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Full length article

Development of a highly sensitive reporter gene cell line for detecting estrogenic activity (the ER Isjaki assay)

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ABSTRACT

Monitoring of estrogens in water sources faces significant challenges, as proposed changes in the European Union regulation for environmental protection of water bodies, compromise the ability of conventional analytical methods to detect low concentrations of estrogens. The proposed changes involve the decrease of the environmental quality standards for 17β-estradiol, estrone and 17α-ethinyl estradiol in surface waters and the obligation to monitor estrogenic substances in water bodies, using effect-based methods. In this study, the optimal experimental conditions for developing a novel and highly sensitive reporter gene assay were established. For this purpose, optimization of transfection plasmid concentration, exposure time and basement membrane matrix effect as well as assessment of assay reproducibility and relative effect potency of natural estrogens and estrogenic substances were conducted. With the optimal experimental conditions set as 5 ng per well in 96-well uncoated plates for plasmid transfection and 24 h exposure to treatments, the assay yielded an average sensitivity, measured as effect level 20 % for 17β-estradiol, estrone and 17α-ethinyl estradiol of 0.29, 1.36 and 0.02 pM, respectively. The assay showed a reproducibility variation of approximately 20 % and was able to differentiate the relative effect potency between 17α-ethinyl estradiol and 17β-estradiol with the capacity of detecting 17α-ethinyl estradiol with a high relative effect potency. Moreover, this assay is approximately 10–100 times more sensitive compared to the current state-of-the-art *in vitro* assays used to measure estrogenicity, indicating that the assay can be used to detect 17β-estradiol, estrone and 17α-ethinyl estradiol at the low levels needed to meet regulatory standards.

1. Introduction

Fresh water sources used to produce drinking water are under continuous pressure due to anthropogenic activities and increased urbanization. As a result, thousands of chemicals from domestic use, agricultural activities and industrial discharges are released into the environment. Among these substances, estrogenic compounds are a particularly challenging group of environmental pollutants (Gómez et al. 2021). Natural and synthetic estrogens, such as the endogenous sex hormones 17β-estradiol (E2), estrone (E1) and the synthetic contraceptive drug 17α-ethinyl estradiol (EE2), are very potent and cause adverse effects in the environment (Pereira et al. 2011) at very low concentrations ranging from ng/L to pg/L (Gómez et al. 2021). Moreover, other less potent estrogenic compounds such as the isoflavones genistein and daidzein found in soya-based products (Soukup et al. 2016), as well as the estrogenic compounds nonylphenol and bisphenol A (BPA) are known to activate the estrogen receptor (ER), thus triggering potential adverse effects on reproduction (Brand et al. 2013; Zhao et al. 2022).

A main source of estrogenic contamination in the aquatic environment is the discharge of wastewater effluents from wastewater treatment plants (Jarošová et al. 2014). Data have shown that removal efficiency of estrogens in wastewater treatment plants is insufficient in many cases, as natural estrogens in surface waters have been detected at pg/L concentrations (Ciślak et al. 2023), and estrogenic compounds have been detected in concentrations ranging from ng/L up to μg/L (Pereira et al. 2011; Dopp et al. 2021). Although the process for estrogen removal in drinking water treatment plants with advanced methods has proven efficiency in many cases (Choi et al. 2006), conventional wastewater and drinking water treatment plants are not designed to completely remove estrogens (Völker et al. 2019; Lundqvist et al. 2024).

To date, targeted chemical analysis is the most commonly used method to quantify the presence of well-known chemicals in surface and drinking water. However, this detection method does not account for

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unknown chemicals, the presence of chemicals that are bioactive below the chemical detection limit and mixture effects (Neale et al. 2022). Environmental quality standards (EQS) for E2, EE2 and E1 were proposed in 2022 in the European Union Commission's proposal for amending the Water Framework Directive, the Groundwater Directive, and the Directive on Environmental Quality Standards (European Commission, 2022). The proposed annual average EQS in inland waters were 0.18, 0.017 and 0.36 ng/L for E2, EE2 and E1, respectively, and the corresponding annual average EQS proposed for other surface waters were 0.009, 0.0016 and 0.018 ng/L. Such low levels are unlikely to be detected by chemical analyses. The lowering of the EQS was aimed at further protecting aquatic organisms and human health, given that estrogens are known to elicit adverse effects at very low concentrations (Ciślak et al. 2023). Commercially available methods for chemical analyses of E2, EE2 and E1 in water often have a limit of quantification around 1 to 5 ng/L, which means that they cannot be used for monitoring the compliance with EQS values, further highlighting the urgent need for more sensitive methods to detect estrogens in environmental samples.

In recent years, the use of effect-based methods as a tool to monitor estrogenic activity in complex environmental samples such as wastewater and drinking water, has gained noticeable popularity given their high sensitivity and capability for assessing the mixture effect of known and unknown substances (Escher et al. 2015). Among the several estrogenic activity assays for assessing water quality, yeast and mammalian reporter gene assays are commonly used (Könemann et al. 2018). For instance, the A-YES yeast bioassay sensitivity, defined from the effect concentration 10 % (EC_{10}) is around 12 ng E2/L (Leusch et al. 2017). Mammalian bioassays, also defined from the EC_{10}, such as ERα-CALUX (0.19 ng E2/L) (Escher et al. 2018), ERα-GeneBLAzer (1.23 ng E2/L) (Neale et al. 2023), T47D-KBluc (0.085 ng E2/L) (Leusch et al. 2017) and MELN (0.68 ng E2/L) (Escher et al. 2018) display higher sensitivity compared to yeast-based assays, for which these assays are preferred for water monitoring. Furthermore, it is a common practice to pre-concentrate samples using solid phase extraction to increase the analysis sensitivity (Neale et al. 2018). However, the proposed lowering of the EQSs for E2 and EE2 highlights the need for even more sensitive assays for estrogenic monitoring, to ensure that there are methods available to monitor these pollutants in the regulatory relevant concentrations.

