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Critical parameters in the MCF-7 cell proliferation bioassay (E-Screen)

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The MCF-7 cell proliferation bioassay has grown in popularity as a rapid test for detecting potentially oestrogenic compounds. Several MCF-7 cell sublines with different sensitivities to oestrogens are currently used, with maximal proliferation responses ranging from two- to 10-fold above those of hormone-free controls. In the highly responsive MCF-7 BUS cell line, we evaluated critical assay parameters for test performance, including growth conditions, initial seeding densities and differences in growth stimulation in medium containing human serum or fetal calf serum as well as appropriate solvents for oestrogen-mimicking compounds. Modifications significantly reduced the labour-intensive steps and overall assay costs without affecting the sensitivity of the assay. Using this optimized test regimen, the responsiveness of treated MCF-7 BUS cells was consistently increased up to 11-fold over hormone-free controls. The specificity was characterized by examining the effects of oestradiol-17β, the anti-oestrogen ICI 182,780, and dieldrin, a recognized xeno-oestrogen. The improved proliferation bioassay will be a useful tool in identifying potential xeno-oestrogens.

Keywords: E-Screen, cell proliferation, MCF-7 cells, xeno-oestrogens, SRB assay, dieldrin.

Abbreviations: CT-FCS, charcoal-dextran treated fetal calf serum; CT-HuS, charcoal-dextran treated human serum; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; E2, oestradiol-17β; EDTA, ethylene diamine tetra-acetic acid; ER, oestrogen receptor; ERE, oestrogen responsive element; EtOH, ethanol; FCS, fetal calf serum; PBS, phosphate buffered saline; SRB, sulphorhodamine B; TCA, trichloroacetic acid.

Introduction

Exposure to xeno-oestrogens has been linked to abnormal sexual development in wildlife, developmental disorders of the male reproductive tract, and increasing incidence of breast and testicular cancer in humans (Fry 1995, Sumpter 1995, Folmar *et al*. 1996, Toppari *et al*. 1996). A battery of *in vitro* tests has recently been suggested as screening tools for suspected oestrogenic chemicals (US Environmental Protection Agency 1998). Assays include oestrogen receptor (ER) binding, ER-dependent transcription systems and proliferation of oestrogen-dependent cell lines (e.g. MCF-7 cells). Because human exposures often include many potential xeno-oestrogens, the joint effect may be difficult to predict. Efforts have therefore been initiated to develop a biomarker using a cell-based system to assess the oestrogenic activity of a serum sample. The MCF-7 cell proliferation bioassay (E-

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Screen) originally described by Soto *et al.* (1995) has grown in popularity as a rapid test for detecting weakly oestrogenic compounds. The E-Screen assay is based on the ability of oestrogenic compounds to induce proliferation of the oestrogen-sensitive human breast cancer cell line MCF-7. The proliferative response of MCF-7 cells is a sensitive endpoint suitable for screening chemicals because of the fairly reproducible and stable oestrogen sensitivity (Fang *et al.* 2000). Furthermore, oestrogen agonists and antagonists can be differentiated using this method (Soto *et al.* 1998). These features make the E-Screen assay one of the most sensitive assays available for assessing and comparing the oestrogenicity of oestrogen-mimicking compounds.

The maximal proliferative responses of MCF-7 cells induced by oestradiol-17β (E2) vary between laboratories from approximately two-fold up to approximately 10-fold above those of hormone-free controls (Soto *et al.* 1995, Brotons *et al.* 1995, Villalobos *et al.* 1995, Andersen *et al.* 1999, Schafer *et al.* 1999, Lin and Garry 2000). Widely varying test regimens and numerous MCF-7 cell sublines may explain this variability in test results. Wild type MCF-7 cultures contain cells with varying oestrogen response characteristics. Clones of the cells can be obtained by elaborate selection procedures in which cells with certain phenotypic characteristics become predominant. These MCF-7 cell sublines are often used since they display a more consistent proliferative response to E2 over a range of many passages than wild type MCF-7 cultures. To detect low potency oestrogenic compounds it is essential to use a cell subline with high responsitivity, as a limited proliferation relative to controls may be difficult to distinguish from experimental deviations. Comparative studies have shown that the MCF-7 BUS cell subline is the most responsive subline (Villalobos *et al.* 1995, Schafer *et al.* 1999), and therefore is highly suitable for the E-Screen assay. However, a large amount of variability in the maximal proliferative response has been reported even when the same cell subline is used. An interlaboratory comparative study of a number of *in vitro* oestrogenicity assays, including the E-Screen assay, demonstrated that, with a standardized cell line and protocol, good agreement can be achieved with most test compounds (Andersen *et al.* 1999).

This study addresses key issues important for the performance of the E-Screen assay. The protocol originally described by Soto and co-workers (1995) includes some labour-intensive steps and requires relatively large amounts of test material. The purpose of the present study was to describe a modified E-Screen assay that would (i) reduce labour and time and therefore costs, (ii) improve the utility of the assay by decreasing the amount of test material required, and (iii) increase the ability to detect low potency oestrogenic compounds.

