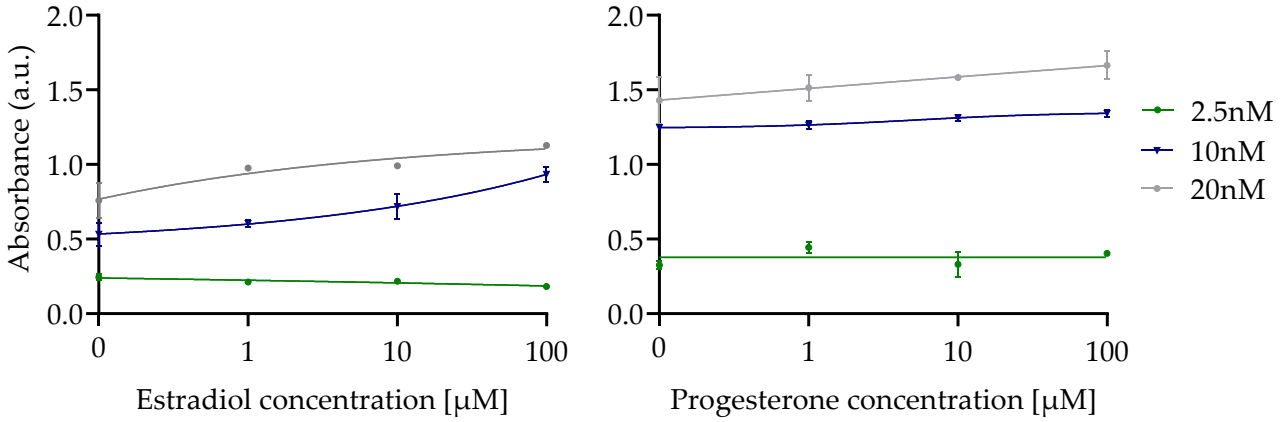

Figure S6.- Competition assay results for progesterone and estradiol detection using SiMAG amine beads for its immobilization. The graph illustrates the system response using P5 and E28 different aptamers concentrations (20nM, 10nM and 2.5nM) as a function of the corresponding steroid concentration.

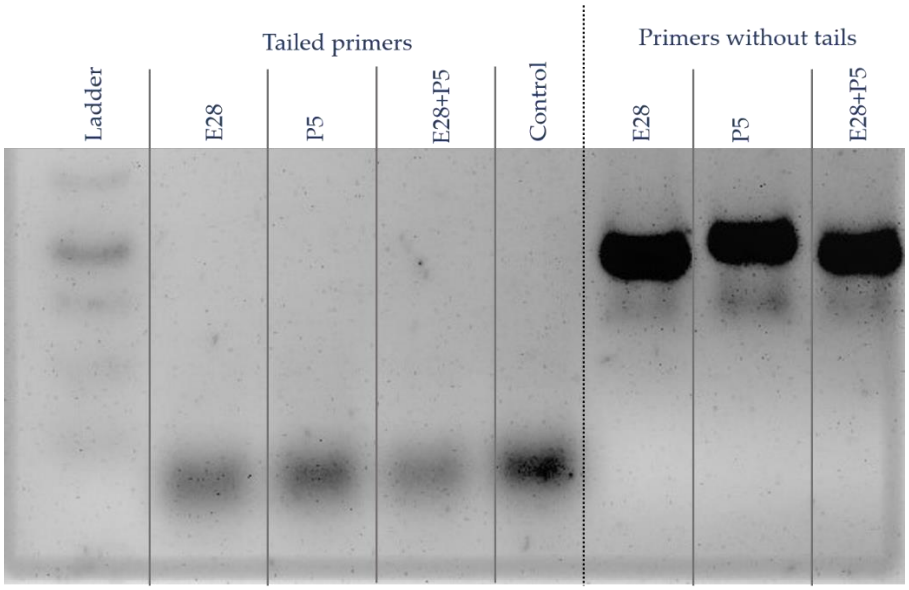

Figure S7.- Electrophoresis results of PCR simultaneous amplification using tailed and non-tailed primers.

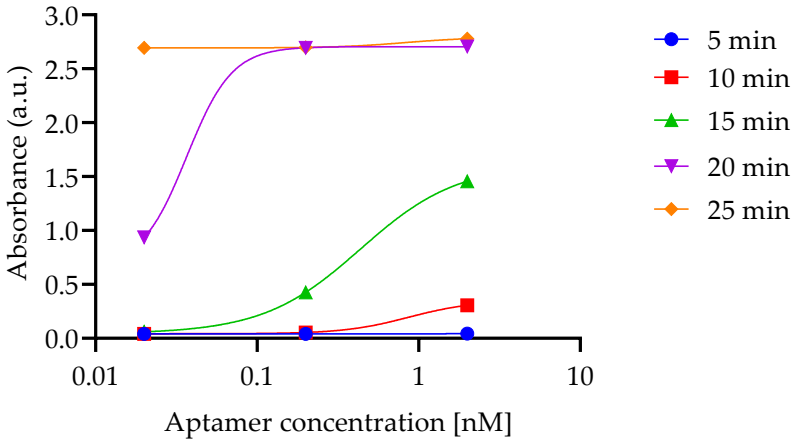

Figure S8.- ELONA results of different RPA optimization times by evaluating different amplification times.

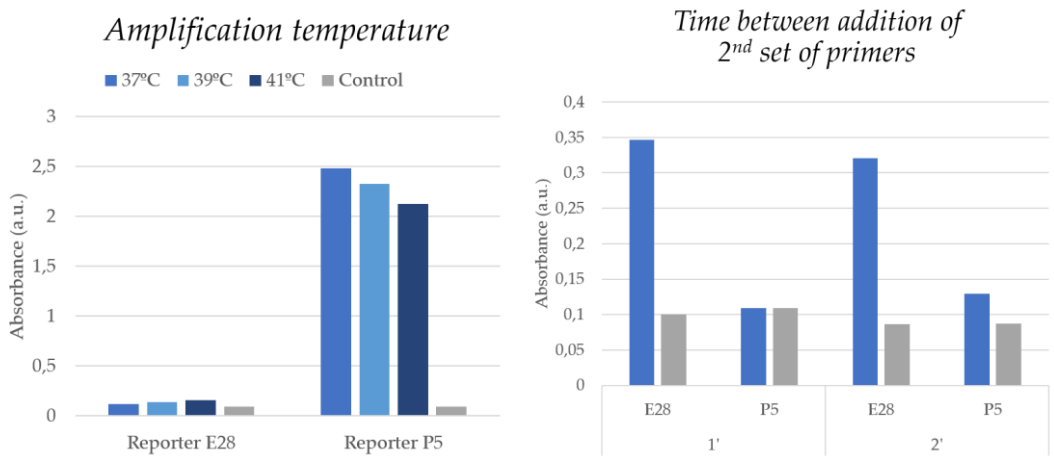

Figure S9.- ELONA results of RPA optimization of sequential experiment by changing the amplification temperature and the time between addition of the second set of primers.