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Detection of hormone receptor ligands in yeast by fluorogenic methods

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Abstract

Yeast-based bioassays are becoming widespread tools for detection and quantification of ligands for vertebrate nuclear hormone receptors, including estrogens, progestans, androgens and dioxin-like compounds, both for agonistic and for antagonistic effects. These systems rely on the monitoring of transcription rates of reporter genes whose expression in yeast depends on binding of receptors to their natural or xenobiotic ligands. Among the different methods to quantify reporter gene transcription, those based on fluorescence detection are fast, very sensitive and reproducible. We propose a fast method for ligand detection for different vertebrate receptors in yeast, based on the use of fluorogenic substrates for the widely used reporter β-galactosidase gene. In this method, β-galactosidase activity is calculated from kinetic data, rather than from end-point measurements, which increases accuracy and facilitates the statistical analysis of the data. It also provides statistically rigorous procedures to distinguish between active and inactive compounds and to evaluate the fitness of the data to alternative models of dose/response mechanisms. All these features combined configure a flexible, fast (less than three hours for some systems) and reproducible method to evaluate the presence of potential endocrine disruptors in the environment.

Keywords: Endocrine disruption; Xenoestrogen; Hormonal response; Nuclear receptors; β-galactosidase; Reporter genes

1. Introduction

The availability of genetically engineered yeast strains expressing and monitoring the activity of vertebrate hormone receptors has been a major advance on the detection of putative endocrine disruption activity in the environment as well as on the screening of the ever-increasing number of new chemical compounds appearing in the market every year [1–4]. Although yeast-based bioassays do not provide a precise chemical characterisation of the hormone–receptor ligands, their low cost and easiness of handling makes them a first choice when testing large numbers of ill-characterised samples or compounds. Yeast bioassays have been used to correlate the presence of suspected or bona fide endocrine disruptors and estrogenic activity in environmental samples [5–7], and to establish relationships between chemical structures and affinity for selected vertebrate hormone receptors for different chemical compounds and their chemical derivatives [8–11].

Yeast-based bioassays, also known as recombinant yeast assays (RYA), consist of an engineered yeast strain harbouring two foreign genetic elements: one expressing a vertebrate nuclear receptor and a second one in which a convenient gene whose expression is easy to follow (the reporter gene) is under transcriptional control of the expressed nuclear receptor. In this work, we used the gene *Lac*Z from *E. coli*, which encodes the enzyme β-galactosidase. The reporter gene is constructed in a way that this enzyme will only be expressed in the presence of the receptor–ligand complex. This setup imitates the mechanism by which many hormone-responsive genes are regulated in vertebrates [12,13]; the fundamental similarity of all eukaryotes ensures that the same mechanism also works in yeast. The expression of the reporter gene is then monitored by its enzymatic activity by using convenient substrates whose products are easy to detect and quantify. The kinetics of the appearance of this product is directly related to the amount of enzyme produced, which is in turn related through a typical dose-response

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sigmoidal curve to the effective concentration of estrogenic compound(s). Two characteristics of the yeast cell contribute to the success of the RYA. First, yeast has no endogenous system homologous to vertebrate nuclear receptors that could interfere with the assay. Second, the folding and post translational processing of vertebrate protein in yeast is very similar to the one from mammalian cells, which results in the preservation of the native receptor structure when expressed in yeast. This is of paramount interest for our purposes, since the correct structure of the ligand-binding domain of the receptor determines the specificity of the system, that is, its capability to distinguish between ligands and non-ligands [12]. This latter point has been tested in numerous reports comparing ligand activity of different compounds in yeast and in mammalian systems. Although some differences do occur, RYA always stands as a reliable method to detect and characterise vertebrate receptor ligands [1,3,8,14,15,10].

In this work, we will present an integrated protocol to optimise these processes, both in terms of reproducibility and in reducing at maximum the time of the assay. The protocol takes advantage of the availability of fluorogenic substrates for β-galactosidase and of the possibility to use in situ permeabilised yeast cells in a 96-well plate assay. It also describes improved methods of quantification of β-galactosidase enzymatic activity, fitting of data to a theoretical dose/response curve and the automatic and precise determination of limits of detection. Once set, the process does not require user's supervision, opening the possibility to automatization and robotization. We present here data from three β-galactosidase-based yeast systems (estrogen receptor, progesterone receptor and aryl hydrocarbon receptor), but the method can be adapted to most existing yeast bioassays based on enzymatic detection. The whole process can be completed in less than three hours, including incubation and detection times; in some cases, the rate-limiting step is the biological response of the yeast cell to the presence of the ligand.

2. Material and methods

2.1. RYA systems

2.1.1. Estrogen-receptor system (ER-RYA)

Yeast strain BY4741 (MAT**a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) from EUROSCARF, Frankfurt, Germany was transformed with plasmids pH5HE0 and pVitBX2 as described [2]. Expression plasmid pH5HE0 contains the human estrogen hormone receptor HE0 [12] cloned into the constitutive yeast expression vector pAAH5 [16]. The reporter plasmid pVITB2x contains two copies of the pseudo-palindromic estrogen responsive element ERE2 from *X. laevis* vitellogenin B1gene (5′-AGTCACTGTGACC-3′, [12]) inserted into the unique *Kpn*I site of pSFLΔ-178K [2].