Materials and methods

Materials

E2 and sulphorhodamine B (SRB) were purchased from Sigma-Aldrich (Vallensbaek Strand, Denmark), dieldrin from Ehrenstorfer (Augsburg, Germany), ethanol (EtOH) from Fluka, and dimethylsulphoxide (DMSO) from Bie & Berntsen (Roedovre, Denmark). The oestrogen antagonist ICI 182,780 was kindly provided by Astra Zeneca (Cheshire, UK). All media ingredients were purchased from In Vitro (Fredensborg, Denmark), except insulin (Sigma-Aldrich) and gentamicin sulphate (DuraScan, Odense, Denmark). The charcoal-dextran treated fetal calf serum (CT-FCS) batch originated from the same lot (Lot no. 416632) as the fetal calf serum (FCS) batch (In Vitro, Fredensborg, Denmark). FCS and CT-FCS from the same batch were used in all experiments. Charcoal-dextran treated human serum (CT-HuS) was prepared from outdated plasma as described

in Sonnenschein *et al.* (1995). The comparative study of 96-well plates included Nunclon Delta TC (catalogue no. 167008) (Nunc, Roskilde, Denmark), Greiner TC (catalogue no. 676001) (Bie & Berntsen), Linbro TC (catalogue no. 7600305) (In Vitro), Falcon Standard TC and Falcon Primaria TC (catalogue nos 3072 and 3872, respectively) (Becton Dickinson, Broendby, Denmark).

MCF-7 BUS cell subline

The MCF-7 BUS cells were kindly provided by Dr Soto, Tufts University School of Medicine, Boston, Massachusetts, USA. A cell bank with cells at passage 114 was established, and all experiments were performed with cells at passage 116–121. Cells were maintained in 75 cm^2 culture flasks (Greiner, In Vitro) and were trypsinized with 0.05% trypsin/0.53 mM ethylene diamine tetra-acetic acid (EDTA) and re-seeded at a 1:3 split ratio in maintenance medium (Dulbecco's modified Eagle's medium [DMEM], formula no. 01-050-1A, In Vitro) containing 10% FCS supplemented with 4 mM glutamine, 15 mM HEPES and 54 µg/l gentamicin sulphate) every 2–3 days before reaching confluence. The cultures were regularly tested for *Mycoplasma* contamination and were found to be *Mycoplasma*-free.

E-Screen assay

Stock cultures of MCF-7 BUS cells in maintenance medium in an atmosphere of 5% CO_2/95% air under saturating humidity at 37°C were seeded in 96-well plates (200 µl/well). After 24 h the medium was carefully aspirated, wells were briefly washed with 100 µl oestrogen-free medium, and filled with 200 µl oestrogen-free medium. The oestrogen-free medium consisted of phenol red–free DMEM (formula no. 01-053-1A, In Vitro) containing 10% CT-FCS supplemented with 4 mM glutamine, 20 mM HEPES, 0.1% sodium bicarbonate and 3 µg/l insulin. The compounds to be tested were added from EtOH stocks (final EtOH concentration $< 0.5\%$ in all experiments).

SRB assay

Cell proliferation was assessed after 7 days in culture (late exponential phase) using the SRB colorimetric assay (Skehan *et al.* 1990). The medium was carefully aspirated to avoid cell detachment and the cells were subsequently incubated at 4°C for 30 min in 10% (w/v) trichloroacetic acid (TCA). Following incubation, the TCA was discarded and the wells were washed five times under a gentle stream of tap water and air-dried completely. The TCA-fixed cells were stained in a shaker for 10 min with 100 µl 0.4% (w/v) SRB dissolved in 1% acetic acid. Following incubation, the supernatant was discarded and unbound dye was removed by rinsing the wells five times with 1% acetic acid. After complete drying, bound dye was solubilized with 10 mM Tris base, pH 10.5, for 20 min on a gyratory shaker, and the dye intensity was read at 490 nm in a MRX microtitre plate reader (Dynex Technologies, UK).

Transient transfection assay

MCF-7 BUS cells were plated in 24-well plates 24 h before transfection. The medium was changed 1 h prior to the transfection. Each well was transfected with 1 µg oestrogen responsive element (ERE)-luciferase reporter plasmid DNA and 0.3 µg β-galactosidase control plasmid using the calcium phosphate method. Five hours later the cells were shocked with 12.5% glycerol in phosphate buffered saline (PBS), pH 7.3, at room temperature, and fresh oestrogen-free medium was added together with the indicated compound or solvent alone. After 24 h the cells were harvested and the luciferase and β-galactosidase activities measured in a Berthold MicroLumat LB96P luminometer using a commercial kit (Galacto-Light, Tropix). The inducible luciferase values were normalized to the constitutive β-galactosidase values.

Statistical analysis

To establish concentration response relationships, at least three independent experiments were performed. Within each experiment three or six culture wells per concentration were used to establish mean values. Cell proliferation was normalized to the hormone-free controls in each experiment to correct for differences in the initial seeding density. In the experiments with dieldrin, the results were further normalized to the proliferative effect of 10 pM E2 on the same plate to correct for differences between experiments, and data are therefore presented as proliferative effect relative to E2. The concentration response curve for dieldrin was generated using GraphPad Prism and fitted to the asymmetric Hill function. Homogeneity of variance was tested by Bartlett's test, and the data was log transformed if required before conducting analysis of variance (ANOVA). If the ANOVA test showed a significant difference ($p < 0.05$), the Tukey's multiple comparisons test was used to identify differences between means.

