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Fluorescence Site-encoded DNA Addressable Hapten-Microarray for Anabolic Androgenic Steroids

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ABSTRACT

A new strategy for the immunochemical screening of small organic molecules is reported based on the use of *hapten-microarrays*. Using DNA- directed immobilization strategies we have been able to convert a DNA chip into a *hapten-microarray* taking advantage of all the benefits of the structural and electrostatic homogeneous properties fo the DNA in comparison to proteins. Moreover, the hapten-microarray uses hapten-oligonucleotide probes instead of protein, avoiding the limitations derived from preparing stechiometrically defined protein-oligonucleotide bioconjugates. As proof-of concept, we show here the development of a microarray for anabolic androgenic steroid (AAS) analysis. The microchip is able to detect several illegal substances with sufficient detectability to be use as screening method according to the World Antidoping Agency (WADA) and the European Commision (EC) regulations in the sport and food safety fields, respectively. The results shown here corroborates the universal possibilities of the DNA-chip, in this case opening the possibility to develop *hapten-microarrays* for small organic molecule immunochemical determinations.

INTRODUCTION

Microarrays are defined as bidimensional molecular receptor arrays that allow simultaneous automation of assays, aimed to know specific recognition of biological markers. The use of this technology permits simultaneous detection of a large number of substances being ideal for high-throughput analysis. Multiplexation, miniaturization and detectability are goals pursued by many researchers. Improvement in the analytical efficiency and the consequent reduction of the necessary time and cost of the analysis are some of the advantages of multiplexing. Technological advances in micro (nano)biotechnology have provided the possibility to develop multiplexing bioassays through two strategies: a) achieving spatial multiplexing (planar microarrays) or b) using multiple quantitation tags (non-planar microarrays). The first case, in which the identity of the target analyte is encoded by its location with a secondary reporter providing quantitative data (i.e., fluorescent dye), is the most widely used technology. Relevant growing areas such as proteomics (large-scale study of proteins, particularly their structures and functions), genomics (study of the genome of an organism, investigation of single genes, their functions and roles) and pharmacogenomics (study of expression in individual genes relevant to disease susceptibility as well as drug response at cellular, tissue, individual or population levels) are possible thanks to the availability of protein and DNA microarrays based on this principle (more information can be found in recent reviews and books dealing with this topic [1; 2; 3; 4]. Thus, the technology behind the well-known Affymetrix' GeneChip®, started in the late 1980's as result of the advances in the semiconductor manufacturing techniques and the combinatorial chemistry [5]. Today these arrays are considered standard tools for analyzing complex genetic information. Main users are companies working in fields such as pharmaceutical, biotechnology, agrochemicals and diagnostics, in addition to academic, government and other non-profit research institutes to analyze the relationship between genes and human health.

Besides genomics, *protein microarrays* represent a big challenge in diagnostics. For example, microarrays can be very useful for drug development, allowing the analysis of the interactions of chemicals or important pharmacological targets with proteins. It is suggested that protein arrays may have a strong impact on interaction screening assays as the mode of action of therapeutically interesting drugs. However, protein microarray technology is not as straightforward as DNA technology due to the molecular variability and complex nature of proteins (different hydrophobicities, acidic or basic characters, functionality, etc.). Unlike

nucleic acids, which are relatively homogeneous in terms of structural and electrostatic properties, proteins can be extremely diverse regarding chemical structure and biological properties. Preventing protein denaturation and maintaining structural conformations are key issues in microarray technology (see reference [6] for a recent review on immobilization strategies). This is the reason because DNA microarrays are much more standardized.

An alternative to circumvent some of the limitations of the protein microarray technology consists on the use of oligonucleotide probes with well-known sequences and their subsequent hybridization with their complementary oligonucleotides previously immobilized on the surface. This strategy, known as DNA-Directed immobilization (DDI), has been used to spatially assemble mixtures of molecular components, such as nanoparticles, proteins and polypeptides [7; 8; 9; 10; 11; 12; 13]. It not only provides greater immobilization efficiency than conventional adsorption techniques [8], but also allows for reversible immobilization of biomolecules allowing development of reusable microarrays and biosensor chips. In combination with antibodies, DDI may also provide a useful strategy to construct *antibody microarrays*, expanding the number of substances that can be analyzed considering the wide variety of selectivities provided by the antibodies and their exceptional features as natural bioreceptors[14]. However, DDI strategies to immobilize proteins would ideally require a 1:1 oligonucleotide:protein molar ratio plus a careful control of the site in which the oligonucleotide is attached, which is very difficult to achieve by the usual chemical bioconjugation procedures. In this context, site-specific labeling strategies of recombinant proteins with DNA oligonucleotides and peptide nucleic acid (PNA) strands has been reported[6; 15; 16; 17; 18; 19].

In this context, this paper reports an alternative strategy to the DDI antibody-microarrays for small organic molecule determination. These targets are frequently analyzed under competitive immunochemical configurations on indirect formats, by immobilizing a haptenized protein on the surface of a microplate, a microchip or a transducer. Thus, using this format protein microarrays have been reported for the immunochemical analysis of steroids in urine samples [20; 21] or antibiotics [22; 23]. However these approaches also suffer from the above mentioned draw-backs and limitations of the protein-arrays. In contrast, *hapten-microarrays* could be excellent screening platforms for the immunochemical analyses of small organic molecules, such as metabolites, drugs, etc, avoiding the problems derived from the immobilization of the proteins without the need to construct site and stechiometrically defined DNA-protein conjugates.