2.1.2. Progesterone-receptor system (LPR-RYA)

Plasmid pLPR contains a fusion construct between the LexA-DNA-binding domain (amino acids 1–202) and the ligand-binding domain (amino acids 676–934) of the human progesterone receptor in the expression plasmid pLexA202 from Clontech (BD Biosciences, Palo Alto, CA, USA). This chimeric construct was tested in the yeast strain L40 (MATa*, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-LacZ* [17]), which contains eight copies of the LexA-DNA recognition sequence in front of a CYC1-lacZ fusion integrated into the genome. Also, the integrated LexA-HIS3 fusion was previously deleted by homologous recombination with the G418-resistance cassette pFA6-kanMX4 [18], flanked by convenient HIS3-coding sequences.

2.1.3. Aryl hydrocarbon receptor system (YCM-RYA)

Strain YCM4 was a generous gift from Charles A. Miller [19]. This strain is a derivative of W303a (MAT**a**, *ade2-1, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1*), which harbor two chromosomally integrated constructs. One of the constructs co-expresses human aryl hydrocarbon receptor and ARNT genes under the Gal1-10 promoter. The second integrated construct is the pDRE23-Z reporter, encompassing three XRE5 sequence and the CYC1-LacZ fusion (more information in the original paper [19]). To perform the assay, YCM4 cells were grown in galactose overnight to express both AhR and ARNT.

2.2. Recombinant yeast assay

Transformed clones were grown overnight in minimal medium (6.7 g/L yeast nitrogen base without amino acids, DIFCO, Basel, Switzerland; 20 g/L glucose, supplemented with 0.1 g/L of prototrophic markers as required). For fluorogenic determination, the final culture was adjusted to an optical density (OD) of 0.1 and split into 75-, 50- or 25-μL aliquots (see below) in a 96-well polypropylene microtiter plate (NUNC™, Roskilde, Denmark [10]). We performed a serial dilution scheme by dispensing 5 μL-samples into wells on the first column (which contained 75 μL of culture). Subsequent columns (2–11) contained 50 μL of culture; the last column (12) contained 25 μL of culture. Serial dilutions were made by sequentially transferring 25 μL from the previous well to the next one; at the end, all wells contained 50 μL and different dilutions of the samples, with dilution factors 1:30, 1:90, 1:270, 1:540, etc. In such a scheme, a typical 96-well plate fits eight serial dilution series. Plates were incubated for 2–4 h (depending on the RYA system) at 30 °C under mild shaking. After incubation, 50 μL Y-PER™ (PIERCE™, Rockford, IL, USA) were added to each well and further incubated at 30 °C for 30 min. Afterwards, 50 μL of assay buffer supplemented with 0.1% 2-mercaptoethanol and 0.5% of the 4-methylumbelliferone β-D-galactopyranoside (MuGal) solution (both from the FluorAce™ beta-galactosidase Reporter Assay Kit, Bio–Rad Laboratories, Hercules, CA, USA) were added to the lysed cells. After brief centrifugation, plates were read in a Victor^3 Wallac spectrofluorometer (Perkin Elmer Inc., Wellesley, MA, USA), at 355 nm excitation and 460 nm emission wavelengths. Fluorescence was recorded for 15–20 min (one measurement per min); β-galactosidase activity values were calculated as rates of the increment of arbitrary fluorescence units with time, using standard linear regression methods. To test anti-ligand (inhibitory) activity in YCM-RYA systems, yeast cultures were incubated for 4 h with 0.5 μM of β-naphthoflavone and different concentrations of genistein, and processed as before.

The β-galactosidase activity was determined also using the chromogenic substrate *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG). The protocol was similar to the one described above, but for the increasing of the OD of the culture to 0.5, the incubation time up to 16 h (overnight) and the use of appropriate assay buffer (buffer Z, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM HCl, 1 mM $MgSO_4$, pH 7.0, 0.5% 2-mercaptoethanol, 25 μL) and substrate (25 μL of 4 mg/mL ONPG in water). The chromogenic reaction was stopped when negative controls (methanol) begun turning yellow (30–90 min) by addition of 50 μL of 1 M Na_2CO_3. Times of addition of ONPG (initial time, T_i) and of Na_2CO_3 (final time, T_f) were recorded. Plates were then read in a microtiter photometric reader at 420 nm (A_{420}). β-galactosidase activity (traditionally known as Miller units) were calculated according to the equation:

$$\text{Miller units} = \frac{1000 \times A_{420}}{A_{600} \times V \times (t_f - t_i)}$$

where V and A_{600} represent the volume and the optical density of the culture at 600 nm, respectively.