Results

Replacement of human serum with FCS

Replacement of human serum with FCS did not affect the growth rate of MCF-7 BUS cells nor did it alter the appearance of the cells determined by microscopic examination (data not shown). We also examined whether the CT-HuS prepared in our own laboratory could be replaced by a commercially prepared CT-FCS in the study of oestrogen-dependent proliferation of MCF-7 BUS cells. Parallel concentration response curves to E2 were obtained with cells cultured in oestrogen-free medium containing either 10% CT-HuS or 10% CT-FCS. Cell proliferation increased dose-dependently in both media. In CT-FCS medium the cell proliferation of control cells was very low, whereas control cells cultured in CT-HuS medium exhibited a two-fold increase in cell number, indicating the presence of oestrogen in the CT-HuS. Since the proliferative effect is calculated as the ratio between the proliferation of E2-treated and hormone-free controls, the low proliferation of the hormone-free control cells cultured in CT-FCS medium led to a higher maximal proliferative effect than that in cells cultured in CT-HuS medium (table 1). The CT-FCS batch was further characterized by culturing cells in CT-FCS medium in the presence of E2 and increasing concentrations of the anti-oestrogen ICI 182,780. The proliferation induced by 1 nM E2 was reduced to the hormone-free control level by addition of an excess concentration of ICI 182,780 (figure 1A), and cells treated with 1 nM ICI 182,780 did not show any difference in proliferative effect compared with hormone-free controls (figure 1B).

Miniaturization of the E-Screen assay

Comparative studies between 24-well plates and 96-well plates demonstrated no significant difference in proliferative effect (data not shown). But cells from the peripheral wells seeded in 96-well plates unexpectedly aggregated toward the outer edge of the plate and gave rise to higher optical densities than cells growing in the central wells (figure 2A). This edge effect was especially pronounced in corner wells. The effect gave rise to large variations between wells. A comparative study of several 96-well plates (see the Materials and methods section for details) was consistent with the initial observation of an edge effect. Since the edge effect was least pronounced on the Nunclon Delta microwell plates, these plates were selected for further studies.

Table 1. E2-induced cell proliferation in medium containing CT-HuS or CT-FCS.

	Proliferative effect	
Treatment	CT-HuS	CT-FCS
Control	1.0 ± 0.1	1.0 ± 0.1
1 pM E2	1.5 ± 0.1	1.8 ± 0.1
10 pM E2	3.2 ± 0.1	4.7 ± 0.2
100 pM E2	5.3 ± 0.3	7.0 ± 0.3

Cells (3000 cells/well) were cultivated in the absence or presence of E2 (1, 10 and 100 pM). After 7 days of culture, cell proliferation was measured with the SRB assay using 150 µl/Tris base well. Results are expressed as the mean proliferative effect ±SD ($n = 3$).

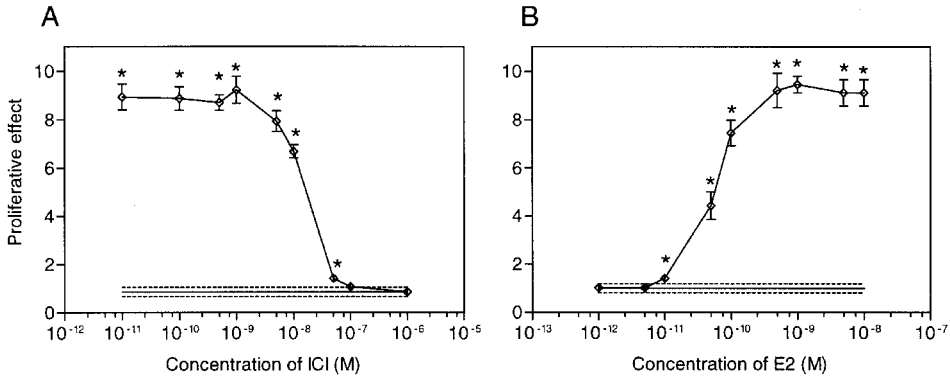

Figure 1. Concentration response curves to (A) 1 nM E2 and increasing concentrations of ICI 182,780 or (B) 1 nM ICI 182,780 and increasing concentrations of E2, supplemented with oestrogen-free medium containing 10% CT-FCS. MCF-7 BUS cells (3000 cells/well) were seeded in microwell plates. After 7 days of culture, cell proliferation was measured with the SRB assay using 150 µl Tris base/well. Results are expressed as the mean proliferative effect ± SD ($n = 3$). Results from control cells are indicated by the horizontal solid line. *Values differ significantly from control ($p < 0.05$).

To avoid temperature differences between the peripheral and the central wells, all plates were preheated in the incubator prior to seeding of the cells. This change reduced the edge effect, and it was further reduced when plates were not stacked during experiments. Moreover, handling procedures outside the incubator, including microscopic inspections of the cells during experiments, were kept to a minimum. Despite these precautions, a small edge effect remained in the outermost peripheral wells. Therefore, the outermost peripheral wells were not used for cell culture, but PBS was added to these wells to increase homogeneity between the remaining 60 wells. As seen in figure 2B, introduction of these initiatives eliminated the edge effect.

Preconditioning treatment in oestrogen-free medium

We examined whether preconditioning of MCF-7 BUS cells for extended periods of time in oestrogen-free medium prior to experiments affected their sensitivity (the lowest observed effect level, $p < 0.05$) and responsitivity (the maximal proliferative effect) to E2. The sensitivity was 1 pM E2 irrespective of the preconditioning periods (table 2), but the preconditioning treatment negatively affected the responsitivity. Thus, non-preconditioned cells showed a maximal proliferative effect to E2 that was approximately 25% higher than that induced following preconditioning for 1 day and approximately 50% higher than that induced following preconditioning for 3 or 6 days (table 2).

