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Simultaneous detection of three sex steroid hormone classes using a novel yeast-based biosensor†

Running title: Simultaneous detection of different sex hormones

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Abstract

A biosensor detecting estrogens, progestogens and androgens in complex samples and in a single step is described. Three *Arxula adeninivorans* yeast strains were created, each strain producing a different recombinant human hormone receptor and a different fluorescent reporter protein. These strains were then mixed to create G1212/YRC102-hHR-fluo, the biological component of the biosensor. During incubation with G1212/YRC102-hHR-fluo, hormones present in a sample bind to their target receptor, which leads to the production of a specific fluorescent protein. Three fluorescence scans of the yeast suspension determine which fluorescence protein has been produced, thus revealing which hormone receptor (estrogen, progesterone and androgen) has been activated by the hormones or hormone mimics present in the sample. The biosensor has similar sensitivities to the existing *A. adeninivorans* cell-based assays. The detection of the three hormone classes in one single experiment reduces the labour and time required to assay for the three hormone classes. The biosensor was also trialed with animal serum samples for the detection of progestogens; androgens and estrogens and gave results that correlated well with ELISA analysis in case of progestogens. These results highlight the potential usefulness of the biosensor for comprehensive determination of hormone status in samples from veterinary origin.

Keywords: hormone assay, *Arxula adeninivorans*, fluorescence, marmoset serum

Introduction

In vertebrates, the three sex steroid hormone classes, estrogens, androgens and progestogens, play an essential role in sex differentiation and reproduction by binding to the estrogen, androgen or progesterone receptors respectively (Leblebicioglu et al. 2013). However, other synthetic or natural compounds called endocrine disruptors (EDC's) can interact with these receptors and they are suspected to present physiological risks for humans and aquatic organisms (Casals-Casas and Desvergne 2011; Fent 2015; Kabir et al. 2015). The identification of EDC's in a large variety of environmental samples (Bain et al. 2014; Viglino et al. 2011), foods (Plotan et al. 2013; Seo et al. 2005), medical samples (Vitku et al. 2015) and cosmetics (Kunz et al. 2006) has been facilitated by an increasing number of biosensors dedicated to the detection of these molecules. As a result the production and marketing of several molecules have been regulated due to their endocrine disrupting properties, for example, nonylphenol and nonylphenol ethoxylates in the European Union (Soares et al. 2008). The development of cell-based biosensors has been particularly useful as these devices can determine the total biological effect of a molecule or mixture of molecules, while analytical detection methods such as GC-MS or LC-MS can only quantify known molecules and do not give an indication of biological effect. Because cell-based biosensors take into account the concentration and the affinity of each EDC for the hormonal receptor, they determine the total hormonal equivalent concentration, which is more useful in assessing the potential risk of a sample than the addition of concentrations of a limited number of molecules. Most of these biosensors use yeast as the biological host for recombinantly produced vertebrate hormone receptors and a thorough description of the newly developed biosensors can be found in the review by Adeniran et al. (2014).

Cell-based biosensors can be used to assay several types of sample from wastewater treatment plant influent and effluent (Balsinger et al. 2010; Garcia-Reyero et al. 2001) to sera (Bellem et al. 2011) and personal care products (Nakama et al. 2007). These assays generally serve as preliminary screen for estrogenic, progestogenic or androgenic activity. Once a sample has demonstrated

hormonal activity with one of these tests, subsequent analyses can be performed to identify the molecules generating the effect. Thus to obtain a complete picture of a sample's sex steroid activity, the use of three different cell-based biosensors for each hormone group is necessary.

A. adeninivorans has already been demonstrated to be able to produce recombinant human hormone receptors and is the biological host of the commercially available biosensors, A-YES (*Arxula* Yeast Estrogen Screen), A-YAS (*Arxula* Yeast Androgen Screen) and A-YPS (*Arxula* Yeast Progesterone Screen) (Chamas et al. 2015; Gerlach et al. 2014; Kaiser et al. 2010). In these three assays, a similar strategy is used to transform the receptor-ligand binding into a measurable signal. Phytase K protein from *Klebsiella* sp. ARS1 (Sajidan et al. 2004) was used as reporter protein and transformed a substrate to give a chromogenic signal. These assays generally involve the use of two different plates, a deep-well plate for yeast incubation under vigorous shaking at 30 °C and a plastic microtiter plate for detection. As A-YES, A-YAS and A-YPS use the same reporter protein, a simultaneous incubation of a sample with the three bioassays is not possible. To overcome this limitation, the replacement of the enzyme reporter with fluorescent reporter proteins was attempted. The use of fluorescent proteins as reporters in yeast (Beck et al. 2005; Beck et al. 2008b) and in *A. adeninivorans* in particular (Chamas et al. 2015; Pham et al. 2016) has been demonstrated.

The use of different fluorescent reporter proteins would allow the simultaneous incubation of a sample with strains producing different receptor proteins to give the simultaneous detection of estrogen, progestogens and androgens. Thus three new yeast strains were created, each constitutively producing a different human hormone receptor, which control the production of different coloured fluorescent reporter proteins: CFP (Cyan Fluorescence Protein), GFP (Green Fluorescent Protein) and DsRed (*Discosoma* Red fluorescent protein). These strains were mixed and the resulting cell suspension served as the biological component of the biosensor. After incubation with a sample, three fluorescence scans of the cell suspension at the excitation/emission wavelengths for CFP, GFP and DsRed allows the determination of the activity of estrogens, androgens and progestogens. The main advantage of this biosensor is that performing a single

assay, rather than three separate assays, reduces the time and reagents required. With one incubation step, the complete sex hormone profile of a sample can be determined. The main challenge for this biosensor is effective differentiation between the fluorescence emitted by the three reporter proteins.

This biosensor will be particularly useful in veterinary medicine because sex hormone levels can give useful information regarding animal health and/or reproductive status (Queiroga et al. 2015; Wiltbank et al. 2014). For example, progesterone and 17β-estradiol concentrations in marmoset (*Callithrix jacchus*) serum was quantified by ELISA to monitor the ovarian cycle (Chambers and Hearn 1979; Harlow et al. 1983). Standard detection methods involves the use of analytical detection methods, radioassays or ELISA directed against each hormone. Serum samples from marmosets were used to trial the biosensor in a biological context. The simultaneous determination of estrogens, progestogens and androgens in the serum with a single assay would present a significant advantage for monitoring the sex hormone status of an individual at a cost and analysis time that is much less than that for LC-MS or GC-MS analysis.

In this work, the construction of the biosensor by using existing *A. adeninivorans*–specific molecular cloning tools will be first described and then the biosensor will be tested with standard hormones. As this study is the first example of mixing different *A. adeninivorans* strains to detect different hormones classes, special attention will be given to the comparison between assay performance when the strains are incubated either separately or mixed with the standard hormones. Finally, this work will determine if marmoset serum samples can be effectively analysed with this biosensor and produce results comparable to analysis by ELISA.