To our knowledge, no records on the application of DDI approach to the multiplexed to construction *hapten-microarrays* have been reported. Thus, in an attempt to exploit the strengths of the DNA hybridization and the microarray technology, the aim of this work has been to develop a universal microarray platform for small organic molecule analysis, using DDI in combination with hapten-oligonucleotide conjugates and antibodies. Control on the stechiometry and conjugation site of these conjugates could be straight forward in comparison to the DNA-protein constructs. As proof of concept, we have focused on the development of a microarray for anabolic androgenic steroids (AAS) determination. These substances are illegally used to improve athletic performance in sports and to increase meat production in the agro-alimentary field, and are completely prohibited by the WADA (World AntiDoping Agency) [24] and the European Community through Directive 96/23/EC and Directive 2003/74/EC [25; 26]. For this reason both organizations have established regulations and requirements in order to control the use of these substances, which calls for screening methods able to meet the requirements and to improve the health status of the population and the fair competition on athletic events.

EXPERIMENTAL SECTION

Reagents and Immunoreagents. The immunoreagents for Stanozolol (St), Boldenone (B) and Tetrahydrogestrinone (THG) used in this study have described before [27; 28; 29]. Other immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). The "As cocktail" are a combination of the three antiserums (St: As147, diluted 4000 times; THG: As170 diluted 2000 times; B: As138 diluted 16000 times, all in PBST). Tetrahydrogestrinone was synthesized in our laboratory [28] and other analytes were purchased from Sequoia Research Products, Ltd. (Oxford, UK). Stocks solutions wee prepared 10mM concentration in DMSO. The synthesis of the oligonucleotides (*N1down, N2down and N3down)* and of the hapten-oligonucleotide conjugates (St: 8-*N1up* THG: hG-*N2up,* and B: 13-*N3up*)will be described elsewhere.The "H-OLG cocktail" is a mixture of the three conjugates at 0.01 μg/mL. Other chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Buffers: PBS is 0.01 M phosphate buffer in a 0.8% saline solution (137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl), and the pH is 7.5. PBST is PBS with 0.05% Tween 20. Printing buffer consist in 150mM Sodium Phosphate (pH 8.5)/0.01% Sodium Dodecyl Sulphate. Blocking solution is 2% BSA in

PBS. Hybridization buffer is 10 mM TRIS, 1mM EDTA, 1M NaCl (pH 7.2). The final washing buffer is with 0.1 x SSC (15 mM NaCl + 1.5 mM sodium citrate), 0.05% SDS (pH 7.5).

General Methods, Matherials and Instruments. The poly-L-lysine coated microscope slides are purchased from Polysciences, Inc. The pH and the conductivity of all buffers and solutions were measured with a 540 GLP pH meter and a LF 340, conductimeter (WTW, Weilheim, Germany), respectively. ***Microarray Printing*****.** Oligonucleotide chains ($N_{1\text{-}3}$down, 200 µg/mL in printing buffer) were spotted onto poly-L-lysine substrates using a BioOdissey Calligrapher MiniArrayer (Bio-Rad Laboratories, inc. USA) provided of a high humidity chamber, maintained for 30 minutes at room temperature and finally dried in an oven for 30 minutes at 60°C. Each glass slide contains 24 wells. A 5x3 spot matrix was printed on each well with five spots replicates for each oligonucleotide *Nxdown*. **Scanner:** Measurements were recorded on a ScanArray® Gx PLUS (Perkin Elmer, USA) with a Cy3 optical filter with 5 µm resolution. The laser power and PMT were set to 90% and 70%, respectively. The spots were measured by F543_Mean-B543 (Mean Cy3 foreground intensity minus mean Cy3 background intensity). Fluorescence intensity values are expressed normalized or in relative units as average and standard deviation of three replicate wells. The competitive curves were analyzed with a four parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism v 4 (GraphPad Software Inc., San Diego, CA). The standard curves were fitted to a four parameter equation according to the following formula: $Y = [(A - B)/1 - (x/C)^D] + B$, where *A* is the maximal fluorescence, *B* is the minimum fluorescence, *C* is the concentration producing 50% of the difference between A and B (or IC_{50}), and *D* is the slope at the inflection point of the sigmoid curve. The limit of detection (LOD) is defined as the concentration producing 90% of the maximum fluorescence (IC_{90}).

Preparation of the Hapten-Microarray. The slides were placed on a microplate microarray ArrayIt® hardware system allowing 96-well formatted experimentation with up to four glass substrate slides (Telechem International Inc.). The system is provided of a silicon gasket that demarcates 24 wells for slide. Before starting the assay the slides were blocked (100 μL/well blocking solution) for 30 min and washed four times with PBST. Once ready, the H-OLG cocktail was added (0.01 µg/mL in the hybridization buffer, 100 μL/well in all cases) and after 30 min of incubation at RT, the slide were washed and dried. In this way, the chip was ready for use.

Multianalyte Immunoassay on the Microchips. Standard (0.1 nM-1 µM (St), 0.1 nM-1 µM (THG) and 0.5 nM-5 µM (B), all in PBST) or the samples were added to the wells (50 μL/well in PBST) followed by the "AS Cocktail" (50 µL/well, PBST) and incubated for 30 min at RT. The slides were washed and the anti-IgG-TRITC solution (1/250 in PBST, 100 μL/well) was added. After incubation step of 30 min at RT the slide is washed with the final washing buffer, dried with N_2 and read with the scanner.

Semi-quantitative Study. Blind samples were prepared in PBST and measured using the multianalyte microarray protocol described above. Negative and positive control solutions with a concentration of the AAS at the MRPL value (according to WADA 10 µ L^{-1}: 30,5 nM for St, 32 nM for THG and 35 nM for B) were also measured on the same slide. In order to obtain response within the linear range of St and THG, the samples and also the St CTR-MRPL and the THG CTR-MRPL were diluted (6 times in the St assay and 10 times in the THG assay) in PBST. Analyses were performed in triplicate.

Specificity of the chip. The response of other AAS and related steroids MRPLs concentration was also evaluated in triplicates and measured with the microarray.