Effect of seeding density on proliferative response

MCF-7 BUS cells were seeded at concentrations ranging from 1500 to 6000 cells/well in 96-well plates followed by administration of a range of E2 concentrations 24 h later (figure 3). After 7 days in culture, an initial seeding density of

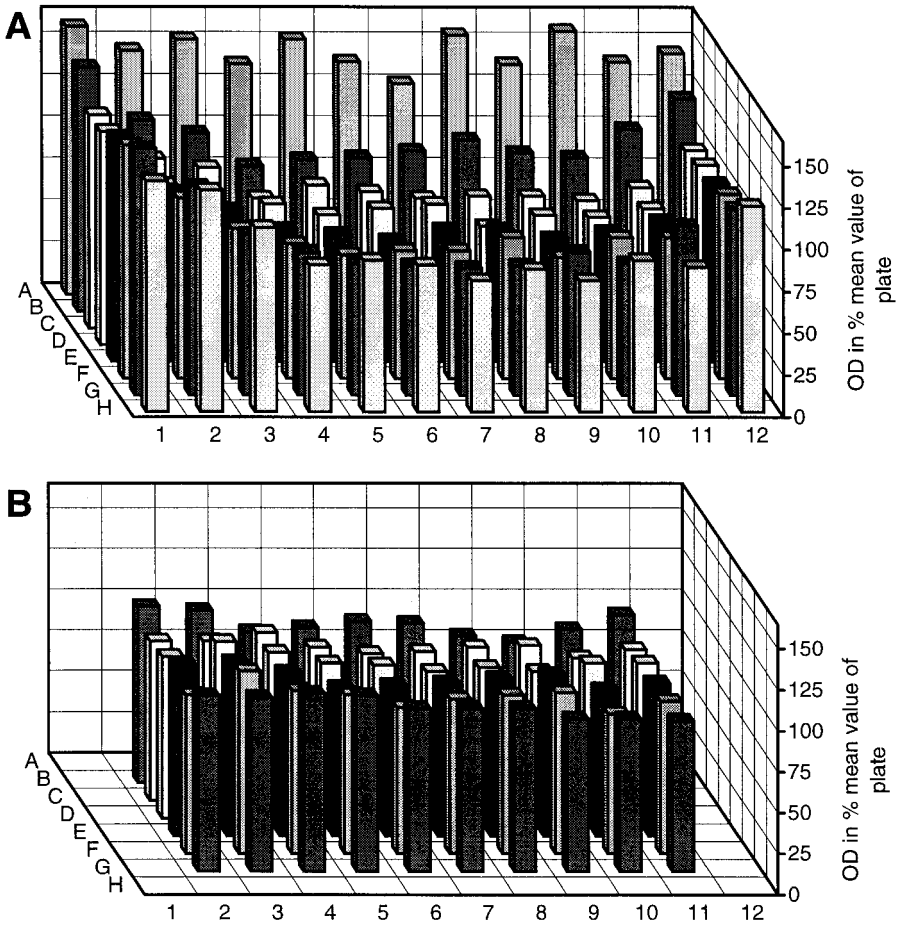

Figure 2. Block diagrams of the optical density readings in (A) Linbro TC or (B) Nunclon Delta microwell plates, illustrating the edge effect before and after optimization of assay parameters, respectively. Each column represents the optical density of the respective well as a percentage of the plate mean value. Note in (A) that the edge effect is most pronounced in the corner wells, A1 giving the maximum value (160%), while the central well E5 gives the minimum value (72%).

1500 cells/well resulted in a maximal proliferative effect of 4.3 ± 1.3-fold, 3000 cells/well resulted in a significantly higher maximal proliferative effect of 7.9 ± 0.7-fold ($p < 0.05$), and at a seeding density of 4500 cells/well the maximal proliferative effect was significantly increased yet again to 10.3 ± 0.7-fold ($p < 0.05$). A seeding density of 6000 cells/well did not significantly increase the maximal proliferative effect further.

Effect of solvents

Serial dilutions of E2 in CT-FCS-containing medium were tested to assess the effect of the solvents and mixtures of solvents in concentrations up to 1% EtOH or up to 0.5% DMSO. Instead of using a commercial cytotoxicity assay, which measures the cytosolic release of enzymes from damaged cells into the supernatant

Table 2. Effect of preconditioning treatment on E2-induced cell proliferation.

	Proliferative effect	
Duration (days)	1 pM E2	100 pM E2
0	1.5 ± 0.1	8.1 ± 0.9
1	1.7 ± 0.1	6.1 ± 1.5
3	1.5 ± 0.2	4.6 ± 0.3
6	1.7 ± 0.1	4.8 ± 0.5

After preconditioning treatment in oestrogen-free medium, the cells (3000 cells/well) were cultivated in the presence of E2. After 7 days of culture, cell proliferation was measured with the SRB assay using 150 µl/Tris base well. Results are expressed as the mean proliferative effect ±SD ($n = 3$).

or, alternatively, the metabolic activity in living cells, we evaluated the effect on E2-induced cell proliferation because this is the hallmark of oestrogen action in MCF-7 cells. Cell yields obtained from cells cultured in the presence of up to 1% EtOH were comparable with those obtained when similar inocula were cultured in CT-FCS-containing medium without EtOH, indicating the absence of an effect. Though gross cytotoxic effects were not seen on microscopic examination, effects were observed in medium containing 0.5% DMSO and in mixtures above 0.5% EtOH:0.25% DMSO. Addition of 0.1 nM E2 to these preparations resulted in a lower increase in cell yields than that in CT-FCS-containing medium supplemented with 0.1 nM E2 alone.