Both Miller units and fluorescence units (U) could also be related to the maximal (set to 100) and minimal (set to 0) values from the positive (saturating concentration of ligand) and negative (methanol) controls, respectively, and expressed as relative units, RU:

$$\text{RU} = 100 \times \frac{U - U_{\min}}{U_{\max} - U_{\min}}$$

2.3. Mathematical modelling

The simplest model to describe dose/response curves assumes equilibrium between hormone-free and hormone-loaded hormone receptor molecules in solution,

$$R + h \overset{K_d}{\rightleftarrows} hR$$

where R represents the concentration of hormone-free receptor molecules, h the hormone concentration, hR the concentration of the hormone-loaded receptor molecule, and K_d is the dissociation constant. This particular model assumes a single agonist molecule binding to each receptor molecule, as it has been demonstrated by numerous physical and crystallographic data [12,13]. Assuming hormone concentration much larger than receptor concentration, the fraction of receptor bound to the hormone Φ_r can be described by the Hill equation

$$\Phi_r = \frac{[hR]}{R_t} = \frac{1}{1 + (K_d/[h])} \tag{1}$$

In which R_t is the total receptor concentration (bound and free) [20].

The Hill equation only describes the equilibrium for the hormone–receptor interaction. It is frequently assumed that this is the rate-limiting step in the cascade of events going from the presence of a given concentration of hormone and the final physiological effects—in our case, expression of β-galactosidase. However, there are many observations in complex systems that indicate a further modulation of the hormonal response due to the subsequent steps of the hormone–receptor complex dimerisation, binding to multiple DNA sites and cooperative activation of transcription. The corresponding mathematical methods are much less developed, but in most cases, they introduce a "cooperativity coefficient", p. In this work, we will test two of these approaches: a cooperative version of the Hill equation:

$$\Phi_r = \frac{1}{1 + \left(\frac{K_d}{[h]}\right)^p} \tag{2}$$

which assumes that more than a single hormone molecule is necessary to produce an active receptor configuration (e.g., considering the "active" receptor a dimmer molecule showing two hormone binding sites); and the so-called "modified Hill equation",

$$\Phi_r = \frac{1}{\left(1 + \frac{K_d}{[h]}\right)^p} \tag{3}$$

This has been empirically shown to describe the relationship between ligand/receptor interactions and physiological response in complex systems [20].

2.4. Data analysis

Eqs. (1)–(3) can be re-formulated to express the variation of the physiological response to a given concentration of ligand to give Eqs. (1′), (2′) and (3′), as follows:

$$U_L = \frac{A}{1 + \frac{K}{[L]}} + B \tag{1′}$$

$$U_L = \frac{A}{1 + \left(\frac{K}{[L]}\right)^p} + B \tag{2′}$$

$$U_L = \frac{A}{\left(1 + \frac{K}{[L]}\right)^p} + B \tag{3′}$$

In which U_L represents the magnitude of the physiological response (in our case, β-galactosidase units), "B" is the basal level (without ligand), "A" represents the increment of response from the basal level to the saturating ligand concentration, and K is the apparent K_d. In Eq. (1′), K is equivalent to EC_{50}, the concentration of ligand that ellicits 50% of the maximal response. Parameter "p" is the previously described "cooperativity factor." All these parameters were calculated from dose/response curve data by non-linear regression methods. Data analysis, statistics, and linear and non-linear equation fitting were performed using the SPSS for Windows package (12.01 version, www.spss.com).

To calculate anti-ligand activity, Eqs. (1′) and (2′) can be re-formulated as:

$$U_L = B - \frac{A}{1 + \frac{K}{[L]}} \tag{1″}$$

$$U_L = B - \frac{A}{1 + \left(\frac{K}{[L]}\right)^p} \tag{2″}$$

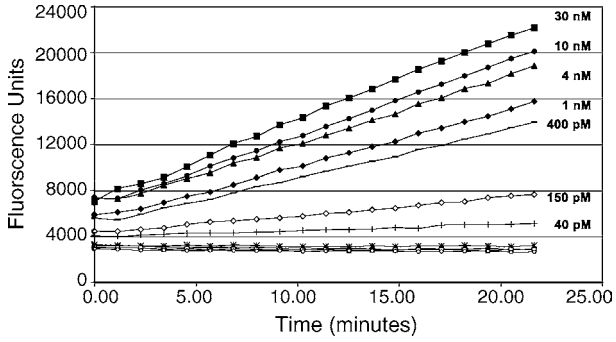

Fig. 1. Linear plots of the increase on fluorescence (in fluorescence units, FU) with time for different estradiol concentrations in the ER-RYA system. Concentrations of estradiol are indicated on the right margin. β-galactosidase activity values were calculated from the slopes of these linear plots by standard linear regression methods.