Decision limit (CCα) and detection capability (CCß). Blank samples (25 PBST solutions) were analyzed for 3 days to calculate the decision limit and the signal to noise ratio at the window in which the lower concentration of the analyte is expected. The CCα was calculated by subtracting 2.33 times the corresponding standard deviation to the average signal obtained when measuring the blank samples (α = 1 %) following by interpolation of this value in the calibration curve of each staroid. CCß was determined, in the same way, using the equation CCß = CCα (signal) - 1.64 × SD, being SD the standard deviation obtained for above blanks.

RESULTS AND DISCUSSION

We present here the development of a universal bioanalytic platform for immunochemical analysis of different substances, particularly small organic molecules. The approach uses the advantages of the DNA microarrays combined with the excellent features of the antibodies as bioreceptors. *Antibody microarrays* have the potential to revolutionize protein diagnostics[9; 14; 30]. The major problems in the fabrication of antibody arrays, however, concern the reproducibility and homogeneity of the attachment of the proteins on the solid substrate.

DNA-directed immobilization (DDI) strategies allow to convert an array of DNA oligomers into an *antibody microarray*[14], but a vey précis control of the stechiometry and the site of conjugation of the single stranded DNA to the protein is required[17]. In this work we have applied the DDI strategy to create a *hapten-microarray* through the use of well-defined hapten-oligonucleotide conjugates. This type of microarrays could allow determination of small organic molecules under competitive conditions, using the well-know indirect immunoassay format. As proof of concept we have intended to develop a microarray platform for androgenic anabolic steroids (AAS) determination, substances illegally used to improve athletic performance and also to increase meat production in the food field. These substances are completely prohibited by the WADA [24; 31] and the European Community [25; 26].

To reach this objective the microarray chip has been prepared with oligonucleotide chains of different sequences (*N1down, N2down* and *N3down*, see figure 1) immobilized on the microarray substrate (microarray-chip) using poly-lysine or glycidoxy propyl sylane (GOPS) derivatized glass slides demarcated with 24 wells, by spotting a 5 x 3 matrix of the three oligonucleotides on each well (*N1down, N2down* and *N3down*, five replicates/ each per well, see figure 2). The slides were in this manner used for the preparation of the AAS *hapten-microarray*, by hybridization of the oligonucleotide chains with their complementary sequence using a cocktail of hapten-oligonucleotide conjugates (H-OLG cocktail). In this case, the hapten-oligonucleotide conjugates (8-*N1up*, hG-*N2up* and 13-*N3up,* see figure 1), contained the complementary sequence of the immobilized oligonucleotides, covalently attached to the St (stanozolol), THG (tetrahydrogestrinone) and B (boldenone) haptens, respectively. Hybridzation on the surface of the DNA-chip affords a *hapten-microarrray* in which each hapten has been immobilized on an organized manner, through hybridization of the matching single stranded DNA chains.

Following, the hapten-microarray can be used for measurements by following the steps shown in the figure 2. First, a mixture of the sample (or standards) and a cocktail with the antibodies (AS cocktail) is added, and after 30 min incubation, visualization of the antibody-hapten-oligonucleotide interaction was accomplished using Anti-IgG-TRITC.

Selectivity of the Hybridization Step was assessed by using microarrays prepared as described above and incubating separately each chip with one of the oligonucleotide-hapten conjugates (8-*N1up*, hG-*N2up* and 13-*N3up*). After a washing step, the “AS cocktail” was added, followed by the anti-IgG-TRITC . As it can be observed in figure 3 fluorescent signals were only observed

on those spots in which the complementari oligonucleotide was immobilized, demonstrating the high specificity of this step. Moreover, specific antibody binding only occurred on those spots in which hybridization had taken place.

Selectivity of the Antibody Binding Step has been demonstrated before on ELISA formats in which haptenized proteins were inmobilized on the micropaltes [28; 29]. However, to ensure the specificity of the signal for the hapten-oligonucleotide conjugates, each mciroarray chip was incubated with the H-OLG coktail and in the next step, the antibodies were added separately on each microchip, followed by the anti-IgG-TRITC. In this case, the greater signals were observed on those spots that contained the matching hapten-oligonucleotide conjugate. However, surprinsingly shared-recognition was observed between St and THG . Thus, a marked recognition of the the **8-***N1up* (Stanozolol) by As170 (specific for Tetrahydrogestrinone) was observed and viceversa, although the recognition of As147 (specific for Stanozolol) for **hG-***N2up* (Tetrahydrogestrinone) was lower (see figure 4). Although this shared-recognition effect could be a drawback for the method we decided to go forward in order to evaluate recognition of the free analytes in the microarray.

Analysis of Stanozolol, Tetrahydrogestrinone and Boldenone with the Microarray. The hapten-microarray was exposed to solutions containing the analytes mixed the As cocktail, incubated for 30 min, and revealed with antiIgG- TRITC. As it can be observed in figure 5, the shared recognition was not observed when using the As cocktail, instead of indidual antibodies This could be explained by the greater affinity of each antibody for its counter hapten-oligonucleotide in the microarray. In the presence of the corresponding antibody, binding of other antibodies with lower affinity is prevented. Moreover, the presence of the analyte inhibited the binding of the corresponding antibody to the hapten-oligonucleotide on a specific manner, although a slight inhibition in the THG assay was observed in presence of St and *viceversa*, probably due to the high concentrations assayed in these initial experiments.