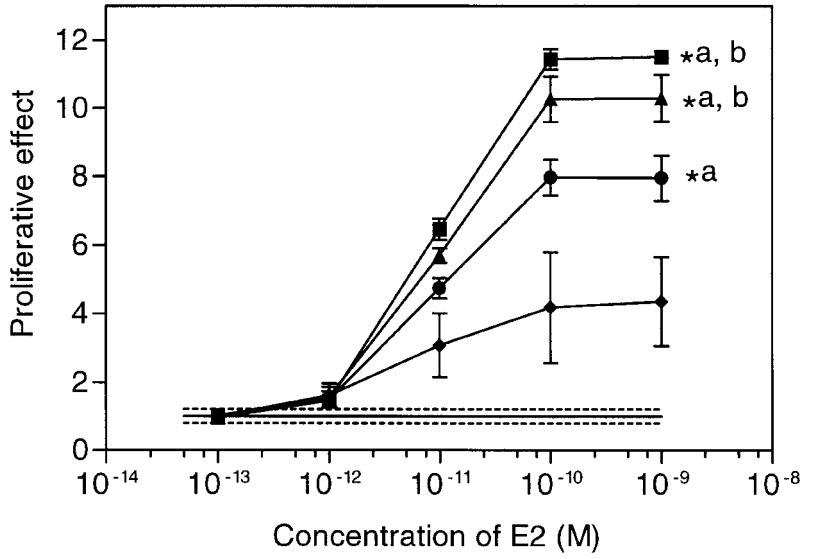

Figure 3. The proliferative response to E2 depends on the initial seeding density. MCF-7 BUS cells at densities of 1500 (◆), 3000 (●), 4500 (▲) and 6000 (■) cells/well were seeded in microwell plates and treated with E2. After 7 days of culture, cell proliferation was measured with the SRB assay using 150 µl Tris base/well. Results are expressed as the mean proliferative effect ±SD ($n = 4$). Results from control cells are indicated by the horizontal solid line. ^{*a,*b} Values differ significantly from 1500 and 3000 cells/well, respectively ($p < 0.05$).

Simplification of the SRB assay for measurement of cell proliferation

The induction of cell proliferation was monitored using the SRB colorimetric assay (Skehan *et al.* 1990). We examined whether the optical density could be read directly in the culture plate with the cells present in the wells. After SRB staining of the TCA-fixed cells, 200 µl Tris base was added to each well. From the first set of plates 150 µl solubilized dye was then transferred to a new 96-well Nunclon Delta plate and from the second set of plates 50 µl solubilized dye per well was removed to ensure identical volumes of solubilized dye per well in the two sets of plates. No difference in optical density was observed between the two sets of plates (figure 4). In the same manner, the effect of cells on the optical density readings in successively smaller volumes of solubilized dye were analysed, and again no significant difference was observed (data not shown). However, the volume of Tris base markedly influenced the optical readings. Thus, increasing the volume of Tris base from 80 µl/well to 200 µl/well significantly increased the maximal proliferative effect from 5.0 ± 0.2-fold to 8.6 ± 0.5-fold ($p < 0.05$) (figure 5).

Analysis of dieldrin using the optimized E-Screen assay

Using our optimized E-Screen assay we analysed dieldrin at concentrations from 0.1-100 µM. Moreover, between-days variation was tested in three independent experiments carried out over a period of several months. As seen in figure 6, agreement between experiments was excellent and demonstrated the robustness of the protocol. Dieldrin produced concentration-response curves with a significant proliferative response at 5 µM, a maximal proliferative effect at 25 µM and a 50%

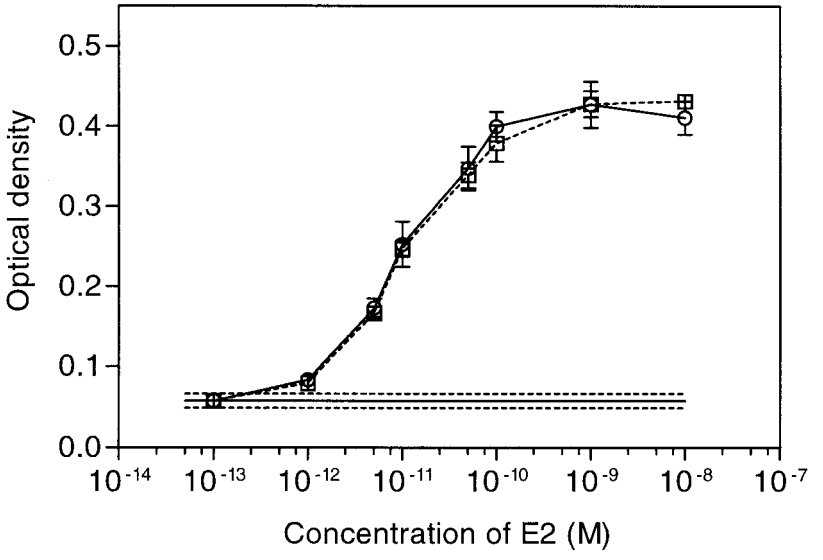

Figure 4. The presence of cells has no influence on the optical density readings. MCF-7 BUS cells (3000 cells/well) were seeded in microwell plates and treated with E2. After 7 days of culture, cell proliferation was measured with the SRB assay. The optical density at 490 nm was measured in aliquots (150 µl/well) from the culture plate (□) and directly in the culture plate with 150 µl Tris base/well (○). Results are expressed as the mean optical density ±SD ($n = 4$). Results from control cells are indicated by the horizontal solid line.