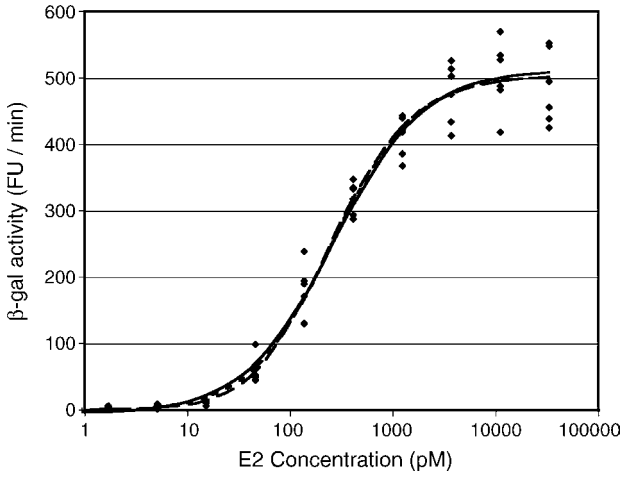

Fig. 2. Dose/response curve for estradiol (E2) in the ER-RYA system. The *Y*-axis represent β-galactosidase activity in FU/min, diamonds represent values from six independent determinations for each estradiol concentration. Graphs indicate theoretical curves as defined by Eqs. (1′) (continuous line), (2′) (long dashes) and (3′) (short dashes); the corresponding parameters, calculated by non-linear regression methods) are shown in Table 1.

where "*B*" is the β-galactosidase activity obtained with the positive effector alone (in our case, β-naphthoflavone), and "B–A" equals to the residual activity at infinite concentrations of the inhibitor. In Eq. (1″), *K* is equivalent to IC_{50}, the concentration of inhibitor that reduces the effector response to 50%.

3. Results and discussion

3.1. Dose-response curves for estrogen receptor RYA

The ER-RYA system is based in episomal, high-copy plasmids, which ensure 10–20 copies of both receptor expressing and reporter plasmids in each cell [2]. In addition, the estrogen receptor is expressed from the very strong, 1.5 Kb version of *ADH1* promoter. In these conditions, the response to estrogen agonist is extremely high, which allows incubation times as short as two hours, which is the time we estimated minimal for the induction process to occur in yeast. Fig. 1 shows the kinetics of formation of the fluorogenic product for different estradiol concentrations. At saturating substrate concentrations, the appearance of the fluorescent product followed a linear correlation with time, with a slope equivalent to β-galactosidase activity values in fluorescent units per minute (FU/min). The variation on β-galactosidase activity at different estradiol concentrations (each tested in sextuplicate) is represented in Fig. 2. The figure also shows non-linear fitting of the data to Eqs. (1′), (2′) and (3′) (see Section 2); the calculated values of the parameters for each model are shown in Table 1. Data showed a good fit to all three equations, whose prediction of the relevant parameters closely coincided. It is particularly relevant that Eqs. (2′) and (3′) predicted a value close to 1 for the cooperativity parameter *p*. In fact, a value for $p = 1$ (indicating no cooperativity between hormone–receptor complex) are consistent with the prediction of both models at 95% confidence (Table 1). The main conclusion from these data is that the simple, non-cooperative Hill equation fits adequately to the data, indicating that the hormone–receptor equilibrium is actually the rate-limiting process in this synthetic system and that the putative cooperative interaction of hormone-loaded receptors did not influence the dynamics of the hormone response. The *K* values obtained from the three models are similar, from 175 to 267 pM; in the case of Eq. (1), this would coincide with the dissociation constant for estradiol. The calculated value (267.64 pM, 95% confidence interval 220.18–315.11 pM) is very close to the values obtained in ONPG-based systems, both by us and by other groups [2,21].

3.2. Progesterone receptor RYA

The LPR-RYA system we used is based on a yeast strain L40 (a strain used routinely for two-hybrid studies), in which a lexA-LacZ fusion is integrated into the genome. The effector molecule

Table 1
Results of fitting estradiol dose/response data to three alternative variants of the Hill equation

Model	Parameters								
	A		*B*		*K*		*P*		R^2
	Value	95% confidence limits	Value	95% confidence limits	Value	95% confidence limits	Value	95% confidence limits	
Hill (1)	−5.77	(−16.67/5.52)	518.60	(448.38/538.81)	267.64	(220.18/315.11)	1.00[a]	–	0.979
Cooperative Hill (2)	−2.55	(−14.84/9.75)	505.70	(476.98/539.46)	256.55	(212.22/300.88)	1.10	(0.91/1.28)	0.980
Modified Hill (3)	0.97	(−11.3/13.23)	503.54	(479.80/527.25)	174.79	(132.50/251.08)	1.55	(0.68/2.43)	0.981

[a] Set as invariant in this model.