Standards at different concentrations of the three AAS were measured using the above mentioned protocol affording calibration curves such as those shown in figure 6. As it can be observed, the detectability accomplished is in compliance with the WADA and EC requirements regarding the MPRL proposed by those agencies (see microchip features in the table). Thus, the experiments performed demonstrate that St, THG and B can be detected in buffer with a limits of detection of 0.32, 0.14 and 0.48 μg L^{-1}, respectively, when the MRPL values proposed by WADA are 10 μ L^{-1}

Preliminary Validation Study. In order to assess *microarray precision,* inter- and intra-slide variability was evaluted measuring samples spiked at zero concentration (15 blank samples) and at the minimum required performance limit (MRPL, 45 samples, 15 for each steroid). Analyses were performed using 5 spots/chip in 3 chips/slide in 3 different microarray slides for each hormone. Results obtained are shown in table 1. The intra-slide variability refers to the variability between chips in one same microarray slide aand the inter-slide variability it refers to the variability between different microarray slides. As it is shwon, the coefficient of variations (% CV) obtained were quite low considreing the microarray is addresse to perform screening.

The *specificity* of the microarray was evaluated by measuring mixtures of different AAS, doping substances and dietary supplements (see figure 7). The results shown that mixtures of the analytes do not affect the recognition profile of the individual assays. Moreover, other illegal substances, in addition of St, THG and B, could also be detected with this microarray due to the immunoassay cross-reactity, which expands de potential of the actual microchip configuration for screening purposes. Taking into account that the use of these substances is completely prohibilited, any unknown sample with a signal below the CTR-zero could be a non-compliant sample that would need to be confirmed with a reference analytical method

With the aim to perform a preliminary evaluation the microchip, blind spikded samples of the three steroids at different concentrations (from 100 to 15 nM for each steroid in buffer), were analyzed with the hapten-microarray and the response compared with that provided by the controls at zero (CTR-zero) and at the MRPL (CTR-MRPL) concentration of each steroid, according to WADA. The objective of this experiment was to proof if the microarray could respond to the WADA requirements by detecting all samples with vaules over the MRPL. Results shown in figure 8 demonstrate that the *hapten-microarray* developed in this study can clearly detect samples that contain AAS even below the MRPL. It must be noticed that the MRPL values is an indication of the detectability that the method should accomplished, but since the use of AAS is completely prohibited, any sample giving a positive answer, even if the level is below the MRPL, could be subjet of the necessary actions if confirmed by reference method.

Finally, a series of experiments were performed following to the criteria of the EC Commission Decision 2002/657/EC regarding qualitative screening methods in order to determine the

decision limit (CCα) and the detection capability (CCß) of the AAS *hapten-microarray.* Following the procedure described in the experimental section, the CCα values obtained were 1.01 µg L^{-1} for St, 0.52 µg L^{-1} for THG and 4.89 µg L^{-1} for B. (see figure 9). Similarly, the detection capability CCß was found to be 1.79 µg L^{-1}, 2.71 µg L^{-1} and 21.98 µg L^{-1} for St, THG and B, respectively. Thus, while the CCα and CCß values obtained for the St and THG were in compliance with the WADA and EC requirements, the B values did not, which was unexpected considering the LOD values calculated from the calibration curves. Moreover, as can see in figure 8, the microarray can, in our hands distinguish very well the samples that contain B, which does not match with the result obtained using the procedure suggested by the EC. This disagreement could be related to the competitive nature of this type of immunochemical methods, for which perhaps other calculation procedures should have been used.

CONCLUSIONS

Immunochemical methods show great potential for diagnostics and screening purposes in many fields. Combining with the microarray tecnnology it may extraordinarily increase the analytical capabilities of those methods. Major concerns are around the homogeneous and reproducible immobilization of the proteins to the surfaces of the microarrays which may compromisie their functionality. To circumvent this limitations DNA-directed immobilization methods have open the possibility to develop universal platforms for the analysis of a great varitey of substances (proteins, peptides, cells) using the microarray technology. The results presented here are in support of the universality of this approach. Thus, a hapten-microarray has been developed by immobilizing hapten-oligonucleotide conjugates in which the single stranded DNA sequence is complementary to the oligonucleotide immobilized on the chip. This approach, opens the door to use this universal platform for the analysis of small organic molecules by microarray. Moreover, at difference of the protein-DNA probes used to build protein microarrays, hapten-oligonucleotide conjugates can be easily prepared with very well defined structure and stechiometry.

As a proof-of concept, we have developed a hapten-microarray platform for the detection of AAS using a site-encoded configuration where the identity of the target analyte is encoded by its location in the detection platform. The assay can be run in about 3 hours and provides a specific response on a different microarray section depending on the target analyte. This platform is able to detect and identify these hormones in blind samples, and allows the

discrimination between compliant, non-compliant or blank samples. The strategy presented here is universal and it could be used to detect other target analytes of interest in the biomedical, food and environmental fields.

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FIGURE LEGENDS

Figure 1: Chemical structures and sequences of the oligonucleotides immobilized on the microchip and the hapten-oligonucleotide conjugates, which complementary sequence, used to build the hapten-microarray.

Figure 2: Scheme of the preparation and use of the hapten-microarray

Figure 3: Results from the specificity of the hybridization studies. A) To each well, only one hapten oligonucleotide was added (a different on each microchip) followed by the AS cocktail B) Situation on each chip after a washing step followed by the addition of the antiIgG-TRITC. C) Images obatined with the scanner. D) Bar graph showing the quantified fluorescent signal of each spot. Bars show the average and standard deviation of signals recorded from 15 spots and 3 chips.

Figure 4: Results from the specificity studies of the antibodies towards the hapten-oligonucleotide conjugates. A) Hybridization of the chip with the H-OLG cocktail followed by the addition of the antibodies individually (a different antibody to each microchip). B) Situation on each chip after a washing step followed by the addition of the antiIgG-TRITC. C) Images obatined with the scanner. D) Bar graph showing the quantified fluorescent signal of each spot. Bars show the average and standard deviation of signals recorded from 15 spots and 3 chips.