Material and Methods

Chemicals

Progesterone (CAS 57-83-0), 17β-estradiol (CAS 50-28-2) and 5α-dihydrotestosterone (CAS 521-18-6) were all purchased from Sigma-Aldrich (Steinheim, Germany). All hormones were dissolved in ethanol at a stock concentration of 1g/l.

Strains

Escherichia coli XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F' proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]), obtained from Stratagene (La Jolla, USA), served as a host strain for bacterial transformations involved in the yeast plasmid construction. The cells were grown on LB media (Sigma-Aldrich, Saint Louis, USA) supplemented with ampicillin, 100 µg/ml (AppliChem GmbH, Darmstadt, Germany) or kanamycin, 50 µg/ml (Carl Roth GmbH, Karlsruhe, Germany) for selection.

The tryptophan-auxotrophic mutant *A. adeninivorans* G1212 [*aleu2 atrp1::ALEU2*], deposited in the strain collection of the Department of Biology of the University of Greifswald, was used as the host cell (Steinborn et al. 2007). Agar plates were prepared by the addition of 1.6 % (w/v) agar to liquid LB media (Rose et al. 1990; Tanaka 1967).

Modification of the DsRed protein

In two earlier publications (Chamas et al. 2015; Pham et al. 2016) the use of the fluorescent protein DsRed in *A. adeninivorans* was described. To accelerate the maturation of the DsRed protein, substitution of three amino acids (H41T, N42Q, and A145P) were introduced using a two-step polymerase chain reaction as described in the work of Bevis and Glick (2002). The mutated version of the gene was designated DsRed2.

Plasmid construction

The three vectors for yeast rDNA integration, Xplor2.2-hER-DsRed2, Xplor2.2-hPR-CFP and Xplor2.2-hAR-GFP, were constructed in *E. coli* using the same strategy used by Chamas et al. (2015) and Pham et al. (2016). The ORF (Open Reading Frame) of the three *human hormone*

receptor genes in an *A. adeninivorans* codon-optimized form (Life Technologies GmbH Darmstadt, Germany) was inserted between the constitutive *A. adeninivorans*-derived *TEF1* promoter and the *S. cerevisiae*-derived *PHO5* terminator of the pBS-TEF1-PHO5-EBN plasmid. The fluorescent reporter gene was placed under the control of the maltose-inducible *GAA* promoter (*Arxula*-derived glucoamylase promoter) as described by Hahn et al. (2006) containing either two copies of an Estrogen Response Element (2xERE) or two copies of a Hormone Response Element (2xHRE) to construct the three reporter modules: *2xERE-GAA*$^{-107}$ promoter – *DsRed2* gene - *PHO5* terminator, *2xHRE-GAA*$^{-107}$ promoter – *CFP* gene - *PHO5* terminator and *2xHRE-GAA*$^{-107}$ promoter – *GFP* gene - *PHO5* terminator. HRE is known to be the target site of both androgen and progesterone receptors (Hahn et al. 2006).

Each hormone receptor module and reporter module construct was then inserted into the plasmid Xplor2.2, generating the plasmids Xplor2.2-hER-DsRed2, Xplor2.2-hPR-CFP and Xplor2.2-hAR-GFP. All plasmids also contained a selection marker module (*ALEU2* promoter – *ATRP1m* gene – *ATRP1* terminator) for tryptophan-auxotrophic mutants and two 25S rDNA target sequences for preferential integration of the recombinant DNA, either as a single copy or as multiple copies into rDNA clusters of the *A. adeninivorans* genome by homologous recombination.

All three plasmids, Xplor2.2-hER-DsRed2, Xplor2.2-hPR-CFP and Xplor2.2-hAR-GFP, were constructed in *E. coli* and then digested with the restriction enzyme *Asc*I (Thermo Scientific, Rockford, USA) to remove the *E. coli* sequence. The resulting linear cassettes YRC102-hER-DsRed2, YRC102-hPR-CFP and YRC102-hAR-GFP were then transformed into the *A. adeninivorans* tryptophan-auxotrophic mutant strain G1212. Isolation of plasmid DNA and DNA restrictions were carried out as described previously (Wartmann et al. 2002).

Transformation procedures

E. coli and *A. adeninivorans* cells were transformed according to Böer et al. (2009). Stabilization of the yeast transformants was performed by passaging on selective and non-selective media (Klabunde et al. 2003).

Microtiter plate assays

For assays with single yeast strains, *A. adeninivorans* G1212/YRC102-hER-DsRed2, G1212/YRC102-hPR-CFP and G1212/YRC102-hAR-GFP were cultivated for 24 h in yeast minimal medium supplemented with glucose (YMM-glucose) at 30 °C until the OD_{620nm} reached approximately 1. The cells were pelleted by centrifugation at 4,000 x g for 10 min and the pellet was resuspended in an equal volume of fresh yeast minimal medium supplemented with maltose (YMM-maltose). 250 µl of this cell suspension and 2.5 µl of the hormone standard were added to each well of a 96 well polymer base black microtiter plate (Thermo Scientific Nunc, Rochester, USA). The plate was sealed with Labsolute® self-adhesive foil (Th. Geyer, Berlin, Germany) and incubated without shaking for 18 h at 37 °C. The fluorescence of the cell suspensions was measured with a Tecan Sunrise™ reader M200 pro (Tecan, Switzerland) with the following $\lambda_{excitation}$ / $\lambda_{emission}$ wavelengths: 445 nm /485 nm for G1212/YRC102-hPR-CFP, 485 nm /515 nm for G1212/YRC102-hAR-GFP and 542 nm /582 nm for G1212/YRC102-hER-DsRed2. Gain was set to "optimal" for all experiments. The total assay duration of all incubation steps was 42 h. All experiments were performed in triplicate.

To perform simultaneous hormone detection, the three yeast strains were cultivated for 24 h in separate flasks of YMM-glucose at 30 °C until the OD_{620nm} reached approximately 3. The cells were pelleted by centrifugation at 4,000 x g for 10 min, the pellet was resuspended in an equal volume of fresh YMM-maltose and the three cell suspensions were combined. This mix was named G1212/YRC102-hHR-fluo and served as the biocomponent of the steroid detection system. Fluorescent detection was performed as described in the previous paragraph.

Serum hormone extraction and detection

Serum samples (200 µl) were collected from 16 female marmosets (*Callithrix jacchus*) and either assayed immediately (ELISA) or stored frozen at -80°C for later analysis (Biosensor). The progesterone content was measured in 50 µl of fresh samples using an in-house standardized ELISA

method by the Institute of Physiological Chemistry, Leipzig, Germany. Progesterone determination was performed in duplicate.