To date, there is a growing interest in continuous monitoring of chemical pollutants in both drinking water and wastewater in the form of sensors, including proposals for effect-based biosensors. For instance, the US EPA has carried out a two-phase challenge to promote the development of effect-based sensors to detect toxicity in water. The technical development of such an effect-based biosensor could be accelerated if certain parts of the analytical process that are difficult to automatize were eliminated. Examples of such parts of the process involve the solid phase extraction and the cell lysis currently used in reporter gene assays.

The aim of this study was to develop a highly sensitive reporter gene assay allowing estrogenic reporter activity to be measured directly in the cell culture medium without requiring cell lysis or sample preconcentration, facilitating the possibility of automation of the detection and for the development of an effect-based biosensor.

2. Materials and methods

2.1. Culturing of MCF-7 cells

MCF-7 breast cancer cells (ATCC-HTB-22) were cultured in Dulbecco's Modified Eagle's medium (DMEM) high glucose, without glutamine and phenol red (GIBCO), supplemented with 10 % fetal bovine serum (FBS, GIBCO) and 50 units/ml of penicillin and 50 μg/ml of streptomycin (Pen-Strep, GIBCO). For estrogenic activity assays, MCF-7 cells were cultured in estrogen-free medium containing DMEM high glucose, without glutamine and phenol red, supplemented with 5 % dextran-coated charcoal treated (DCC, GIBCO) FBS and 50 units/ml of penicillin and 50 μg/ml of streptomycin for 48 h. Cells were cultured in a humidified environment at 37 °C and 5 % CO_2 at seeding density of $2x10^5$ cell/ml. For all experiments, cells were used in culturing passages ranging from passage 3 to 30.

2.2. Test compounds

The following chemicals were used through all the experiments performed in this study:

Compound	Supplier	CAS No.	Purity
17β-Estradiol	Sigma Aldrich	50-28-2	≥ 98 %
Estrone	Sigma Aldrich	53-16-7	≥ 99 %
17α-Ethynylestradiol	Sigma Aldrich	57-63-6	≥ 98 %
Daidzein	Sigma Aldrich	486-66-8	≥ 98 %
Genistein	Sigma Aldrich	446-72-0	≥ 98 %
Nonylphenol	Sigma Aldrich, PESTANAL®	84852-15-3	≥ 99 %
Bisphenol-A	Sigma Aldrich	80-05-7	≥ 99 %
Ethanol	Solveco Sweden	64-17-5	99.5 %
Dimethyl sulfoxide	Sigma Aldrich	67-68-5	≥ 99.9 %

2.3. pNL2.3-ERE[secNluc/Hygro] plasmid

The reporter gene plasmid was constructed by inserting four tandem copies of the synthetic estrogen response element (ERE) oligonucleotide, EREc38 (Tyulmenkov et al. 2000) into the pNL2.3 vector (Promega, Madison). The 38-bp ERE consensus sequence 5′ CCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG 3′ was synthesized and inserted into the provided pNL2.3 vector (pNL2.3[secNluc/Hygro] Vector GenBank® Accession Number JQ513380) by GenScript Biotech (Netherlands) to obtain the ER plasmid (*pNL2.3-ERE)*. To develop the ER-bioassay, MCF-7 cells were chosen for transfecting the pNL2.3-ERE [secNluc/Hygro] reporter gene plasmid since this cell line is ER positive, implying that ERα and β are naturally expressed in these cells (Liu et al. 1995; Powell et al. 2001). For the reporter assay, a secreted reporter was preferred to allow reporter detection without cell lysis. Therefore, the plasmid pNL2.3, which has an N-terminal section signal sewed to the NanoLuc luciferase, was chosen. Nano-Glo® luciferase Assay system (Promega) was used to measure luminescence from NanoLuc luciferase secreted directly into the cell culture medium.

2.4. Establishment of estrogen receptor-sensitive reporter assay in MCF-7 cells

Cells were seeded in transparent 96-well plates (Costar® Corning Incorporated) at a density of 10,000 cells per well in estrogen-free medium. The cells were incubated for 48 h (to ~ 50 % cell confluence) before they were transiently transfected with the estrogen receptor-sensitive reporter plasmid (*pNL2.3-ERE*). For all the assays, the plasmid DNA was delivered in 10 μl of Opti-MEM (1x) reduced serum medium (GIBCO, Thermo Fisher) with 0.3 μL Lipofectamine® 2000 reagent (Invitrogen, Thermo Fisher) per well, as recommended by the manufacturer.

Following transfection, cells were incubated for 24 h and then exposed to test compounds and ethanol vehicle control. After 24 h, the Nano-Glo® Luciferase Assay System (Promega) was performed in accordance with the manufacturer's protocol. In short, preparations for the Nano-Luc activity measurement were done by gently mixing the cell medium (to avoid gradient formation in the cell culture wells), and then transferring equal volumes (10 μL) of Milli-Q water, cell medium suspension and Nano-Glo® Luciferase Assay reagent altogether into a white-transparent bottom well-plate. Luminescence was measured using the Spark® Multimode Microplate Reader. Luminometer settings for the readings were set as wavelength range from 370 and 700 nm, integration time per well of 1000 ms and dynamic range between 10^7-10^9. The

luciferase activity was expressed as fold change compared to the vehicle control.

E2 dissolved in ethanol was used as a reference compound and analyzed in a dilution series with concentrations ranging from 0.0007 pM to 10 nM in 15-fold dilutions (except for plasmid optimization experiments). The final vehicle concentration was 1 % and equal in both all exposure groups and in the vehicle control.

2.4.1. Plasmid concentration and exposure time optimization

To optimize the sensitivity and overall quality of the assay, different plasmid concentrations and exposure times were evaluated. The plasmid concentration was optimized by transfecting the cells with 5, 10 and 15 ng per well of the plasmid, respectively. The transfection was performed as described above. The exposure time was optimized by exposing the cells to E2 for 6, 8, 16, 24 and 48 h, respectively. For both optimization experiments, cells were exposed to E2 ranging from 0.0007 pM to 10 nM in 3 or 15 fold dilution series. Luminescence was measured as described above. The effect concentration 20 % (EC_{20}) of E2 was then calculated as a measurement of assay sensitivity and maximum assay fold change, Z-factor and precision as other quality parameters.