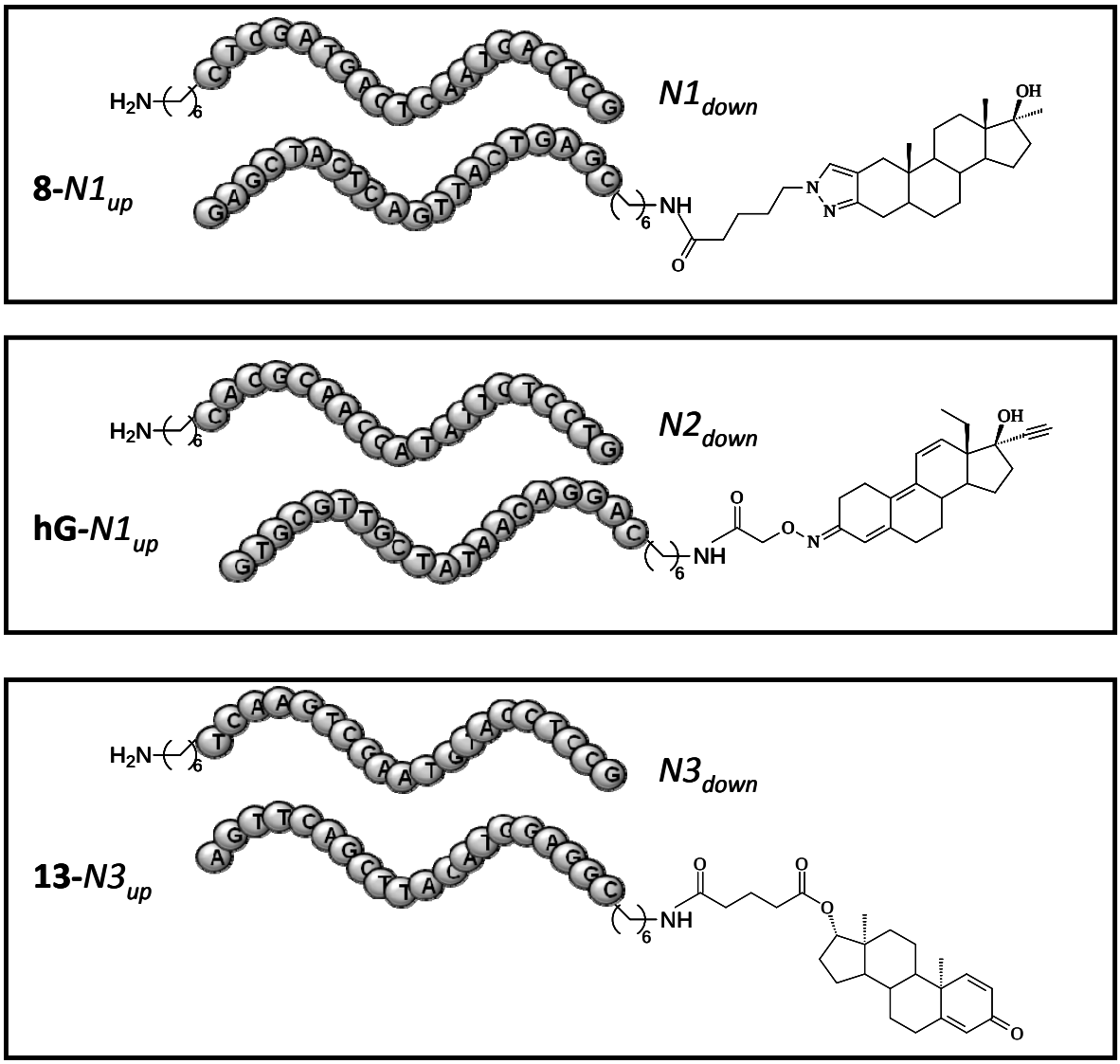
Figure 5: Fluorescent signal recorded from the chips when a) no analyte, b) 1 µM Stanozolol, c) 1 µM Tetrahydrogestrinone and c) 5 µM Boldenone. Bars show the average and standard deviation of signals recorded from 15 spots and 3 chips.

Figure 6: A) Graph showing the standard curves obtained with the microarray. Black line (Stanozolol), grey line (Tetrahydrogestrinone) and dotted line (Boldenone). B) List of parameters for the individual steroids using the hapten microarray. The values shown are the average and standard deviation of signals recorded from 15 spots and 3 chips. C) Image of the hapten microarray.

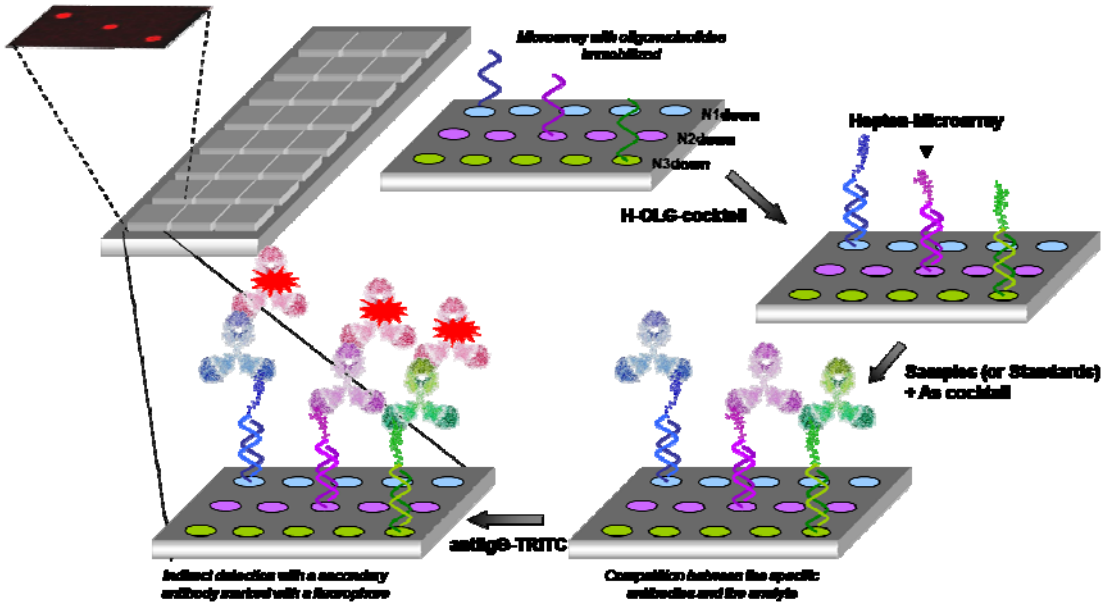
Figure 7: Results obtained from the measurement of the specificity of the microarray. Each analyte is at their MRPL value (10 µg/L) in buffer. The values showed in the graphs are obtained in A) St assay, B) THG assay and C) B assay.