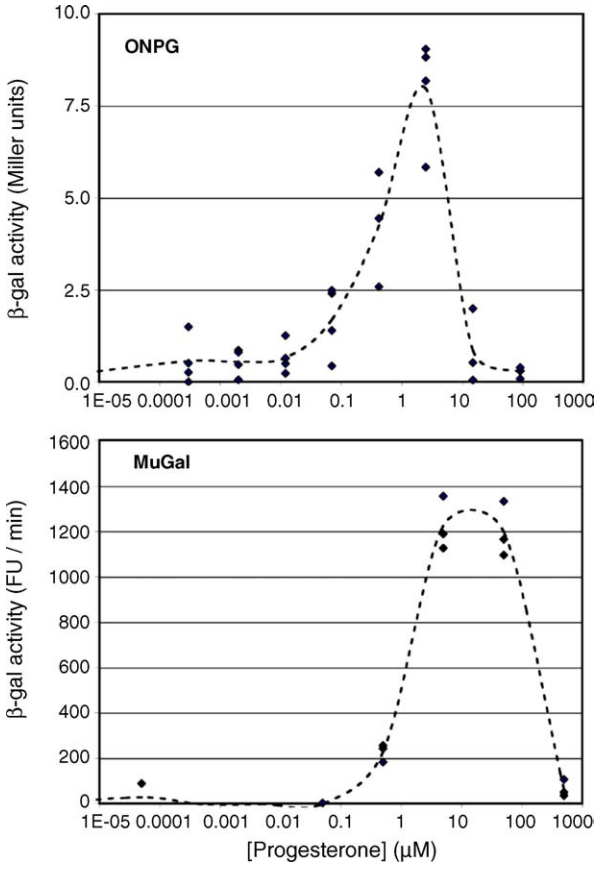

Fig. 3. Dose/response curve for progesterone in the LPR-RYA system. The top panel represent values using the ONPG chromogenic substrate; the lower panel shows results obtained with the fluorogenic MuGal substrate. Discontinuous lines follow average values for each progesterone concentration.

is a chimeric construct encompassing the DNA-binding domain of the bacterial repressor LexA and the Ligand-binding domain of the human progesterone receptor, constitutively expressed from a episomal, high-copy plasmid. This strategy should allow testing a variety of receptors (in fact, any transcriptional activator) on the same strain, by only fusing the corresponding ligand-binding domains to the LexA construct. However, in our hands this system proved to give poor results, both in terms of transcriptional activity and of apparent affinity for the cognate ligand, progesterone. As such, it provides a good model to test the comparative behavior of colorimetric and fluorogenic protocols for low-expression systems. Such a comparative is shown in Fig. 3. On top, there is a dose/response curve for the LPR/L40 system using ONPG chromogenic method; the corresponding results from the fluorogenic assay are shown on the bottom graph. Calculated EC_{50} values for progesterone were similar for both methods (0.54 and 1.32 μM, respectively), both notably higher than the values obtained using the native PR in a similar RYA system (30 nM, [2]). This probably reflects a poor interaction of the chimeric construct and progesterone.

Although the dose/response curves for chromogenic and fluorogenic assays in Fig. 3 are similar, observed toxic effect of progesterone occurred at lower concentration in the chromogenic system. The apparent higher toxicity of progesterone in the chro-

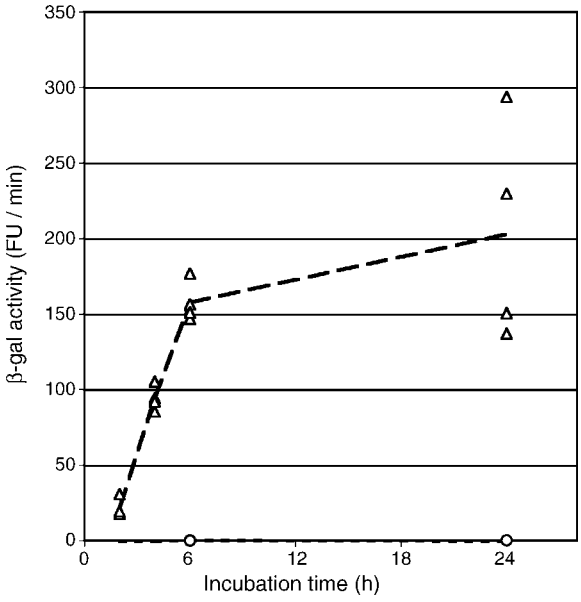

Fig. 4. Variation of β-galactosidase activity (in FU/min) for the LPR-RYA system at different incubation times with 5 μM of progesterone (triangles). Discontinuous line indicates average values for each time (in quadruplicate). Circles indicate basal levels, from cultures incubated with no progesterone added.

mogenic assay is probably related to the need of an overnight incubation, due to the low sensitivity of the system. In contrast, fluorogenic detection of β-galactosidase activity allowed shorter incubation times, diminishing the toxic effects. Fig. 4 shows β-galactosidase activity values from fluorogenic assays after 2, 4, 6 and 24 h of incubation with 5 μM progesterone. A similar graph could not be produced using ONPG since it only detected β-galactosidase activity after an overnight incubation (not shown). The data indicate that the activity levels off at 6 h, whereas further incubation only increases variability among replicates, most probably due to differences of growth and/or viability. The highest variability of ONPG data on Fig. 3 relative to the fluorogenic data on the same figure probably could also be explained by the requirement for an overnight incubation.