Biosensor analysis used 25 µl aliquots of thawed samples. These were aliquoted into tubes and 150 µl diethylether was added to each tube. The tubes were vortexed for 2 min followed by 10 min centrifugation at 3,000 x g to obtain phase separation. The tubes were then immersed in liquid nitrogen for 2 sec to freeze the aqueous phase. The organic phase was immediately collected in a new glass tube. The aqueous phase was then thawed and twice extracted with 150 µl diethyether. All organic phases were collected into the same tube and the diethylether was allowed to evaporate at room temperature until dryness was achieved. The extracted samples were resuspended in 50 µl water:methanol (60:40) and 2.5 µl of this resuspended sample was used for the bioassay (total volume 250 µl). This correspond to a 1:200 dilution of the original sample. The hormone activity (estrogens, progestogens and androgens) was quantified by measuring the fluorescence of G1212/YRC102-hHR-fluo cells. The experiments were performed in triplicate with three measurements per plate. A calibration curve using 7 mixtures of the three hormone standards plus a blank was established for each plate. The final concentrations of 17β-estradiol, progesterone and 5α -dihydrotestosterone used for the calibrations were in the range of 16.4-150 ng/l, 20.2-400 ng/l and 54.6-500 ng/l respectively, thus covering the working range of the assay.

Calculation of the hormone equivalent concentration and statistical analysis

To model the relationship between fluorescence and concentration of the hormone or hormone-like compound in the sample, the following four-parameter model, which is equivalent to the Hill–Slope model, was used:

$$f(x_i) = y_{ij} = \frac{A - D}{1 + (\frac{x_i}{C})^B} + D + \varepsilon_{ij}$$

Here, y_{ij} denotes the fluorescence values corresponding to the jth measurement at concentration level x_i (concentration of the standard hormone/sample), and ε_{ij} describes the random error with mean zero and standard deviation σ. The four parameters A, B, C and D indicate the unknown

model parameters, which have to be estimated. A represents the mean value of the corrected extinction effects y_{ij} without hormone effects (at spike level 0, bottom curve point), D is the mean value of y_{ij} with maximum hormone effect (curve plateau), C describes the mean effect concentration (curve point of inflection – EC_{50}) at which the hormone effect attains half of its maximum value (A + D)/2, and the parameter B is proportional to the slope at C. In addition, the limit of detection (LoD) and the limit of quantitation (LoQ) were calculated. The LoD is the hormone equivalent concentration from which it can be concluded (at a significance level of α = 0.05), that the sample is not free from hormone activity. The LoD describes the lowest analyte concentration likely to be reliably distinguished from zero. The LoQ corresponds to the lowest concentration of an analyte in a sample that can be detected and quantified with a predefined imprecision (usually 33%). Calculation of these parameters and the statistical analysis was carried out using BioVal® software (QuoData GmbH, Dresden, Germany). All curves were plotted using Sigmaplot version 11.0 (Systat Software Inc., San Jose, USA).

Results

Cloning of G1212/hPR-CFP, G1212/hER-DsRed2 and G1212/hAR-GFP

Construction of yeast strains was based on the Xplor®2 transformation/expression platform as described in previous works (Chamas et al. 2015; Pham et al. 2016). Constitutive expression of each receptor gene was under the control of a *TEF1* promoter and a *PHO5* terminator. The *ATRP1m* selection module assured the selection of positive transformants. The reporter gene module was different for each strain but in each case contained a modified *GAA* promoter possessing two copies of a hormone response element upstream on the reporter gene. A negative control comprising the *A. adeninivorans* mutant G1212 transformed with YRC102 without a hormone receptor ORF was also constructed. A description of all the yeast rDNA integration cassettes (YRC) is shown in Fig. 1.

For each yeast transformation, 96 colonies were selected and stabilized to ensure long-term integration of the cDNA into the *A. adeninivorans* genome. The resulting colonies were tested for fluorescence with their target hormone. A diagram showing how hormone detection can lead to cell culture fluorescence is shown in Fig.2. The transformants showing the highest fluorescence intensity in the presence of their target hormone were used for all further experiments. These strains were designated G1212/YRC102-hER-DsRed2, G1212/YRC102-hPR-CFP and G1212/YRC102-hAR-GFP.

Microtiter plate assay with separate yeast strains

The binding parameters for each receptor were determined by incubating G1212/YRC102-hER-DsRed2, G1212/YRC102-hPR-CFP and G1212/YRC102-hAR-GFP with increasing concentrations of their target hormones, 17β-estradiol, progesterone and 5α-dihydrotestosterone on three separate plates. The fluorescence produced by the reporter protein was then measured for each plate and the values obtained were plotted (Fig. 3). In all three cases, data points follow a four parameter logistic curve from which the EC_{50} can be determined. These values can be found in Table I together with the values obtained in previous work with A-YES, A-YAS and A-YPS assays. For all three strains the EC_{50} values from fluorescence-mediated detection were in the same order of magnitude as the values obtained using the biochemical detection method.

Microtiter plate assay with mixed yeast

The task of the biosensor is to selectively determine which hormone activity is present in a sample. To test this selectivity, the three strains G1212/YRC102-hER-DsRed2, G1212/YRC102-hPR-CFP and G1212/YRC102-hAR-GFP were mixed to produce the biocomponent, G1212/YRC102-hHR-fluo. Before the mixed strain assay could be investigated with a sample containing the three hormone classes, it was essential to show that the detection was specific to the hormone used in the assay. For example it was possible that a sample containing estrogens which inducing high DsRed fluorescence, could lead to a GFP or CFP signal even though androgens or progestogens were not present. Different concentrations of the three hormones alone were incubated with G1212/YRC102-

hHR-fluo and the cell suspension was scanned for three different excitation/emission wavelengths. The fluorescence values are plotted in Fig. 4. In the presence of progesterone, GFP and DsRed scans showed no signal increase regardless of the concentration, whereas CFP scan had an increasing fluorescence signal with increasing progesterone concentration (Fig.4.a). The absence of GFP or DsRed fluorescence means that only the G1212/YRC102-hPR-CFP cells present in the mix reacted and produced its signature wavelength demonstrating that the spectrofluorometer can efficiently differentiate between CFP, GFP and DsRed fluorescence. When G1212/YRC102-hHR-fluo was incubated with increasing concentrations of 5α-dihydrotestosterone, a significant increase in fluorescence could be detected if the cell suspension was scanned for GFP fluorescence but a signal of less than 1.5 times the blank value could also be detected when a CFP or a DsRed scans were performed (Fig. 4.b). Similarly in the presence of 17β-estradiol, DsRed fluorescence was detected whereas GFP and CFP fluorescence never exceeded 1.5 (Fig.4.c).