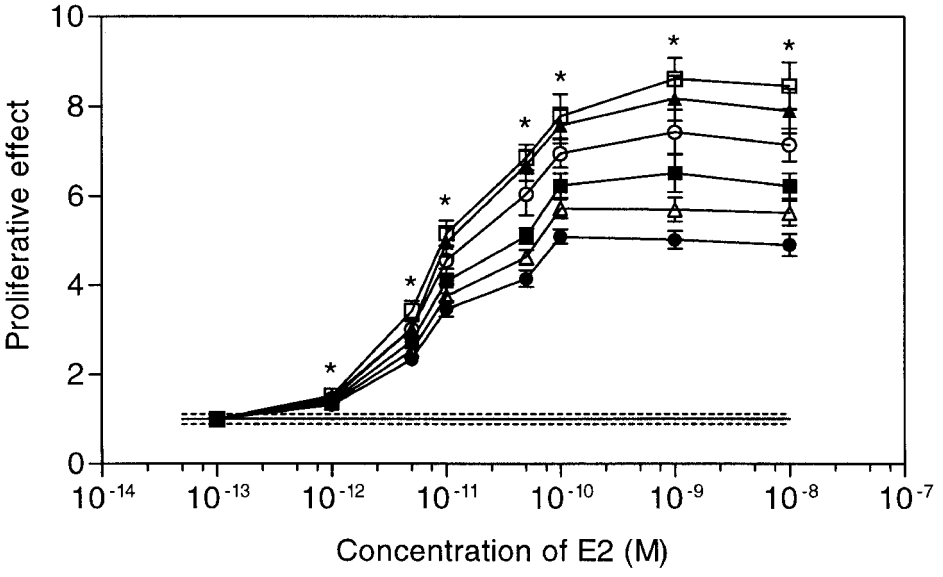

Figure 5. The optical density readings at 490 nm depend on the volume of Tris base. MCF-7 BUS cells (3000 cells/well) were seeded in microwell plates and treated with E2. After 7 days of culture, cell proliferation was measured with the SRB assay using 80 µl (●), 100 µl (△), 120 µl (■), 150 µl (○), 180 µl (▲) and 200 µl (□) Tris base/well. Results are expressed as the mean proliferative effect ±SD ($n = 4$). Results from control cells are indicated by the horizontal solid line. *Values from the 200 µl curve differ significantly from the 80 µl curve ($p < 0.05$).

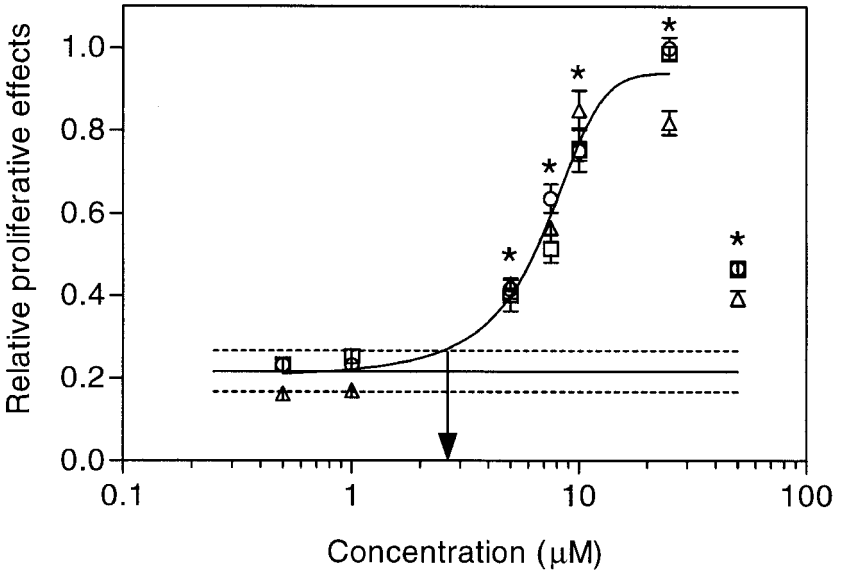

Figure 6. Concentration response curves to dieldrin (open symbols) and controls (horizontal solid line) from three independent experiments. MCF-7 BUS cells (4500 cells/well) were seeded in microwell plates. After 7 days of culture, cell proliferation was measured with the SRB assay using 200 µl Tris base/well. Results given are the proliferative effect relative to 10 pM E2, and are expressed as the mean value ±SD ($n = 3$) from three independent experiments; the curved solid line represents the best fit. Controls are shown as the mean value ±2SD, and the arrow indicates the detection limit (the point at which cell proliferation exceeds the upper control interval). *Values differ significantly from control ($p < 0.05$).

effect concentration (EC_{50}) value of 7.0 µM. A detection limit for dieldrin at 2.5 µM was calculated as described in Payne *et al.* (2000).

To verify that the observed cell proliferation induced by treatment with dieldrin was mediated through specific activation of endogenous ERs, MCF-7 BUS cells were transiently transfected with an oestrogen-responsive reporter plasmid and treated with E2 and dieldrin. As seen in figure 7, treatment with 10 µM dieldrin resulted in a significant ER-mediated transactivation of the reporter gene ($p < 0.05$), comparable to that observed after treatment with 10 nM E2.

Discussion

Interlaboratory comparisons of a number of *in vitro* oestrogenicity assays, including the E-Screen assay, have demonstrated the importance of using a uniform cell line with similar protocols (Andersen *et al.* 1999). We present a further optimization and validation of the E-Screen assay by additional intra-assay comparisons to document the reliability of this assay and to evaluate the influence of several critical steps in the practical assay performance. Further, we introduce substantial modifications and simplifications of the E-Screen assay that do not reduce its sensitivity but increase its responsitivity and repeatability. This assay would now appear to be appropriate for determining the oestrogenicity of complex mixtures, for example as a biomarker for total oestrogenic activity in serum.