3.3. The aryl hydrocarbon receptor RYA

The yeast strain YCM4 was specifically designed to detect ligands of the aryl hydrocarbon receptor (AhR), also called dioxin receptor [19]. We included it in this study to exemplify how the proposed protocol suited pre-made yeast systems. Fig. 5 shows the corresponding dose-response curves for one of the standard known ligands for AhR, β-naphthoflavone, as well as the fitting of theses values to Eq. (1′). The calculated EC_{50} (46.9 nM) is similar to the values reported in the literature for this system [19,22]. AhR-based RYA systems, including YCM4 as and other, show a relatively high basal expression in the absence of ligand, which in turn determines a moderate fold-increase in transcription upon addition of the ligand. In the experiment shown in Fig. 5, β-galactosidase activity in the absence of ligand (basal levels) was calculated as 15 FU/min, whereas the maximal activity at saturating ligand concentration reached 500 FU/min, a 33-fold induction of transcription. This

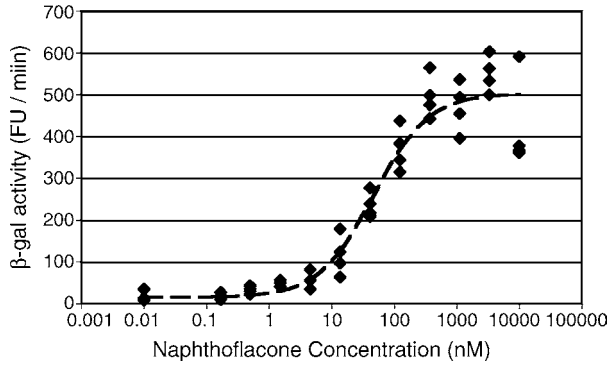

Fig. 5. Dose/response curve for naphthoflavone in the YCM-RYA system. Discontinuous line indicates the predicted curve from Eq. (1′), diamonds represent individual β-galactosidase values (four replicas per dilution point).

value is similar to reported values for different AhR-based RYA systems [19,22]. Fig. 5 also shows the reproducibility of the data at essentially all concentrations of ligand, with the exception of the highest concentration (10 μM), at which there is a possible toxic effect in three out of the four replicates.

3.4. Statistical analysis of detection limits

Limits of detection for kinetic determination of β-galactosidase activity can be calculated as the minimal slope values significantly different from those from negative controls. In the case of the ER-RYA, in which basal β-galactosidase activity is below detection limits, this probability equals to significance parameter σ, which indicates the probability of the calculated slope being zero. Fig. 6 shows a portion of the dose/response curve in Fig. 2 recalculated to plot relative β-galactosidase activity units against estradiol concentration. Symbols are coded relative to the σ values from the corresponding linear regression calculations. Significant slope values ($\sigma \leq 0.05$) corresponded to β-galactosidase values between 7 and 10% of the values at saturating estradiol concentration. We concluded that LOD values calculated with this method coincided approximately with EC_{10}, the ligand concentration which elicits 10% of the maximal

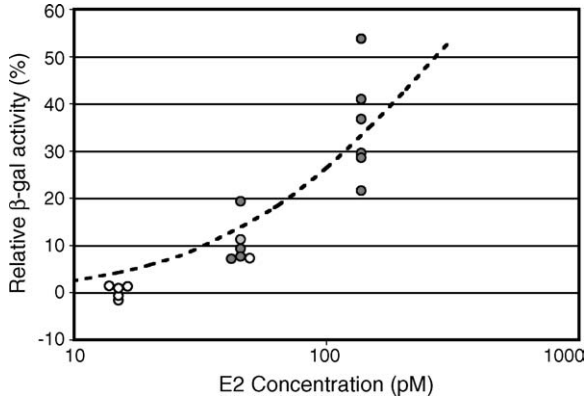

Fig. 6. Plot of relative activity values for ER-RYA at different estradiol concentrations. White, light, and dark grey circles indicate σ values for the corresponding slopes, as determined by the linear regression method: dark grey, $\sigma < 0.01$; light grey, $\sigma < 0.05$; empty circles, $\sigma > 0.05$ (non-significant).

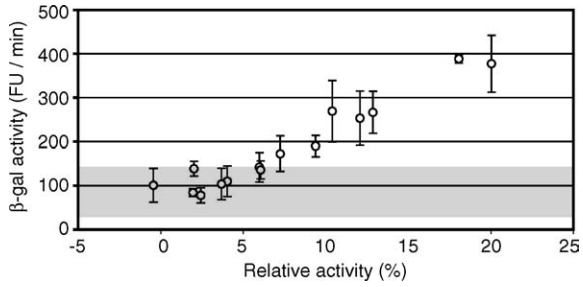

Fig. 7. Plot of relative (% of maximum activity) vs absolute (FU/min) β-galactosidase values for YCM-RYA systems at different β naphthoflavone concentrations. These data correspond to the left portion of the graph in Fig. 5. Bars indicate 95% confidence limits, calculated by non-linear regression methods. The dark sector indicates 95% confidence limits for basal β-galactosidase activity (eight determinations combined). β galactosidase values whose 95% confidence limits did not overlap with basal levels were considered statistically significant.

response. For estradiol, this value was calculated from Eq. (1′) as 34 pM (Table 1). Note that $\sigma \leq 0.001$ for most slope values in Fig. 6, indicating the robustness of the analysis.