Figure8: Results from the measurement of blind samples with the hapten microarray. Fluorescent values below the MRPL, indicate that the concentration is above this value and viceversa. Bars show the average and standard deviation of signals recorded from 15 spots and 3 chips.

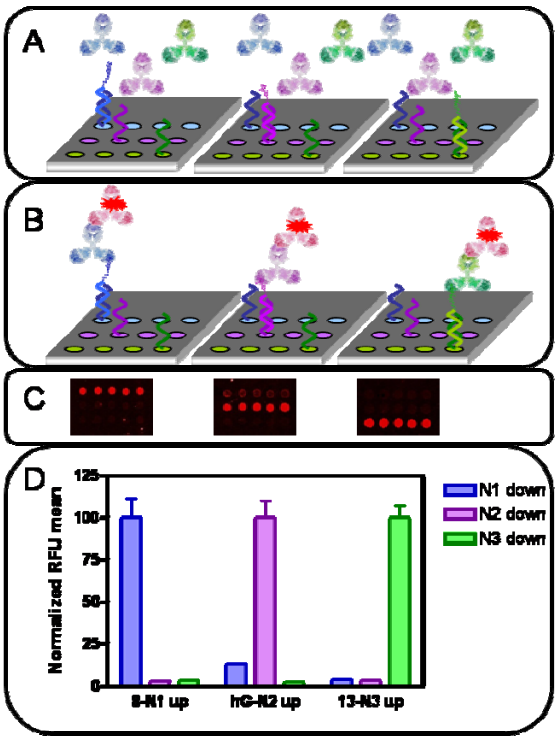
Figure 9: Results from the measurement of 20 negative samples on different chips during three days. The signal obtained at zero concentration and the standard deviation recorded was used to determine the decision limit (CCα) and detection capability (CCß) for each steroid when using the hapten microarray. Se text for more information on how these paramenters were calculated and the results obtained. Each point is the average and standard deviation of signals recorded from 5 spots and 3 chips.