2.4.2. Effects of plate coating

Experiments were carried out to evaluate if the assay sensitivity and overall quality could be improved by pre-coating the culture plates with basement membrane extract. For this procedure, the 96-well plates were pre-coated prior to cell seeding, with 0.1 mg/ml Geltrex Reduced Growth Factor Basement Membrane Matrix (GIBCO) per well as per manufacturer's instructions. Geltrex was prepared in cold PBS and 30 μL of this solution were added to each well. Plates were incubated at 37 °C for one hour and eventually PBS was removed. Next, cells were seeded, transiently transfected, exposed for 24 h to E2 concentrations ranging from 0.0007 pM to 10 nM in 15-fold dilutions and luminescence was measured as previously described. The EC_{20} of E2 was then calculated as a measurement of assay sensitivity as well as the aforementioned quality parameters.

2.5. Relative effect potencies assessment

For determining the assay's sensitivity to other estrogenic substances, the relative effect potencies (REP) for several estrogenic compounds were assessed. ER-activity was tested for EE2 and E1 (dissolved in ethanol) in concentrations ranging from 0.0007 pM to 10 nM in 15-fold dilutions. The estrogenic compounds daidzein, genistein, NP and BPA (dissolved in ethanol) were tested in concentrations ranging from 7 pM to 100 μM in 3-fold dilutions. E2 in concentrations ranging from 0.0007 pM to 10 nM in 15-fold dilutions was used as the reference compound to perform REP calculations for all estrogenic compounds. After transient transfection, cells were incubated with the compounds for 24 h and luminescence was measured as previously described.

2.6. Cell viability

In order to confirm that none of the estrogenic compounds were inducing cytotoxicity, cell viability was assessed using MTS (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega). Following transfection and incubation with the compounds and, 20 μL of CellTiter 96 AQueous One Solution Reagent was added to each well and plates were incubated at 37 °C for one hour and thirty minutes. Absorbance was measured at 490 nm on a Spark® Multimode Microplate Reader. DMSO 15 % was used as a positive control for cytotoxicity.

2.7. Data evaluation

All experiments were performed in at least two independent experiments (inter-assay replicates) with three to six technical replicates per exposure concentration. To quantify ER-activity, luminescence readings from technical replicates were normalized to the average of the vehicle control. The normalized average value from the highest concentration was set as the maximum assay fold change. Next, data was normalized to zero by subtracting the vehicle control mean fold change (1.0) from all data points. Finally, values were normalized to the average response of the highest concentration of each test compound. The maximum assay response was set to 100 %. The mean and standard deviation of all experimental assessments presented in the plots were calculated using the data from all technical replicates per concentration from all the independent experiments.

For cell viability assays, absorbance measurements were normalized to the vehicle control mean, defining 100 % cell viability and cytotoxicity defined as < 80 %. Concentrations below 80 % viability were excluded from further data analysis. The software GraphPad Prism (v. 10.3.0) was used to generate concentration–response curves from normalized data, cell viability bar plots as well as to determine the EC_{20} (parameter used to define the assay's sensitivity) and effect concentration 50 % (EC_{50}) values for all experiments by performing non-linear sigmoidal regression.

To calculate REP, the EC_{20} or EC_{50} value of E2 was divided by the EC_{20} or EC_{50} value of every assessed estrogenic compound. REP values were calculated for every independent experiment. As an evaluation of assay quality, the Z-factor (separation between vehicle control, and highest concentration responses) was calculated using the assay's fold change values. For the Z-factor calculation, the SD (σ) and average (μ) of the vehicle control and highest concentration were used as described by (Zhang et al. 1999) in Equation (1). It is desirable to reach a Z-factor between 0.5 and 1.

$$\mathrm{Z-factor} = 1 - \frac{3x\,(\sigma\,\text{highest concentration} + \sigma\,\text{vehicle control})}{(\mu\,\text{highest concentration} - \mu\,\text{vehicle control})} \quad (1)$$

As a measurement of the assay precision and repeatability, inter-assay variation or coefficient of variation of repeatability (CVr) was calculated using the SD and mean of EC_{20} values as described in Equation (2). It is desirable to have a variation ≤ 20 %.

$$\mathrm{CVr} = \frac{\sigma\ EC_{20}}{\mu\ EC_{20}} \times 100 \quad (2)$$

3. Results

3.1. Cytotoxicity

All test compounds were screened for cytotoxicity. In the case of E2 and EE2, none of them exerted cytotoxicity at any of the tested concentrations (Supplementary Material, **Fig. S1** a and c). For E1, a slight dose-dependent decrease was observed, but since viability at the highest concentrations was not below 80 % these were considered non-cytotoxic (Supplementary Material, **Fig. S1** b). In the case of estrogen-like compounds, daidzein and genistein were not cytotoxic at any of the tested concentrations (Supplementary Material **Fig. S2** a and b). On the other hand, NP elicited cytotoxicity at the highest concentrations (33 000 and 100 000 nM) decreasing cell viability by 23 % and 70 % respectively (Supplementary Material **Fig. S2** c). For BPA, the highest concentration (100 000 nM) exerted cytotoxicity by decreasing viability by 23 % (Supplementary Material **Fig. S2** d). For subsequent experiments, the test compounds' concentrations inducing cytotoxicity greater than 20 % were excluded in order to ensure the quality and reliability of the data.