Data from the YCM-RYA cannot be analysed in the same way as ER-RYA values, as in that case basal β-galactosidase activity levels were high enough (about 15 FU/min) to be statistically significant from zero (not shown, Fig. 5). Fig. 7 shows a plot of relative versus absolute β-galactosidase activity values with indication of the corresponding 95% significance levels. The gray sector in the plot indicates 95% confidence levels for basal transcription (four replicates combined). Differences between basal and tested levels became significant at relative β-galactosidase values higher than 7–10% of the maximal activation, similarly to what observed in the ER-RYA. We concluded that setting LOD values at EC_{10} might be a reasonable value for most RYA determinations. In YCM-RYA system, this corresponds to 5 nM naphthoflavone, similar to the value described for this system [19].

3.5. Analysis of inhibitory activity

The combination of different, fluorogenic RYA systems can also help on the characterization of specific hormone antagonists, i.e., compounds that prevent activation in the presence of a known agonist [10,23]. Genistein is a natural phytoestrogen, which acts as an agonist in ER-RYA (Fig. 8, top), with a calculated EC_{50} value of 2.9 ± 0.7 μM (Table 2). However, when tested in the YCM-RYA system, genistein acted as an antagonist, suppressing activation by β-naphthoflavone, with a calculated IC_{50} value of 29.9 ± 5.9 μM (Fig. 8, bottom). The regression parameters of the inhibition dose/response curve suggest that the antagonistic effect has a strong cooperative effect ($p = 4.5 \pm 3.0$), in contrast to the essentially non-cooperative agonistic response to ER-RYA (Table 2) This dual function of many natural flavones [24] may be central on their proposed beneficial health effect in vivo [25]. In nature, ligand binding to nuclear receptors elicits a delicate imbalance of activatory and inhibitory effects central to the physiological homeostasis of the living being [26]. Although most ligands to nuclear receptors act as pure agonists in RYA

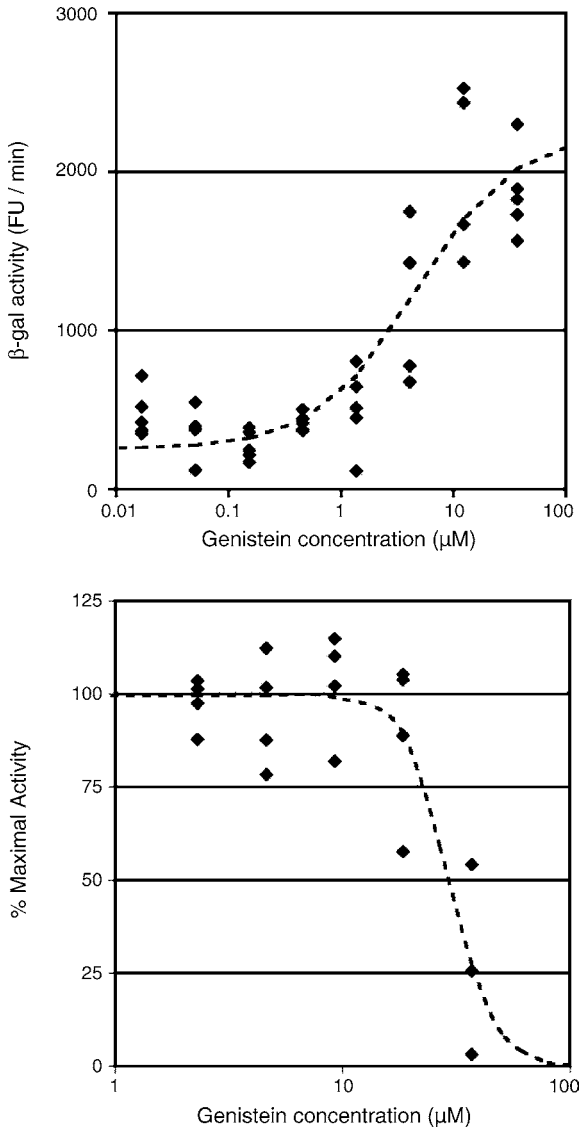

Fig. 8. Top: dose/response curve for genistein in the ER-RYA system. Discontinuous line indicates the predicted curve from Eq. (1′), diamonds represent individual activity values (five replicas for dilution point). Bottom: inhibitory effect of genistein in the YCM-RYA. Yeast cultures were incubated with 0.5 μM of β-naphthoflavone and different concentrations of genistein. Diamonds represent relative activation values, referred to the activity shown by cultures incubated with β-naphthoflavone alone. In both cases, discontinuous graphs represent theoretical dose/response curves as defined by Eqs. (1′) (top) and (2″) (bottom, see Table 2).