Microtiter plate assay with G1212/hHR-fluo and mixed standard hormones

The newly developed biosensor will be used to assay a real sample possibly containing several agonists or antagonists of the three hormone receptors integrated in G1212/YRC102-hHR-fluo. As a consequence, the three strains will be simultaneously activated to produce CFP, GFP and DsRed fluorescence. To test whether this phenomenon affects the performance of the biosensor, an experiment was designed where a mix of 17β-estradiol, progesterone and 5α-dihydrotestosterone was incubated with G1212/YRC102-hHR-fluo. As internal control, the same concentrations of the individual molecules were also incubated with G1212/YRC102-hHR-fluo on the same microtiter plate. After 18 h, the plate was scanned at the fluorescence wavelengths of CFP, GFP and DsRed. The results are presented in Fig. 5 where the red, yellow and orange data points and lines represent, respectively, the wells where progesterone, 17β-estradiol and 5α-dihydrotestosterone were separately present. The green points and line show the fluorescence signal where all three hormones were incubated with G1212/YRC102-hHR-fluo. The results show that mixing the three standards

doesn't modify the general shape of the signal curve obtained with each hormone alone. Visible drifting of the EC_{50} value was not observed thus indicating that the working range of the assay remains similar to the working range of all three separate assays. One difference is that the maximal fluorescence, i.e. the plateau of the sigmoidal curve, is significantly lower in CFP and DsRed fluorescence. However, very high concentrations of the three hormones in the sample give the CFP and DsRed scans a higher fluorescence value than the maximum fluorescence of each hormone alone, thus indicating a small addition effect.

Marmoset serum sex steroid levels

The biosensor was used to detect the levels of the three main sex steroid classes in female marmoset serum samples. These samples were frozen aliquots from previous experiments. The progesterone levels in the sera had been measured by ELISA before freezing making comparison of results possible. As mentioned in the material and methods section, each sample was measured in triplicate in three different plates and the calculated concentrations present in Table II are the means of the average concentrations for each plate. Additionally, the progesterone concentrations determined by ELISA are presented in Table II. For some samples, the fluorescence was inferior or superior to the quantification limits of the calibration curve and these samples were therefore marked as < 1 ng/ml and > 95 ng/ml respectively. In Fig. 6, a comparison between the progesterone concentrations measured by ELISA and the yeast-based biosensor can be seen. In an additional statistical analysis, samples that gave progesterone equivalent concentrations outside the calibration range of the assay were not taken into account. The statistical analysis shows an r^2 value of 0.8329 and that all data points are in the 95 % prediction band, thus indicating a positive correlation between the results obtained with yeast-based bioassay and ELISA.

Discussion

Microtiter plate assay with separate strains

The number of yeast cell-based biosensors is increasing and the detection of steroid hormones is one of their main targets. Since the first report on the use of the human estrogen receptor in yeast to detect estrogens (White et al. 1988), yeast-based biosensors using human progesterone (Chatterjee et al. 2008), androgen (Beck et al. 2008a; Bovee et al. 2007), glucocorticoid (Bovee et al. 2011) and estrogen receptors (Sanseverino et al. 2005) have been reported. Depending on the host organism or the reporter used, the performance of these biosensors in terms of limits of detection, selectivity and robustness have been tuned to match a particular application, thus explaining the broad offering of yeast-based biosensors for hormone detection.

Generally, the limit of detection itself is not the determining criterion for choosing a yeast-based hormone biosensor because an analytical method or immunoassay will generally give more precise information. Biosensors function as a pre-screening step to assay untreated or simply extracted samples from a wide range of origins such as environmental, biological fluids and food extracts. Thus the versatility of yeast biosensors, ease of use and speed are the factors that make the sensor attractive for first line analysis. The use of a fluorescent reporter protein in yeast biosensors has been described in several publications (Beck et al. 2005; Beck et al. 2008a) and more recently in an *A. adeninivorans*-based biosensor (Chamas et al. 2015; Pham et al. 2016). However, the combination of several strains producing different fluorescent reporter proteins has not been reported.

For the success of the biosensor described here, it was essential that the three fluorescent proteins had approximately the same maturation time but have different excitation and emission fluorescence wavelengths. We thus selected proteins that fluoresce in the blue (CFP), green (GFP) and red (DsRed) parts of the visible spectrum to give unequivocal results. GFP, derived from the jellyfish *Aequorea vitoria*, was the first fluorescent protein used in biotechnology. CFP was produced by genetic modification to the GFP gene and DsRed is deriving from the coral

Discosoma. First tests with CFP and GFP indicated that these proteins start to be detectable after 4 h incubation and the cells reach maximum fluorescence at approximately 8 h (data not shown). However, the DsRed protein, required a longer incubation period to develop sufficient fluorescence. The slow maturation time of recombinant DsRed was previously investigated and it was found that three amino acid substitutions in the protein dramatically decreased this maturation time (Bevis and Glick 2002). These three mutations were inserted into DsRed by two-step polymerase chain reaction and the improved reporter protein, designated DsRed2, was used in the construction of the plasmid. Results showed a significant decrease in maturation time with red fluorescence being visible after 3 h incubation (data not shown). Additionally, the fluorescence intensity of DsRed2 was improved by a factor of 4 in comparison to DsRed. This increasing fluorescence was also reported in the work of Bevis and Glick (2002) where these three mutations were described.

The calculated EC_{50} values of 17β-estradiol, progesterone and 5α-dihydrotestosterone for the three strains G1212/YRC102-hER-DsRed2, G1212/YRC102-hPR-CFP and G1212/YRC102-hAR-GFP are similar to the EC_{50} values obtained when using the biochemical tests A-YES, A-YAS or A-YPS. In a previous study with non-modified DsRed, an EC_{50} for progesterone of 237 ng/l was found, which is slightly higher than the value reported here. This reduction in the present paper can be attributed to the use of the enhanced DsRed2 reporter protein and the modification of the assay protocol. Instead of using deep-well plates for incubation and transferring the cell suspension to a measurement plate, the new protocol performs both incubation and measurement in the same plate. Additionally, the incubation at 30 °C under vigorous shaking is replaced by an incubation at 37 °C without shaking. Besides increasing the sensitivity of the assay in comparison to the assay with non-modified DsRed, this new protocol removed the need for a second plate, a temperature controlled shaker and pipetting step.

Microtiter plate assay with mixed strains

After establishing that each strain performs similarly to the currently available biochemical assay, it was then demonstrated that all three strains can function when mixed. Interestingly, the EC_{50} values

for each hormone seem not greatly affected and the assay range remains similar. One notable difference is the lower maximal fluorescence with progesterone and 17β-estradiol. Even though the optical cell density of each strain was the same as in the experiments with separate strains, the plateau of the response curve was two or three times lower when G1212/YRC102-hHR-fluo is used. To explain this phenomenon, it should be considered that, in G1212/YRC1012-hHR-fluo, that while the OD_{620nm} of each strain is kept at 1, the total OD_{620nm} of the three strains is 3. It is possible that the higher total OD_{620nm} of 3 may have an influence on the fluorescence. In preliminary experiments, it was observed that, above OD_{620nm} values of 5, higher cell density leads to decreased fluorescence (data not shown). It is thus important to establish an optimal cell optical density for the assay. This lower maximal fluorescence makes it more difficult to calculate important assay parameters such as limit of detection, limit of quantification or EC_{50} value because the spectrofluorometer has more difficulties to separate the first quantifiable value from the blank. An important point is the fact that a mix of the three hormones progesterone, 17β-estradiol and 5α-dihydrotestosterone incubated with G1212/YRC102-hHR-fluo gives results that are similar to the results of separate incubations. As shown in Fig.5, the general shape of the curve is only marginally modified when G1212/YRC102-hHR-fluo is incubated with the hormone mix.