3.2. Plasmid concentration optimization

Different plasmid concentrations, 5, 10 or 15 ng per well (E2 exposure for 24 h) were tested in order to assess which of these yielded the best sensitivity, highest maximum assay fold change, highest assay quality (Z-factor ≥ 0.5) as well as a CVr of ≤ 20 % in repeatability. Data showed that transient transfection with 5 ng of plasmid per well resulted

in a sensitivity of 0.25 pM (E2 EC_{20}), a maximum assay fold change of 4.0, assay quality with a Z-factor of 0.6 and a CVr of 9 % (Fig. 1 a, Table 1, **Fig. S3** a). On the other hand, plasmid transfection with 10 ng per well, displayed higher sensitivity (0.16 pM) than 5 ng per well. However, the maximum assay fold change (2.5), assay quality (0.1) and repeatability variation (42 %) values (Fig. 1 b, Table 1, **Fig. S3** b) indicated that transfection with this plasmid concentration does not meet the assay requirements to consider it as the optimal transfection concentration. The same trend is observed for plasmid transfection with 15 ng per well, in which an even higher sensitivity of 0.05 pM was observed, but a decreased maximum assay fold change of 1.7, quality factor of −0.8 and CVr of 55 % are observed (Fig. 1 c, Table 1, **Fig. S3** c). In a similar manner to 10 ng per well, this plasmid concentration does not meet the assay requirements to be considered the optimal transfection concentration. Consequently, transfection with 5 ng per well resulted as the optimal concentration to achieve the best sensitivity coupled with the highest assay parameters.

3.3. Exposure time optimization

Several exposure times, 6, 8, 16, 24 and 48 h were assessed after plasmid transfection with 5 ng per well, to determine which of these elicited the best sensitivity, highest maximum assay fold change, highest assay quality (Z-factor $\geq$ 0.5) as well as a variation of $\leq$ 20 % in repeatability. Results showed that E2 exposure for 6 and 8 h exhibited the lowest sensitivity (10.24 pM and 2.11 pM, respectively), maximum assay fold change (1.7 and 2.0) and assay quality (−0.9 and 0.1). However, the CVr was less than 20 % (18 % and 3 %, respectively) (Fig. 2 a and b, Table 1, **Fig. S4** a and b). In contrast, exposure for 16 h displayed a higher sensitivity of 0.13 pM, an increased maximum assay fold change of 2.7 and quality factor of 0.2, as well as a CVr of 10 % (Fig. 2 c, Table 1, **Fig. S4** c). The exposure time of 24 h resulted in a sensitivity of 0.39 pM, a higher maximum assay fold change of 3.6 and assay quality factor of 0.5 and CVr of 11 % (Fig. 2 d, Table 1, **Fig. S4** d). Finally, data from 48 h exposure, exhibited a decreased sensitivity of 1.62 pM, a maximum assay fold change of 4.8, assay quality factor of 0.5 and CVr of 20 % (Fig. 2 e, Table 1, **Fig. S4** e). Although E2 exposure for 24 h was less sensitive compared to the 16-hour exposure, the higher assay fold change and quality showed that data obtained from this exposure time is more reliable. Thereby, we concluded that a 24-hour exposure provided the best balance between assay sensitivity and the other assay quality parameters.

3.4. Effects of plate coating

The effect that matrix basement membrane coating could have on the assay sensitivity after the optimization of plasmid concentration (5 ng per well) and exposure time (24 h) was evaluated by pre-coating 96-well plates with Geltrex basement membrane containing laminin and collagen IV. Data showed that plate pre-coating did not increase the assay's sensitivity as it exhibited an E2 EC_{20} of 0.45 pM, a maximum assay fold change of 2.0, an assay Z-factor of 0.2 and CVr of 3 % (Fig. 3 a, Table 1, **Fig. S5** a) while cell seeding in uncoated plates resulted in a higher sensitivity (0.32 pM) and maximum assay fold change (3.0), quality factor (0.6) and CVr of 7 %. (Table 1). As there were no main advantages with the pre-coating, we concluded that non-coated plates were preferred to achieve the best sensitivity and assay quality parameters.

Table 1
Summary of optimization parameters for MCF-7 cells transiently transfected with the *pNL2.3-ERE* plasmid. The values display EC_{20}, maximum assay fold change, assay quality (Z-Factor) and precision (CVr) for plasmid concentration optimization, treatment exposure optimization and matrix effect. All values are presented as the average of multiple experiments (two independent experiments with three to six technical replicates concentration, n = 6–12). CVr values were calculated from EC_{20} values.

Plasmid concentration optimization	Concentration per well (ng)[a]	E2 EC_{20} (pM)	Maximum assay fold change	Z-factor	CVr (%)
	5*	0.25	4.0	0.6	9
	10	0.16	2.5	0.1	42
	15	0.05	1.7	−0.8	55
Treatment exposure optimization	**Exposure time (hrs)[b]**	**E2 EC_{20} (pM)**	**Maximum assay fold change**	**Z-factor**	**CVr (%)**
	6	10.24	1.7	−0.9	18
	8	2.11	2.0	0.1	3
	16	0.13	2.7	0.2	10
	24*	0.39	3.6	0.5	11
	48	1.62	4.8	0.5	20
Matrix effect	**Coating[b]**	**E2 EC_{20} (pM)**	**Maximum assay fold change**	**Z-factor**	**CVr (%)**
	Non-coated*	0.32	3.0	0.6	7
	Geltrex-coated	0.45	2.0	0.2	3

[a] E2 concentration range 0.0007–10,000 pM in 3-fold dilutions.
[b] E2 concentration range 0.0007–10,000 pM in 15-fold dilutions.
* Optimal assay conditions chosen for further sensitivity and quality assessments.