[23], different variants of RYA have been used to screen for hormone antagonists [2,10,23,27,28]. True antagonists for a given RYA system would display receptor specificity, i.e., they should be neutral or activators in a different RYA system like genistein in the example we show here. Antagonist effects could be easily mimetised or masked by cytotoxic effects, which prevent transcriptional activation to occur by inhibiting vital functions of the yeast cell. Is in this aspect where the use of very short incubation times in fluorogenic RYA methods shows it best advantage for the screening of antagonist effects, for they are typically increased by long exposure times. In the dose/response curves shown in Fig. 8, it is revealing that maximal activation in ER-RYA occurred at genistein concentrations similar to the IC_{50} in YCM-RYA.

4. General discussion

Detection of hormone ligands by yeast-based protocols encounters several problems when testing non-conventional samples. First, many of these samples can only be obtained in small amounts, limiting in this way both the maximal concentrations available and culture volumes. Second, toxicity and ligand activity occur at very similar concentrations in many cases. Third, and most important, yeast-based protocols have to be tailored for their use at medium and large-scale range, to cover the maximum number of samples and compounds at the smallest possible cost in both money and time. We try to cope with these problems by reducing culture volumes to 50 μL and shortening incubation times to the absolute minimum. Small culture volumes allow the use of very little amounts of sample and the use of microtitration plates, opening the possibility to mechanisation of the protocol. Short incubation times, in most cases shorter than division times in the conditions of the assay, imply that the ability of the cell culture to divide does not influence the total activity of the reporter, as opposite to protocols requiring overnight incubation, in which the growth rate of the culture represent a major source of variability. This is probably the main reason why the scatter of values from replicates of a same experiment is typically much smaller in shorter than in longer incubation protocols. It also minimizes the effect of toxic compounds, since only the one able to actively counteract cell metabolism (and not only cell division) would produce false negatives. There are several methods to screen out cytotoxins;

Table 2
Fitting of genistein dose/response data to cooperative and non-coopertive Hill equations

System	Model	Parameters				
		K		P		R^2
		Value (μM)	95% confidence limits	Value	95% confidence limits	
ER-RYA[b]	Hill (1′)	2.91	(1.19/4.62)	1.00[a]	–	0.736
	Cooperative Hill (2′)	2.83	(1.69/3.97)	0.90	(0.60/1.19)	0.736
YCM-RYA[c]	Hill (1″)	38.82	(9.24/68.41)	1.00[a]	–	0.500
	Cooperative Hill (2″)	29.91	(24.00/35.81)	4.52	(1.50/7.53)	0.740

[a] Set as invariant in this model.
[b] Agonistic effect.
[c] Antagonistic effect.

the one we previously proposed of a parallel assay monitoring activation of endogenous yeast promoter [2] is also very efficient and relative little time-consuming in fluorogenic RYA systems (not shown).

The proposed protocol incorporates three major improvements. The key aspect is the use of fluorogenic compounds instead of chromogenic ones, which increases the sensitivity on the detection of β-galactosidase activity by two to three orders of magnitude. This system has been previously reported for bacterial [29,30], mammalian and yeast systems [31]. Fluorescent detection has an intrinsic higher sensitivity when compared to absorbance, which allows short incubation times and small volumes of culture. It also allows the use of cell culture at the early exponential phase of growth (OD_{600} between 0.1 and 0.2); our experience is that these cultures are much more robust and produce better response to stimulus than late-exponential phase cultures (OD_{600} around 1), typically used for chromogenic methods. The combination of fluorescence detection and low OD_{600} cultures allows the precise determination of β-galactosidase activity not by end-point determination (as for Miller units), but by directly measuring the kinetics of appearance of the fluorescent product. This allows precise determination of background β-galactosidase levels, typically very low in many systems, coming from either leakiness of the reporter promoter or the presence of uncharacterized endogenous ligands. These background values were mathematically deducted from the test data in the final calculation of total ligand activity (term "B" in Eqs. (1) and (1′)). This combination of kinetics quantitation of β-galactosidase activity and calculation of total ligand activity by non-linear regression of dose-response curves greatly increases the precision of the assay, whereas allowing automatic processing, as it does not require continuous surveillance by any operator.

A common problem to all methods based in enzymatic reporters in yeast is the relatively low permeability of the yeast cell. The recent report [4] of a GFP-reporter assay for the estrogen receptor RYA that eliminates the need for substrates, since the reporter gene product is itself fluorescent. Although this could be a crucial advantage for some purposes, we believe that the combination of flexibility in the adaptability to different receptors and systems, short incubation times, statistical analysis and high sensitivity for the detection of the gene product is central on the quantitative estimation of estrogenic potential of compounds and environmental samples. The use of in situ permeabilisation of yeast cells is also a factor contributing to increase the precision of the assay. This protocol described in [2] adds a further step (and some 30 min) to the overall procedure. However, we consider that this apparent setback pays off: it allows detection of very weak activity levels and, in our hands, greatly increases reproducibility of replicates.

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