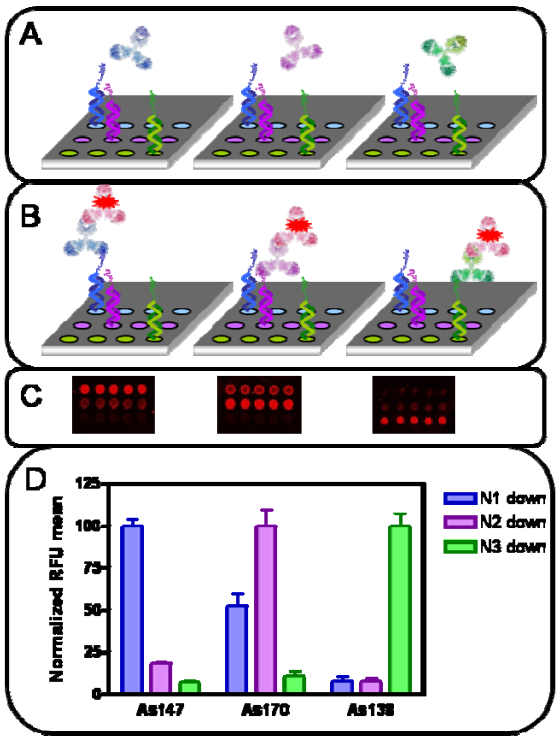
Tort et al. Figure 1



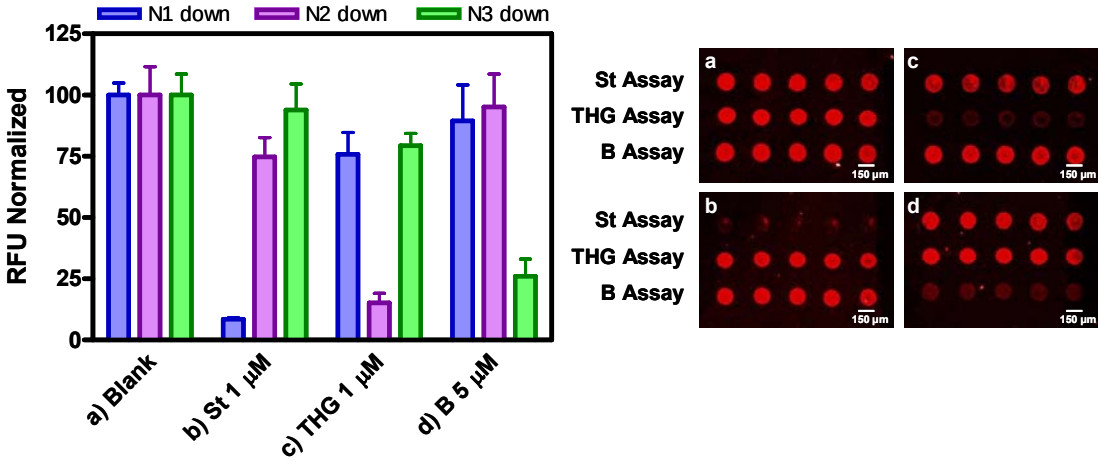
Tort et al. Figure 2



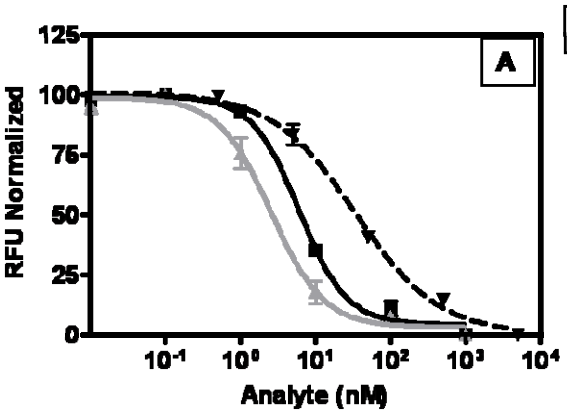
Tort et al. Figure 3



Tort et al. Figure 4

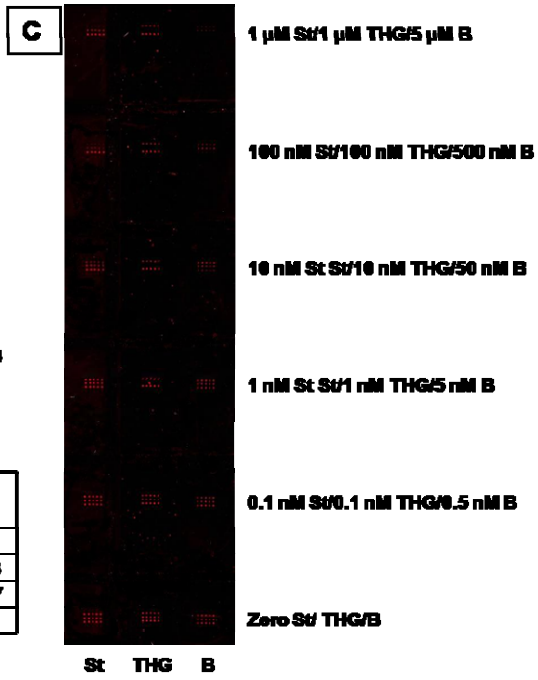


Tort et al. Figure 5

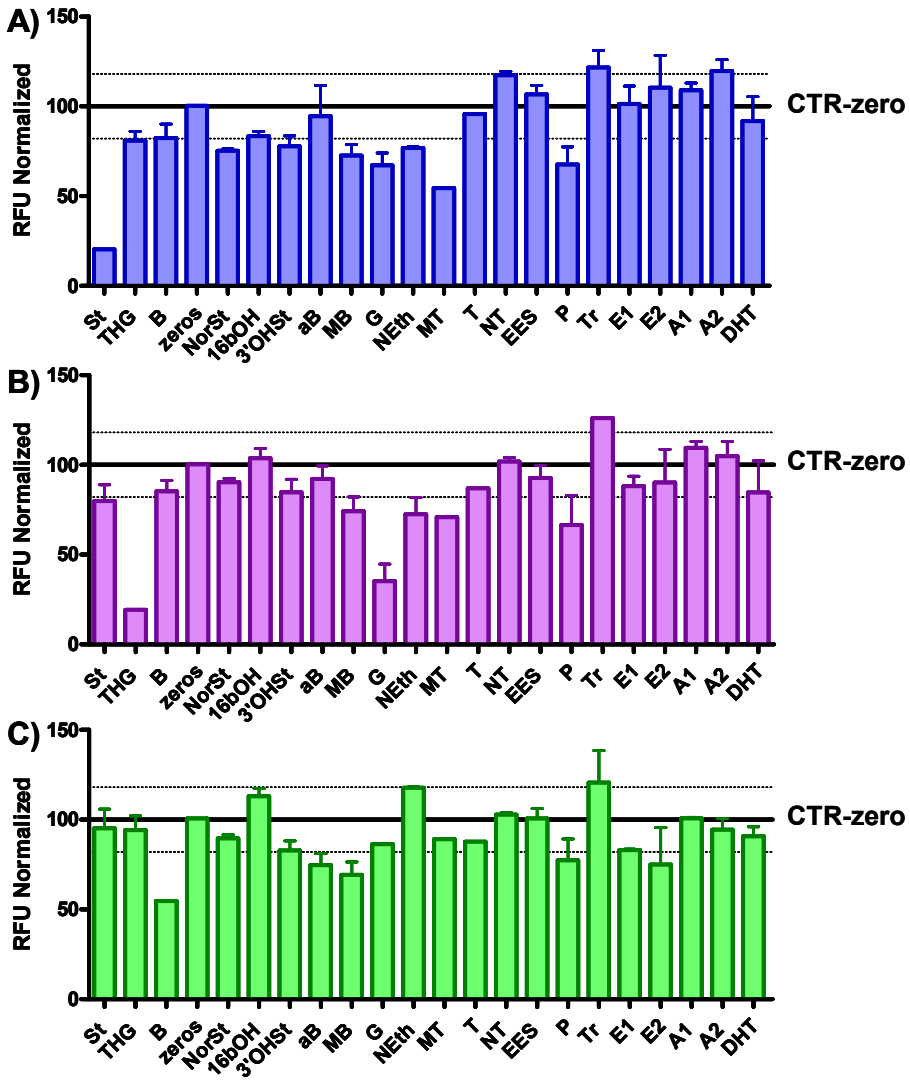


B

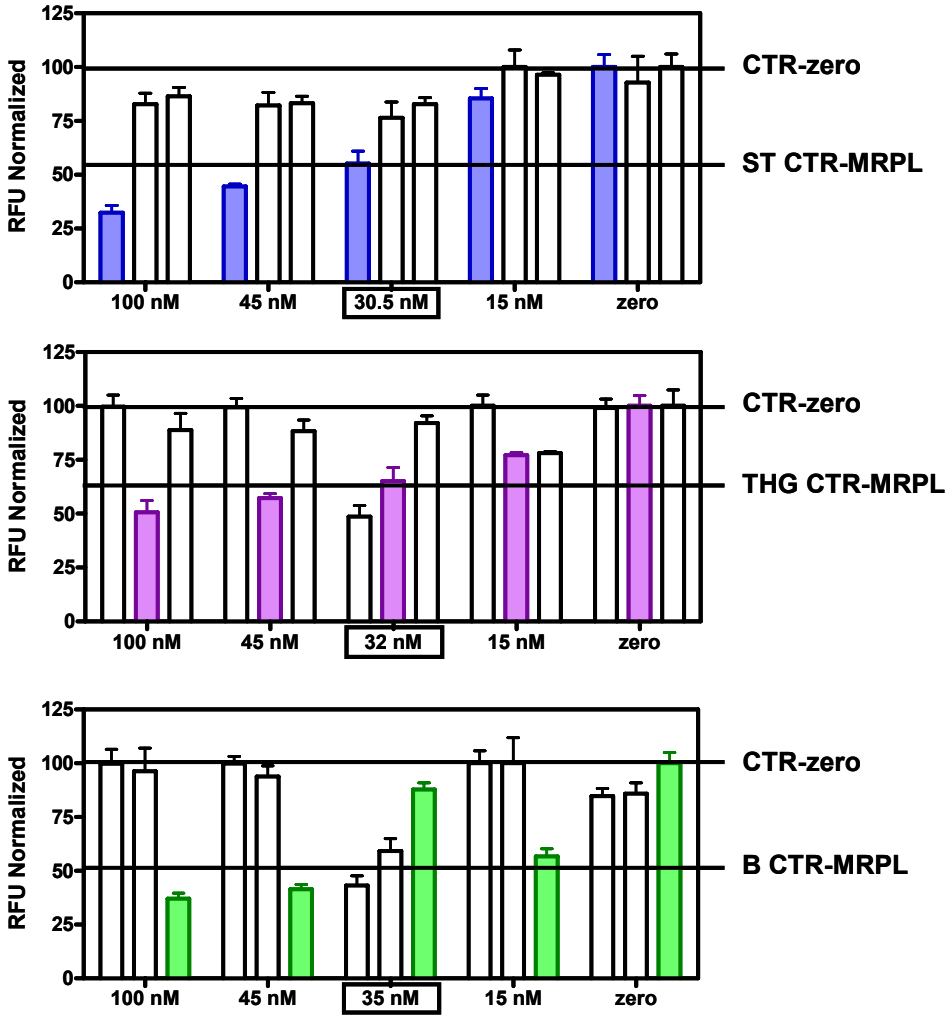
Parameter	St	THG	B
IC_{50} (µg/L)	1,72 ± 0,51	1.14 ± 0.48	7.37 ± 4.21
LOD (µg/L)	0.32 ± 0.19	0.14 ± 0.07	0.48 ± 0.38