3.5. Assay reproducibility

To evaluate the reproducibility of this assay, a dilution-series of E2 was run in eight independent experiments. The results showed that the average sensitivity (E2 EC_{20}) of these eight independent experiments was 0.29 ± 0.063 pM (mean ± SD) with a CVr of 22 %, which is close to

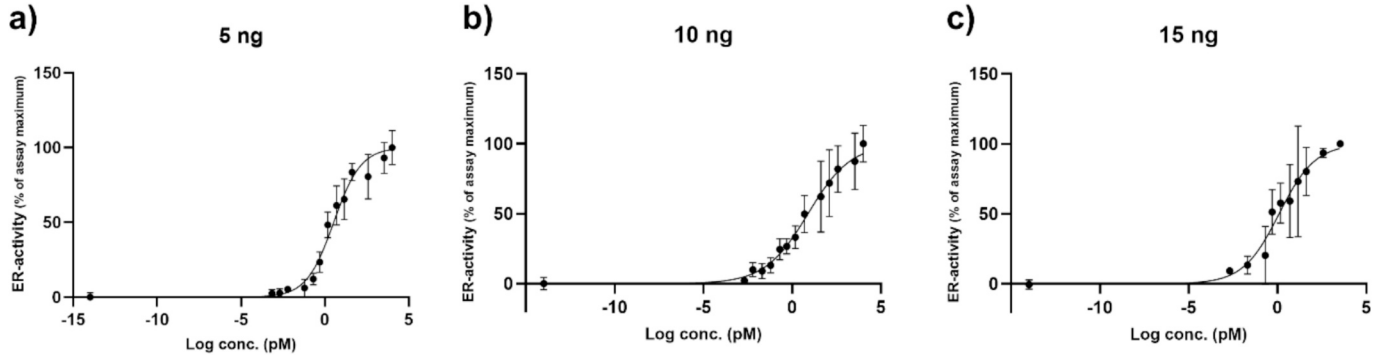

Fig. 1. Plasmid concentration optimization in MCF-7 cells transiently transfected with the *pNL2.3-ERE* plasmid. Three different plasmid concentrations **a)** 5 ng per well, **b)** 10 ng per well and **c)** 15 ng per well were tested in cells exposed for 24 h to E2 in concentrations ranging from 0.0007 pM to 10 nM with a 3-fold dilution factor (two independent experiments with three technical replicates per concentration). Data is presented as mean ± SD, n = 6.

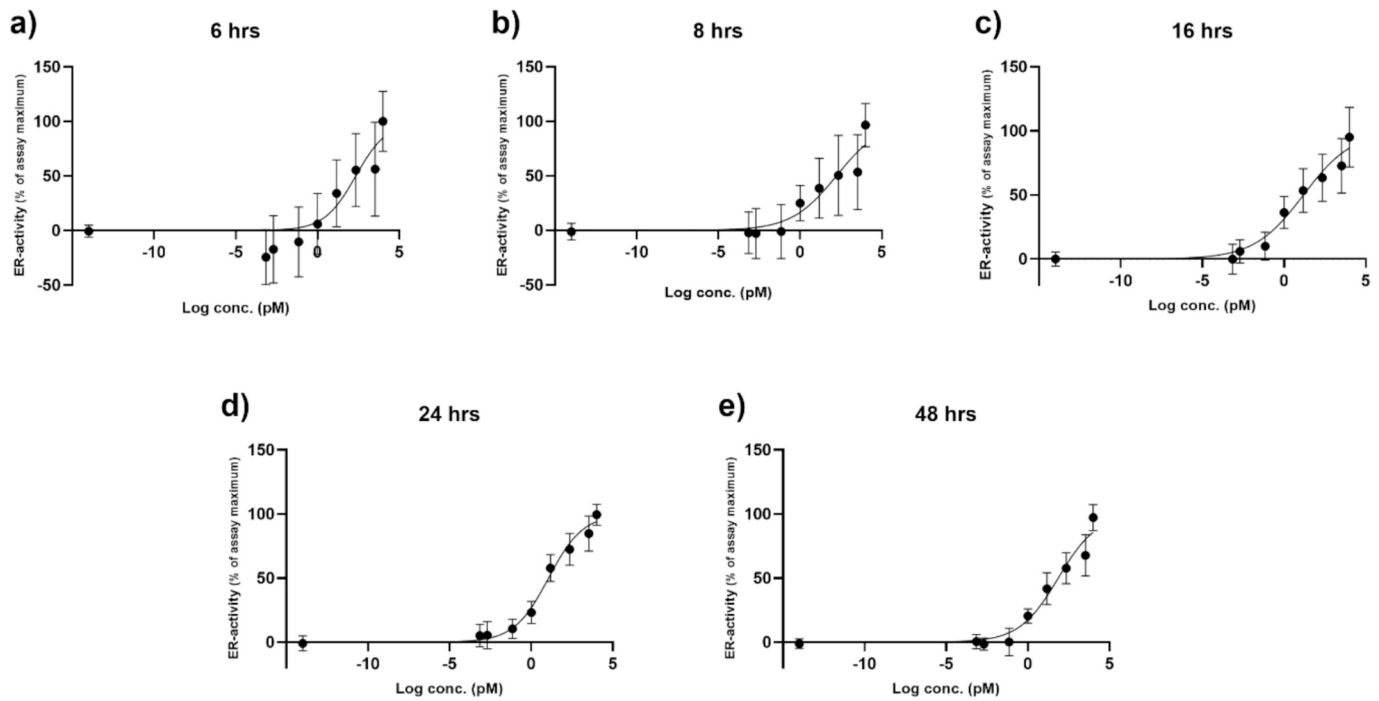

Fig. 2. Treatment exposure optimization. The assays' detection sensitivity for ER-activity was assessed at different exposure times **a)** 6 hrs., **b)** 8 hrs., **c)** 16 hrs. **d)** 24 hrs., and **e)** 48 hrs. in cells transiently transfected with 5 ng per well of plasmid and exposed to E2 in concentrations ranging from 0.0007 pM to 10 nM with a 15-fold dilution factor (two independent experiments with six technical replicates per concentration). Data is presented as mean ± SD, n = 12.