The affinity of 17β-estradiol, progesterone and 5α-dihydrotestosterone for other hormone receptors has already been determined by radioassay and also with yeast-based biosensors for some combinations. Determination of relative binding affinity using radio-immunoassays of progesterone and 5α-dihydrotestosterone for the estrogen receptor was calculated and both molecules were 5,000 times less potent than 17β-estradiol (Scippo et al. 2002). Kaiser et al. (2010) found that 5α-dihydrotestosterone had about 100,000 times less affinity for the estrogen receptor than 17β-estradiol and no affinity for progesterone receptor. Scippo et al. (2002) found relative binding affinities for the human progesterone receptor of 0.5 and 1 for 17β-estradiol and 5α-dihydrotestosterone respectively compared to an affinity of 100 for progesterone. However, yeast-based biosensors containing the progesterone receptor show a 1000 and 100 times lower affinity for

17β-estradiol and 5α-dihydrotestosterone respectively (Chamas et al. 2015). 17β-estradiol and progesterone show moderate affinity to the human androgen receptor in radioassays, with relative binding affinity values of 20% and 10% respectively of the affinity of 5α-dihydrotestosterone (Scippo et al. 2002). In yeast-based biosensors, the affinity of 17β-estradiol for the androgen receptor is reported to be 0.12 times the affinity of 5α-dihydrotestosterone (Gerlach et al. 2014). In our assay format, the maximal concentration of 17β-estradiol is 100 ng/l which corresponds to a signal of 12.5 to 20 ng/l 5α-dihydrotestosterone, which is below the limit of detection calculated for the strain G1212/hAR-GFP.

These experiments show that cross-reactivity between the three hormones is not issue at the concentrations expected in samples. However, a real sample may have a much more complex composition and some compounds may inhibit or modulate the binding of one or more receptors. However, this is a problem in common with other yeast biosensors and to date there have not been reports of any major interferences.

Although these results show that the developed biosensor can effectively detect the three hormone classes, additional experiments including different laboratories and utilizing lyophilized cells instead of fresh cells will be necessary in order to demonstrate the utility of the method. Further work is required to obtain precise determination of the essential parameters such as EC_{50} value, limit of detection and limit of quantification. With the experiments performed here, a preliminary biosensor working range for each hormone has been determined for each hormone; 1 – 150 ng/l for 17β-estradiol, 6 - 500 ng/l for progesterone and 25 – 500 ng/l for 5α-dihydrotestosterone. However, because these characteristics could not be determined precisely, a calibration curve with the three substances was performed with each plate when applying this biosensor to serum samples.

Marmoset serum sex steroid levels

In marmoset serum samples, a positive correlation between ELISA and fluorescence measurements for progesterone was observed even though the absolute concentrations are not entirely similar. However, it should be noted that an ELISA test, only measures the concentration of progesterone

whereas the biosensor gives the progesterone activity, i.e. the activity after influences of agonists or antagonists present in the sample. The concentrations detected with G1212/YRC102-hHR-fluo remains generally lower than the concentration of progesterone (ng/ml range) which agrees with other findings for both estrogens and androgens (Chambers and Hearn 1979). However, this first experiment with real samples did show that the relatively narrow calibration range is a disadvantage of this yeast-based assay. For the 16 samples, five gave progesterone equivalent concentrations values less than the lower quantification limit and two showed progesterone equivalent concentrations values above the upper quantification limit but these results do confirm the usefulness of this yeast-based biosensor as a screening test before more precise analytical determination of hormone concentration is undertaken. By setting threshold concentrations, a sample can in one single experiment be classified as possessing high or low estrogenic, progestogenic or androgenic activity.

It would have been ideal to compare 17β-estradiol and 17α-dihydrotestosterone concentrations with both biosensor and ELISA detection systems. But because ELISA tests for these molecules are not performed routinely in the veterinary medicine, obtaining these concentrations would require fresh marmoset blood samples just for this experiment. Taking a blood sample is a stressful and painful event for a marmoset, hence it was decided to only use frozen blood samples with known progesterone concentrations for this study.

Inclusion of other human receptors could also be envisaged as fluorescent proteins in the yellow, orange, UV and purple wavelengths are available. Strains could be created with recombinant human glucocorticoid, thyroid or mineralocorticoid receptors linked with these fluorescent proteins, which could be incorporated with the existing assay to produce a six-channel biosensor. The main criterion for this biosensor upgrade will be to effectively discriminate between these fluorescences. A limiting parameter will then probably be the performance of the spectrofluorometer. Additionnally, the maximal relative fluorescence for the three reporter proteins shows major differences which can be attributed to the different fluorescence yields of CFP, GFP and DsRed. By selecting enhanced

fluorescent proteins obtained by targeted mutagenesis, the sensitivity of the biosensor can then theoretically be greatly improved, thus allowing precise determination of assay important parameters.

This biosensor could find an important application in environmental field as it has already demonstrated that *A. adeninivorans*-based biosensors do perform well in samples from wastewater origin. These samples often contain not only natural hormones but also xenohormones and the capacity of the biosensor to detect those hormone has to be investigated.

This work presents the initial steps towards the establishment of a new generation of yeast-based biosensors for the detection of sex hormones. It demonstrates the feasibility of mixing different yeast strains in order to simultaneously detect the presence of different sex hormones classes in a sample. In one experiment, three times more informations can be retrieved and, because this strategy can be expanded to other receptors or reporter proteins, this biosensor can potentially simultaneously detect other hormones or biologically relevant compounds.

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Tables

Table I. EC_{50} values for progesterone, 5α-dihydrotestosterone and 17β-estradiol after incubation with strains for enzymatic or fluorescence mediated detection.

ligand	progesterone		5α-dihydrotestosterone		17β-estradiol	
detection method	enzymatic	fluorescence	enzymatic	fluorescence	enzymatic	fluorescence
strain	G1212/YRC102-hPR-phyK	G1212/YRC102-hPR-CFP	G1212/YRC102-hAR-phyK	G1212/YRC102-hAR-GFP	G1212/YRC102-hER-phyK	G1212/YRC102-hER-DsRed2
EC_{50} (ng/l)	151	147	277	164	33	17

Table II. Hormone equivalent concentrations calculated with G1212/YRC102-hHR-fluo and progesterone concentration calculated with ELISA of 16 female marmoset serum samples.