Slope	-1.35 ± 0.17	-1.28 ± 0.30	-0.77 ± 0.07
R^2	0.98 ± 0.01	0.97 ± 0.02	0.99 ± 0.01



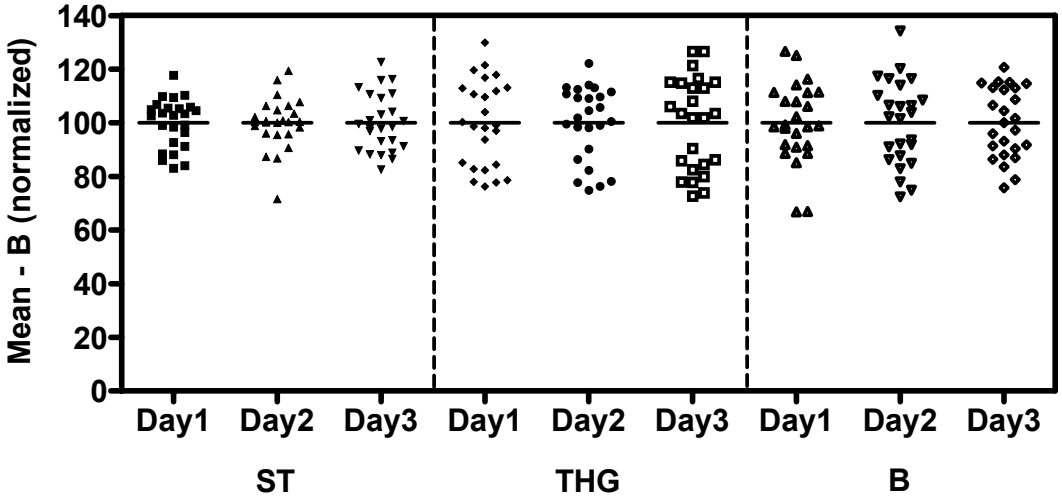
Tort et al. Figure 6



Tort et al. Figure 7



Tort et al. Figure 8



Tort et al, figure 9

Table 1: Microarray precision results[a].

	%CV, Intra-slide		%CV, Inter-slide	
	MRPL	**Zero**	**MRPL**	**Zero**
St assay	**18**	**11**	**17**	**15**
THG assay	**30**	**18**	**15**	**18**
B assay	**14**	**10**	**8**	**11**

[a] The values are the variation coefficients expressed in % (SD/Mean*100).