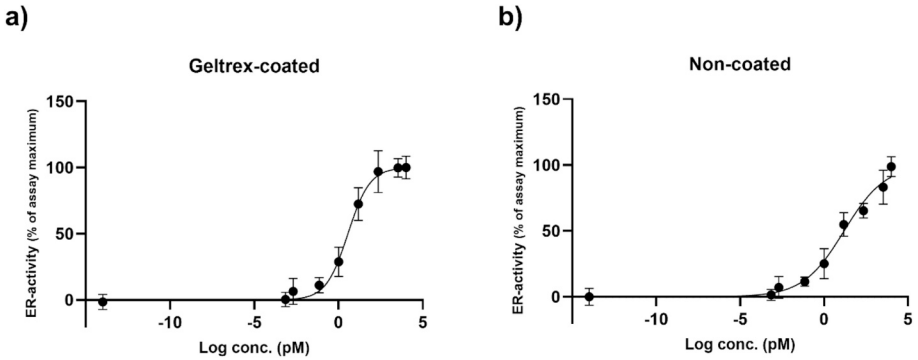

Fig. 3. Effects of plate coating on the assays' detection sensitivity for ER-activity in **a)** 96-well plates pre-coated with 30 μL/well Geltrex basement membrane extract and **b)** non-coated 96-well plates. Cells were transiently transfected with 5 ng per well of plasmid and exposed to E2 for 24 h in concentrations ranging from 0.0007 pM to 10 nM with a 15-fold dilution factor (two independent experiments with six technical replicates concentration). Data is presented as mean ± SD, n = 12.

the acceptable variation limits. Furthermore, the plot in Fig. 4 shows that all E2 dose–response curves intersect at the 20 % effect level, indicating that that the EC_{20} is a suitable metric for this assay.

3.6. Relative effect potencies of estrogens and estrogen-like compounds

To calculate the REPs of the estrogens E1 and EE2, the EC values of these compounds were compared with the EC values of E2 (used to define REP 1) (Supplementary Material **Fig. S6** a, Table 2). For E1, the EC_{20} average was 1.36 pM and the calculated REP (0.25) showed that this estrogen is around 4 times less potent than E2. The EC_{50} average was 57.54 pM and the calculated REP (0.31) was around 3 times less potent than E2. (Supplementary Material **Fig. S6** b, Table 2). In the case of EE2, the average EC_{20} was 0.02 pM and the calculated REP displayed that this well-known contraceptive is approximately 23 times more potent than E2. For the EC_{50} average (2.44 pM), the calculated REP was around 15 times more potent than E2 (Supplementary Material **Fig. S6** c, Table 2). All the experiments with these estrogenic compounds had an average maximum assay fold change of 3.0 and an average Z-factor of 0.5.

The REPs were also calculated for the estrogen-like compounds daidzein, genistein, NP and BPA (Table 3). Overall, our data showed that all the aforementioned compounds were more than 2000 times less potent than E2. For daidzein, the EC_{20} average was 2337 pM and the calculated REP was 0.000147. (Supplementary Material **Fig. S6** d, Table 3). In the case of genistein, the EC_{20} average was 4430 pM and the calculated REP was $7.9x10^{-5}$. (Supplementary Material **Fig. S6** e, Table 3). Data from NP exposure showed that the average EC_{20} was 15547 pM and the REP value was of $2.3x10^{-5}$. (Supplementary Material **Fig. S6** f, Table 3). Finally, the EC_{20} average for BPA was 8580 pM and the calculated REP was $4x10^{-5}$. (Supplementary Material **Fig. S6** g, Table 3).

4. Discussion

The goal of the present study was to establish an estrogen sensitive reporter gene assay that is a) highly sensitive and thereby supporting the

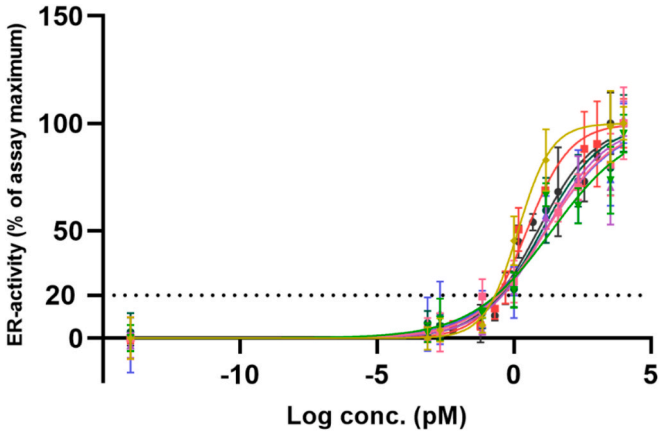

Fig. 4. Reproducibility assessment for ER-activity assay. The dotted line is showing the assay's 20 % effect level. Cells were transiently transfected with 5 ng per well of plasmid and exposed for 24 h to E2 in concentrations ranging from 0.0007 pM to 10 nM with a 3 or 15-fold dilution factor (eight independent experiments with three to six technical replicates per concentration). Data is presented as mean ± SD, n = 3–6.

Table 2
Summary of REP of E1, EE2 and E2 for MCF-7 cells transiently transfected with 5 ng per well of the *pNL2.3-ERE* plasmid and exposed for 24 h to test compounds. The values display EC_{20} in pM, REP (EC_{20}), EC_{50} in pM, REP (EC_{50}), maximum assay fold change and Z-Factor for E1, EE2 and E2. Values display data from individual experiments and the average and SD of all experiments (four independent experiments, six technical replicates per concentration). REP values were calculated by dividing the EC_{20} or EC_{50} value from E2 with the EC_{20} or EC_{50} value from the assessed compound.