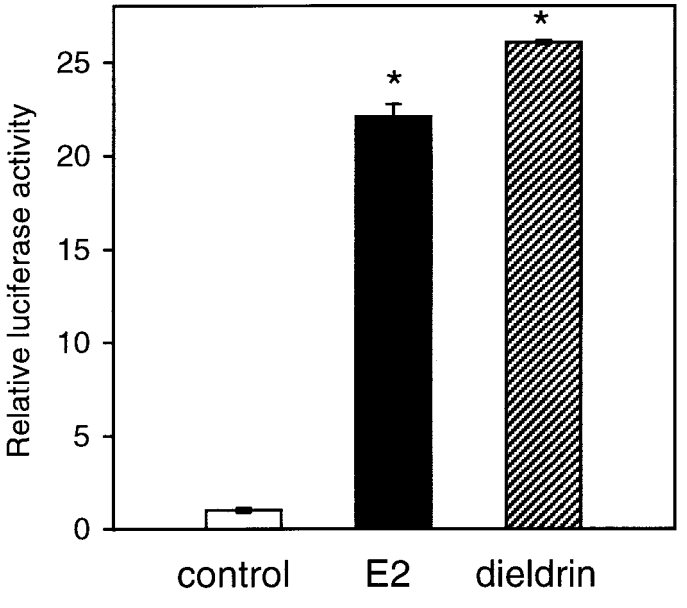

Figure 7. Dieldrin specifically activates ERs in MCF-7 BUS cells. Subconfluent cells were transiently transfected with an ER-responsive reporter plasmid and treated with 10 µM dieldrin, 10 nM E2 or solvent for 24 h. Results are expressed as relative luciferase activity ±SD ($n = 3$) compared with control. *Values differ significantly from control ($p < 0.05$).

MCF-7 cells require oestrogens for growth. Therefore, E-Screen assay conditions require cells to be cultured in oestrogen-free medium. To remove endogenous oestrogens from the cell culture medium, serum is treated with charcoal-dextran, which binds most of the hormones present, followed by repeated centrifugation to remove the charcoal particles and filtration to re-sterilize the serum. Apart from being a rate-limiting step in the preparation of oestrogen-free medium, the charcoal stripping of sera may result in serum batches with variable ability to support the growth of MCF-7 cells in the absence of added E2. The stripping process itself may play a role in inconsistent assay performance due to interlaboratory variations in the stripping technique. The substitution of CT-HuS by commercially prepared CT-FCS is an important simplification and a major step towards the standardization of the E-Screen assay. The CT-FCS batch sustained minimal growth of the MCF-7 BUS cells in oestrogen-free medium, and supported maximal stimulation of cell growth by > 0.1 nM E2. Addition of 1 nM of the anti-oestrogen ICI 182,780 did not reduce the number of cells below that of the hormone-free control cells, indicating that the CT-FCS was free of detectable levels of oestrogens. Furthermore, this substitution did not reduce the sensitivity of the assay. Thus the commercially prepared CT-FCS offered an excellent substitute to the CT-HuS batch.

Pre-assay deprivation of E2 has been shown to increase the proliferative responses of breast cancer cell lines such as MCF-7 SOP (Payne *et al.* 2000), MCF-7 E3 (Desaulniers *et al.* 1998) and T47D (Ruedl *et al.* 1990), possibly due to an upregulation of oestrogen receptors or altered E2 sensitivity associated with alterations in transcriptional regulation. We examined how the maximal proliferative effect was influenced by preconditioning of the cells for varying periods of time in oestrogen-free medium prior to experiments. However, in our hands preconditioning treatment of MCF-7 BUS cells in oestrogen-free medium for up to 6 days prior to the experiments did not improve the sensitivity nor the responsitivity compared with non-conditioned cells. In fact, we observed a decreased responsitivity with prolonged preconditioning. Hence, the inability of preconditioning to increase responsitivity of this particular cell subline is at present unclear, but may be explained by the observation that this cell subline have been shown to constitutively express relatively high levels of ERs compared with T47D and MCF-7 ATCC cells (Schafer *et al.* 1999).

We have been able to modify the E-Screen assay for use in 96-well microwell plates. Performing the E-Screen assay in microwell plates saves culture medium, labour and time. This miniaturization of the E-Screen assay did not reduce the sensitivity nor the responsitivity. When seeding cells prior to experiments it is important to ensure that the monolayer of cells has been properly trypsinized to eliminate variability due to clumping of the cells. This is particularly relevant to E-Screen assays performed in microwell plates. The introduction of microwell plates did initially create problems with a phenomenon known as the edge effect, which led to unacceptable large variations, thus destroying the assay homogeneity in microwell plates. The edge effect has been described in enzyme-linked immunoabsorbent assays performed in microwell plates, where the most probable cause of this effect is thought to be temperature differences between the peripheral and the central wells (Kricka *et al.* 1980, Oliver *et al.* 1981). Although preheating the microwell plates in the incubator prior to seeding of cells decreased the edge effect, a minor difference remained that could possibly be attributed to evaporation. To

avoid evaporation, cell incubators are set at 100% relative humidity, but the efficiency of this is decreased because of the need to check the cells during incubation. Therefore, microscopic inspections of the cells were kept to a minimum and periods of removal from the incubator were kept as short as possible. We did not use the outer, most peripheral wells, but added PBS to these wells to provide a temperature and humidity buffer between the surrounding environment and the central wells. Thus, the microwell E-Screen assay included the 60 central wells out of the total 96 wells.