Sample	Progesterone equivalent concentration (ng/ml)	Progesterone concentration calculated by ELISA (ng/ml)	Androgen equivalent concentration (ng/ml)	Estrogen equivalent concentration (ng/ml)
1	11.1 ± 1.5	30.5	7.8	7.7 ± 0.5
2	37.1 ± 2.3	81.4	5.8	4.8 ± 0.5
3	< 1	3.8	7.4	11.5 ± 0.4
4	< 1	8.3	< 5	7.9 ± 0.5
5	< 1	6.5	8.3 ± 5.4	15.9 ± 1.8
6	18.0 ± 3.1	32.2	26.6 ± 9.0	12.6 ± 1.9
7	1.3 ± 0.1	9.1	< 5	9.7 ± 0.7
8	87.5 ± 5.3	90.9	28.2 ± 2.0	28.0 ± 2.8
9	> 95	96.3	32.8 ± 5.9	17.6 ± 1.5
10	> 95	133	27.9 ± 4.4	16.6 ± 1.0
11	1.9 ± 0.9	12.4	17.5 ± 5.0	19.9 ± 2.0
12	45.8 ± 6.6	69.8	22.2 ± 6.1	17.8 ± 1.0
13	< 1	5.5	9.2	6.8 ± 0.7
14	< 1	2.4	17.9	15.0 ± 0.5
15	4.1 ± 0.4	7.6	28.6 ±6.1	13.3 ± 0.7
16	53.4 ± 16.9	95.4	51 ±2.4	11.2 ± 2.5

Figures

Fig. 1 Physical map of the YRCs (Yeast RDNA integration Cassettes) used in this work. Cassettes contain the selection marker *ATRP1m* fused to the *ALEU2* promoter, the expression module *TEF1* promoter – *receptor* gene – *PHO5* terminator and the two 25S-rDNA sequences for integration in yeast genome. The reporter module consists of *2xHRE-GAA*$^{-107}$ promoter – *CFP* gene – *PHO5* terminator (a), *2xHRE-GAA*$^{-107}$promoter – GFP gene – *PHO5* terminator (b) or *2xERE-GAA*$^{-107}$ promoter – *DsRed2* gene – *PHO5* terminator (c).

Fig. 2 Schematic representation of a modified *A. adeninivorans* cell. In the nucleus, a human hormone receptor gene is constitutively expressed and the protein can be found in cytoplasma (1). When a binding hormone is present, it can cross yeast membrane and bind to the receptor (2). After binding-induced receptor dimerization, this dimer can retranslocate in the nucleus and bind to the hormone response element (HRE) (3), thus activating the *GAA* promoter and allowing expression of the *fluorescence* gene (4). This leads to the production of a fluorescent protein in the cytoplasma.

Fig. 3 Dose-response curves obtained for the ligand progesterone after incubation with *A. adeninivorans* G1212/Xplor2.2-hPR-CFP (a), for the ligand 5α-dihydrotestosterone after incubation with G1212/Xplor2.2-hAR-GFP (b) and for the ligand 17β-estradiol after incubation with G1212/Xplor2.2-hER-DsRed2 (c). Response signal corresponds to the relative fluorescence measurement at the signature wavelengths for CFP (a), GFP (b) and DsRed (c). Data points represent the results obtained for three independent biological replicates. Error-bars represent the standard deviation of each measurement.

Fig. 4 Dose-response curves obtained after the incubation of G1212/YRC102-hHR-fluo (mixed yeasts) with increasing concentrations of progesterone (a), 5α-dihydrotestosterone (b) and 17β-

estradiol (c). Blue, green and red points and line show the cell suspensions relative fluorescence at the signature wavelengths for CFP, GFP and DsRed.

Fig. 5 Relative fluorescence values at the signature wavelengths of CFP (a), GFP (b) and DsRed (c) of G1212/YRC102-hHR-fluo with increasing concentrations of progesterone, 5α-dihydrotestosterone and 17β-estradiol. Red, orange and yellow plots show the cell suspensions relative fluorescence when progesterone, 5α-dihydrotestosterone and 17β-estradiol were incubated alone with G1212/YRC102-hHR-fluo. Green plots show the cell suspensions relative fluorescence when all three hormones were incubated with G1212/YRC102-hHR-fluo.

Fig. 6 Comparison of serum progesterone concentration determined by ELISA (y-axis) and progesterone equivalent concentration determined by G1212/YRC102-hHR-fluo (x-axis). Calculation of correlation coefficient, 95 % confidence band and 95 % prediction band was performed with SigmaPlot.