Compound	EC_{20} (pM)	REP (EC_{20})	EC_{50} (pM)	REP (EC_{50})	Maximum assay fold change	Z-factor
E1	0.88	0.27	55.85	0.24	2.0	0.4
	2.25	0.16	73.84	0.31	3.0	0.4
	1.44	0.24	59.06	0.44	4.0	0.6
	0.86	0.33	60.48	0.24	2.5	0.6
Average	**1.36**	**0.25**	**57.54**	**0.31**	**3.0**	**0.5**
SD	0.65	0.07	7.92	0.09	–	–
EE2	0.01	34.57	0.50	26.30	2.0	0.5
	0.02	21.56	3.98	9.35	2.0	0.5
	0.01	28.15	1.29	17.69	3.0	0.5
	0.04	8.58	4.60	5.65	4.0	0.6
Average	**0.02**	**23.21**	**2.44**	**14.75**	**3.0**	**0.5**
SD	0.01	11.11	2.00	9.20	–	–
E2	0.24	1.0	13.15	1.0	2.0	0.5
	0.37	1.0	23.12	1.0	3.0	0.4
	0.34	1.0	26.90	1.0	2.5	0.4
	0.28	1.0	14.73	1.0	4.5	0.5
Average	**0.31**	**1.0**	**19.22**	**1.0**	**3.0**	**0.5**
SD	0.06	–	9.70	–	–	–

monitoring of estrogenic pollutants at the very low concentrations that are regulatory relevant such as the proposed new EQSs for E2, EE2 and E1, and b) has assay characteristics that facilitate automated assay performance allowing for measurement of reporter activity without cell lysis or sample pre-concentration. We conclude that we have developed an assay that meets both these criteria, with a sensitivity around 10–100 times higher than what has been reported for most other mammalian-based assays (Escher et al. 2015; Escher et al. 2018; Gómez et al. 2021; Robitaille et al. 2022; Neale et al. 2023). Optimized assay conditions resulted in an assay sensitivity (EC_{20} of E2) of 0.29 pM ± 0.06, corresponding to 0.08 ng/L, while maintaining a good assay quality (Z-factor of 0.5) and precision (CVr ≤ 20 %). Although higher Z-factor values (closer to 1.0) are preferred for reporter-gene assays, it is expected that the development of a stably transfected cell line will decrease the variability between technical replicates and thus increase the quality of the assay. Furthermore, it is worth to highlight that the assay developed displays a notably high REP of EE2, a characteristic that is in line with *in vivo* studies carried out in fish of REP of estrogens, while many other cell-based assays show a lower REP of EE2.

Estrogens can cause adverse reproductive effects in aquatic biota at very low concentrations (Nash et al. 2004; Caldwell et al. 2012; Rehberger et al. 2020), which is reflected by the low proposed EQS for E2, EE2 and E1 in the European Union (European Commission, 2022). This has led to a situation where the routinely used targeted chemical analyses are not nearly sensitive enough in regulatory relevant concentration. Mammalian cell-based *in vitro* assays are more sensitive than chemical analysis for detecting these compounds, but also the most commonly used *in vitro* assays are struggling to detect E2 and EE2 in the concentrations needed to monitor the proposed new EQSs. The most commonly used mammalian cell-based *in vitro* assays for estrogenicity have reported sensitivities, based on the EC_{10} of E2, in the range of 0.2 – 2.7 ng/L (Van den Belt et al. 2004; Escher et al. 2018; Neale et al. 2023) with a single assay reporting an EC_{10} of 0.085 ng/L (Neale et al. 2023). While we prefer to evaluate the sensitivity of our assay on the EC_{20} values, we have also calculated the EC_{10} values for E2 and EE2 in our assay, to allow for a direct comparison of sensitivity with literature data. The assay developed here has an EC_{10} of 0.009 ng/L for E2, 0.0004 ng/L for EE2 and 0.04 ng/L for E1, showing that the sensitivity of the developed assay is around 10–100 higher than the currently most used *in vitro* assays for estrogenic compounds.

While it has been reported that estrogenic effects in water samples can be analyzed without sample pre-concentration in some cases (Niss et al. 2018; Johnson et al. 2024), it is common practice to increase the assay sensitivity by pre-concentrating the samples before analysis (Neale et al. 2018). With the sensitivity of the currently available effect-based methods, solid-phase extraction of samples has been essential to allow the detection of estrogens in the low concentrations where they start to exert adverse effects (Neale et al. 2018; Brack et al. 2019). However, to allow for the development of an effect-based biosensor, it would be very beneficial if the need for sample pre-concentration could be avoided. The assay developed here has a sensitivity of 0.08 ng/L for E2 and 0.006 ng/L for EE2, indicating that it is sensitive enough to monitor E2 and EE2 at the proposed EQS levels 0.18 ng/L and 0.017 ng/L, respectively for inland surface waters and even the EE2 EQS level of 0.0016 ng/L in other waters (European Commission, 2022), without pre-concentration of samples. However, sample pre-concentration would still be needed to reach the sensitivity needed to monitor the E2 at the proposed EQS level 0.009 ng/L for other waters (SCHEER, 2022).

The REPs of estrogens vary greatly (Könemann et al. 2018; Robitaille et al. 2022). It has been reported from *in vivo* studies in fish that EE2 is around 30 times more potent than E2 (Van den Belt et al. 2004; Rehberger et al. 2020), but many *in vitro* bioassays for estrogenicity have failed to achieve this high REP for EE2. The assay developed in this study does, unlike previous *in vitro* assays for estrogenicity, show a clearly higher potency of EE2 than E2, similarly to the REP observed *in vivo*. However, data from the EASZY assay based on zebrafish embryos displayed that EE2 is 87 times more potent than E2, which shows a much higher potency than our assay and other fish assays based on vitellogenin and ovarian somatic index (Brion et al. 2019). Our data calculated from EC_{20} showed that EE2 was on average 23 times more potent than E2. Other *in vitro* bioassays for estrogenicity has reported much lower REP values for EE2, for instance HELN-ERα (REP 2.13) (Escande et al. 2006), ER-CALUX (REP 1.86) (Brand et al. 2013), MVNL (REP 1.60) (Van den Belt et al. 2004) and E-SCREEN (REP 1.25) (Gutendorf and Westendorf 2001).