The effect of the initial seeding density on the proliferative response of MCF-7 BUS to E2 was investigated to select the most sensitive proliferation indicator. Depending on the seeding density, the responsitivity could be enhanced from approximately four-fold to approximately 11-fold, thus demonstrating that optimization of this assay parameter is crucial before performing E-Screen measurements.

Instead of directly counting the cells or nuclei, we used an endpoint proportional to the cell number, i.e. the colorimetric SRB assay. The SRB colorimetric assay is based on a simple protein-staining procedure (Skehan *et al.* 1990). It is non-destructive in the sense that it is not necessary to digest the samples, and it allows fixed cultures from which dye has been extracted to be re-stained and saved for future reference. The SRB assay provides a simple, rapid and sensitive method for measuring cell proliferation, and is linear within a wide range of cell numbers (Skehan *et al.* 1990, Pazos *et al.* 1998). Although cell detachment during the fixation or staining step may provide a source of error (Schafer *et al.* 1999), we found that the critical steps are the gentle aspiration of medium and the fixation of the cells with TCA at room temperature. Subsequent incubation at 4°C for 30 min resulted in an effective fixation without any cell loss after washing (see Materials and methods section for further details).

Traditionally, the optical density is measured on aliquots transferred from the actual culture plate to a new 96-well plate. However, the presence of cells has no influence on the optical density readings at 490 nm, and measurement may therefore be performed directly on the microwell culture plates. This represents an important modification of the SRB assay as it removes a labour-intensive step and thus reduces the overall assay costs, which is a highly relevant issue in 'high throughput' screening. However, the optical density readings increased with the amount of Tris base used to extract the bound dye from the cells. Unfortunately, the volume of Tris base is generally not stated in the published accounts of this methodology.

Test chemicals are generally added to the culture medium as stock solutions in EtOH or DMSO. No cytotoxicity was found with concentrations of EtOH up to 1% and of DMSO up to 0.25%. However, when EtOH and DMSO were tested as a mixed solvent preparation using the same concentrations, cytotoxicity was observed. No cytotoxic effect was observed at concentrations up to 0.5% EtOH:0.1% DMSO. These observations stress that it is important to include control groups supplemented with solvent in experiments.

In summary, the optimized E-Screen assay conditions were found to be: (i) no preconditioning of cells prior to experiments; (ii) a seeding density of 4500 cells/well in preheated microwell plates; (iii) a maximum EtOH and DMSO concentration of 1% and 0.25%, respectively, or, if a mixed solvent preparation is used, of 0.5% EtOH:0.1% DMSO; and (iv) in the SRB assay, solubilization of bound dye

with 200 µl Tris base/well and measurement of the optical density directly on the microwell culture plates.

One of the primary uses of the E-Screen assay is for the detection of weakly oestrogenic environmental pollutants. Using our optimized E-Screen assay, we carried out a thorough concentration response analysis of dieldrin, which is known to be a xeno-oestrogen *in vitro* (Soto *et al.* 1995). This organochlorine insecticide is highly lipophilic and bioaccumulates in ecosystems. Even though its use has been restricted in many countries for a decade or more, it can still be found in wildlife and is associated with signs of reproductive impairment (Jorgenson 2001). Moreover, an epidemiological study has shown a positive correlation between breast cancer and serum levels of dieldrin (Hoyer *et al.* 1998), suggesting that xeno-oestrogen exposure increases the incidence of breast cancer. Dieldrin produced concentration response curves with a maximal proliferative response at a concentration of 25 µM and an EC_{50} value of 7.0 µM. From these data we were also able to calculate the lowest concentration at which proliferation increased significantly over control values +2SD. For dieldrin the limit of detection was 2.5 µM. Transient transfection experiments verified that dieldrin induces cell proliferation through an ER-mediated event. Our results thus confirm the oestrogenic activity of dieldrin. Other studies have analysed dieldrin using the E-Screen assay and reported oestrogenic activity at 10 µM (Soto *et al.* 1994, 1995), whereas Wade *et al.* (1997) were able to detect activity at 50 µM but not at 10 µM. Thus, our results demonstrate that our optimized E-Screen assay is a useful assay for detecting effects at low concentrations of weakly oestrogenic compounds. Moreover, the excellent agreement between experiments, which were carried out over a period of several months, demonstrates the robustness of this assay.

In summary, the E-Screen assay is a sensitive and highly reproducible tool for the identification of oestrogenic compounds. This assay was improved in the present study by adapting the assay to microwell plates and by using an optimized SRB procedure as a measure of cell proliferation. The SRB assay is easy and quick, making it ideal for 'high throughput' screening and, combined with the modifications outlined here, it considerably reduces the time and labour-intensive steps required. None of the introduced modifications reduced the sensitivity of the E-Screen assay, which was comparable to that reported in the literature. Our results demonstrate that by utilizing the MCF-7 BUS cell subline for the E-Screen assay, a maximal proliferative effect to E2 of about 11-fold can be achieved under well-defined, reproducible conditions. This maximal proliferative effect of E2 in these cells was observed repeatedly over a period of several months. Because the MCF-7 BUS cell subline is a highly oestrogen-responsive clone when used effectively, it offers a preferable alternative to other MCF-7 clones. Our study thus provides a rationale for optimizing E-Screen assay conditions, and illustrates that, once chosen and adhered to, this assay yields reproducible results for potential use in biomarker studies.

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