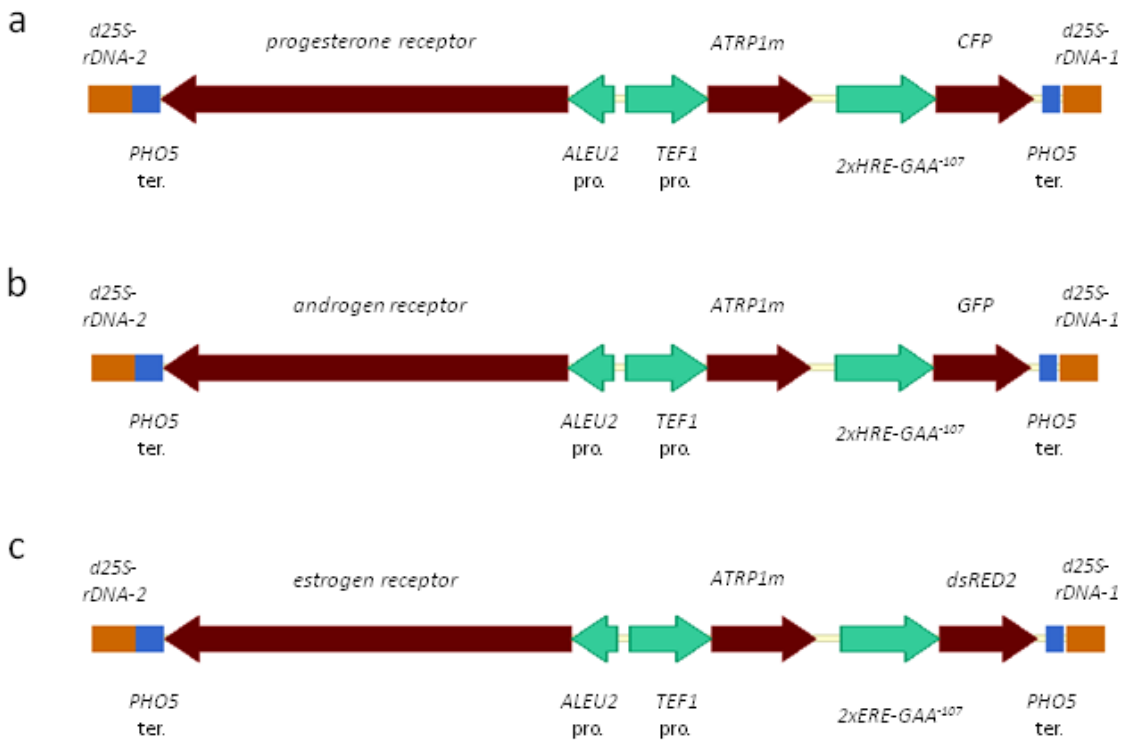

Fig. 1

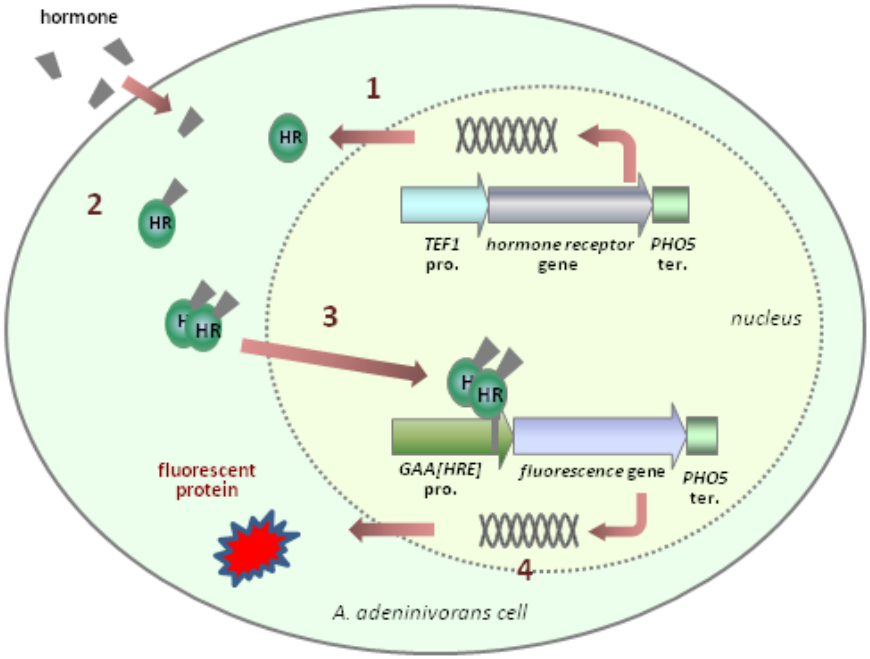

Fig. 2

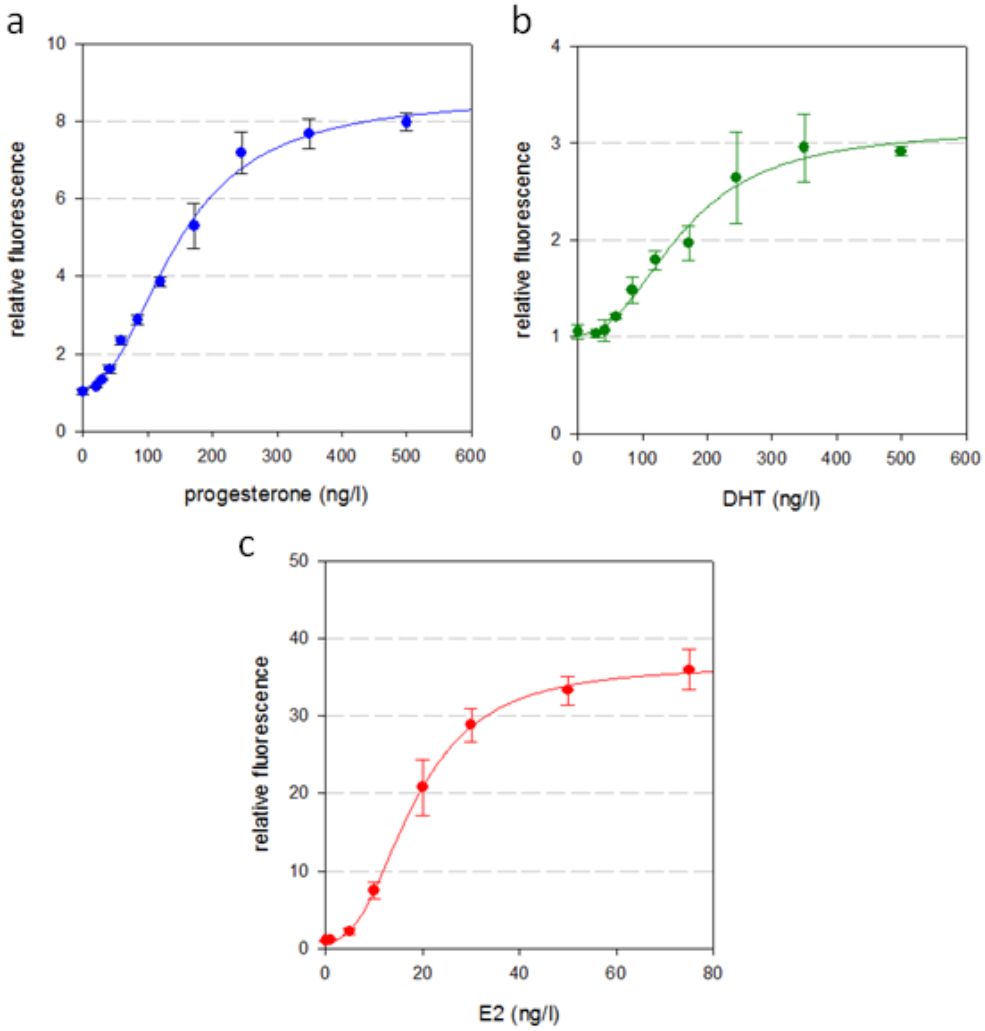

Fig. 3