Regarding the potency of E1, a female sex hormone that is the most frequently detected estrogen with the highest concentrations in various water sources, thus, representing the main driver of ER-activity in water

Table 3
Summary of REP of daidzein, genistein, NP, BPA and E2 for MCF-7 cells transiently transfected with 5 ng per well of the *pNL2.3-ERE* plasmid and exposed for 24 h to test compounds. The values display EC_{20} in pM, REP (EC_{20}), EC_{50} in pM, REP (EC_{50}), maximum assay fold change and Z-Factor for daidzein, genistein, NP, BPA and E2. Values display data from individual experiments and the average and SD of all experiments (three independent experiments, six technical replicates per concentration). REP values were calculated by dividing the EC_{20} or EC_{50} value from E2 with the EC_{20} or EC_{50} value from the assessed compound.

Compound	EC_{20} (pM)	REP (EC_{20})	EC_{50} (pM)	REP (EC_{50})	Maximum assay fold change	Z-factor
Daidzein	2173	0.00013	53,740	0.00027	2.3	0.3
	2526	0.00015	63,880	0.00033	2.4	0.4
	2312	0.00016	42,080	0.00062	2.3	0.6
Average	**2337**	**0.00015**	**53,233**	**0.00049**	**2.3**	**0.4**
SD	178	0.00002	10,909	0.00018	–	–
Genistein	3243	8.7×10^{-5}	43,210	0.00034	2.0	0.3
	5191	7.1×10^{-5}	195,200	0.00011	2.0	0.5
	4855	7.8×10^{-5}	64,610	0.00040	2.0	0.4
Average	**4430**	$\mathbf{7.9 \times 10^{-5}}$	**101,007**	**0.00028**	**2.0**	**0.4**
SD	1041	7.9×10^{-6}	82,273	0.00016	–	–
NP	18,475	1.5×10^{-5}	273,200	0.00005	2.0	0.1
	14,500	2.6×10^{-5}	105,300	0.00020	2.0	0.1
	13,665	2.8×10^{-5}	141,100	0.00018	2.0	0.1
Average	**15,547**	$\mathbf{2.3 \times 10^{-5}}$	**173,200**	**0.00015**	**2.0**	**0.1**
SD	2570	6.7×10^{-6}	88,433	0.00008	–	–
BPA	8992	3.1×10^{-5}	109,700	0.00013	2.0	0.5
	8812	4.2×10^{-5}	150,700	0.00014	3.0	0.7
	7936	4.8×10^{-5}	139,300	0.000188	2.0	0.5
Average	**8580**	$\mathbf{4.0 \times 10^{-5}}$	**133,233**	**0.00015**	**2.3**	**0.6**
SD	565	8.4×10^{-6}	21,163	0.00003	–	–
E2	0.28	1.0	14.73	1.0	3.5	0.5
	0.37	1.0	21.26	1.0	3.0	0.7
	0.38	1.0	26.01	1.0	3.0	0.3
Average	**0.34**	**1.0**	**20.63**	**1.0**	**3.2**	**0.5**
SD	0.05	–	5.67	–	–	–

samples (Conley et al. 2017), our data showed that this estrogen was 4 times less potent than E2. However, different bioassays have reported E1 REP values that can vary from 3 up to 100 times less potency than E2 (REP 0.01 – 0.29) (Gutendorf and Westendorf 2001; Brand et al. 2013; Könemann et al. 2018). Another study using the EASZY assay showed that E1 is around 2 times less potent than E2 *in vivo*, which is similar to our reported data (Brion et al. 2019). Nevertheless, data from the MVNL assay (REP 0.29) (Könemann et al. 2018), shows the closest similarity in potency with our study (REP 0.25). In the case of the potency of the estrogen-like compounds, daidzein, genistein, NP and BPA, our data showed that all these chemicals are more than 2000 times less potent than E2, which is in agreement with reported data on the weak potency of these compounds. For instance, the potency range for daidzein has been reported at REP 0.0001 in HELN-ERα cells, which is in agreement with our data (REP 0.0004) (Escande et al. 2006). Genistein's REP in the ER-CALUX assay (REP 0.0002) (Brand et al. 2013) is also within our data range (REP 0.0003). However, the reported REP of NP (REP 0.00002) and BPA (REP 0.00002) in the ER-CALUX assay (Houtman et al. 2006), showed a lower potency than the results obtained with our assay (NP 0.0001; BPA 0.0002).

To date, all available mammalian-based *in vitro* assays for estrogenicity require cell lysis before measurement of reporter activity. A novel feature in our assay is that the reporter protein is excreted into the cell media, thus allowing measurements without the need for cell lysis. The aforementioned, together with the fact that the cells are sensitive enough to allow detection of E2, EE2 and E1 at regulatory relevant concentrations without the need for sample pre-concentration, is a key characteristic that will be very important to allow automation of the detection system. That in turn, it will be of great importance for the development of an effect-based biosensor that would allow continuous monitoring and rapid detection of estrogenic contaminants in for example wastewater treatment plants and waterworks.

5. Conclusion

In this study, we report the development and optimization of a new estrogen sensitive reporter gene assay that is approximately 10–100 times more sensitive compared to the current state-of-the-art *in vitro* assays used to measure estrogenicity. The high sensitivity indicates that the cells can be used to detect E2, EE2 and E1 at the low levels that are needed for regulator relevance, even without sample pre-concentration. That assay feature, combined with the fact that the reporter activity can be measured without cell lysis, is highlighting that the developed assay is very suitable for the development of an effect-based biosensor as it increases the possibility of automatized analysis. Further, the developed assay has the capacity to detect EE2 with a high REP, in line with the REP observed *in vivo*, something that the current *in vitro* assays for estrogenicity has failed to do.

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CRediT authorship contribution statement

Aline Colonnello Montero: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Geeta Mandava:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Agneta Oskarsson:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Johan Lundqvist:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Johan Lundqvist and Agneta Oskarsson are the founders of and stock-owners in BioCell Analytica Uppsala AB, a company providing effect-based testing services to the water sector. Geeta Mandava is an employee of the same company. Further, Johan Lundqvist and Agneta Oskarsson are the inventors of the Swedish patent *"Effect-Based Biosensor Comprising Reporter Cells for Water Analysis"*.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109635.

Data availability

Data will be made available on request.

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