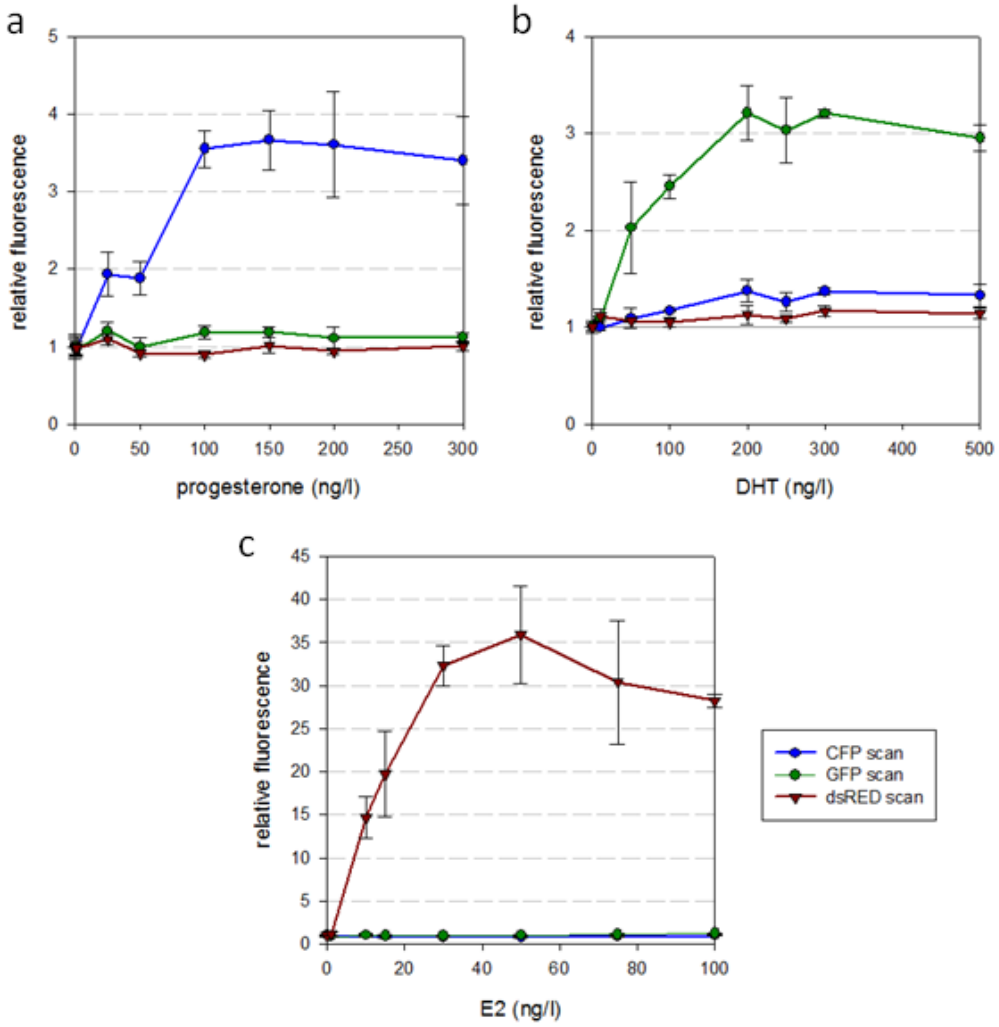

Fig. 4

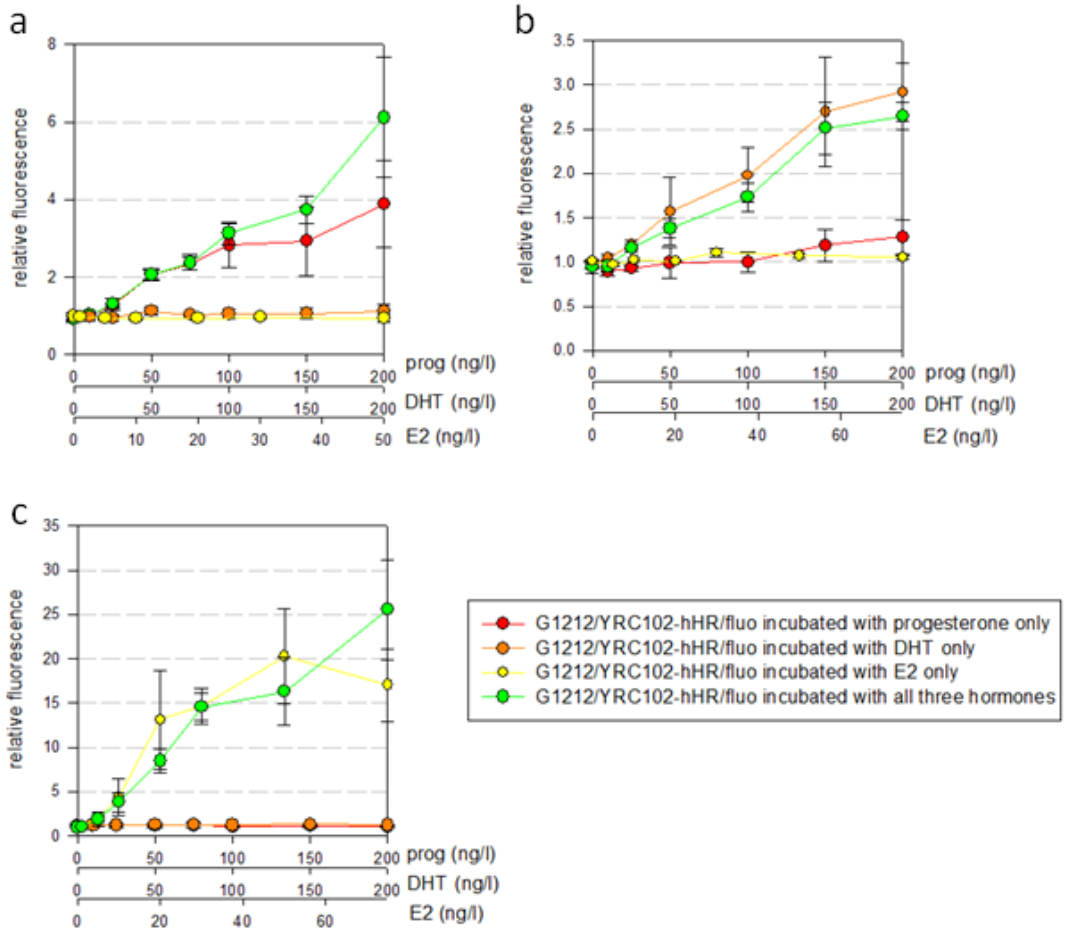

Fig. 5

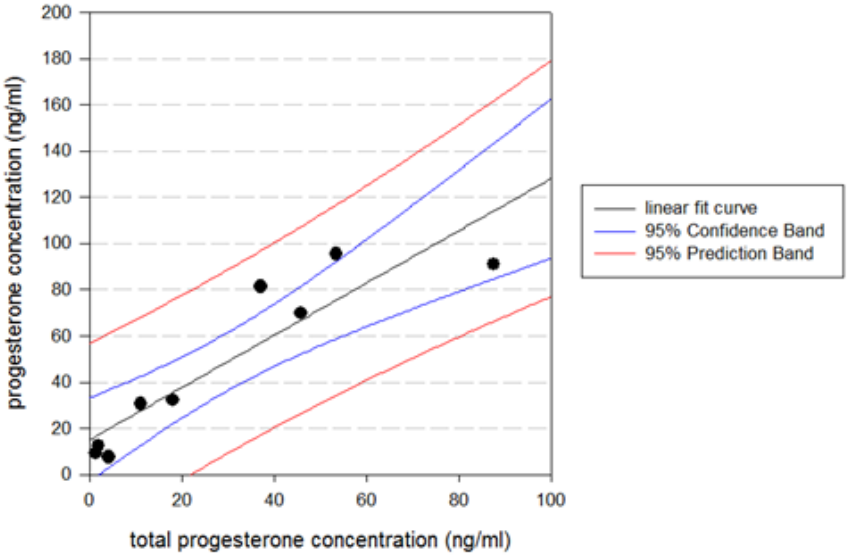

Fig. 6