Issues arising when interpreting results from an *in vitro* assay for estrogenic activity

Beresford, N., Routledge, E. J., Harris, C. A. and Sumpter, J. P.

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Address all correspondence to: **Nicola Beresford**

Department of Biological Sciences

Brunel University

Uxbridge

Middlesex

UB8 3PH

UK

Tel: +44 1895 274000 ext. 2805

Fax: +44 1895 274348

email: Nicola.Beresford@Brunel.ac.uk

ABSTRACT

Concern about possible adverse effects caused by the inadvertent exposure of humans and wildlife to endocrine active chemicals, has led some countries to develop an *in vitro*-*in vivo* screening programme for endocrine effects.

In this paper, a previously described estrogen-inducible recombinant yeast strain (*Saccharomyces cerevisiae*), is used to investigate a number of issues that could potentially lead to the mislabelling of chemicals as endocrine disruptors. The chemicals studied were; 17 -estradiol, dihydrotestosterone, testosterone, estradiol-3-sulfate, 4-nonylphenol, 4-*tert*-octylphenol, 4-*tert*-butylphenol, bisphenol-A, methoxychlor, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane, butyl benzyl phthalate, 4-hydroxytamoxifen, and ICI 182,780.

Alterations in assay methodology (for example, incubation time, initial yeast cell number and the use of different solvents) did not affect the potency of bisphenol-A and 4-nonylphenol relative to 17 -estradiol, but did alter the apparent potency of butyl benzyl phthalate. Other issues (including the metabolic activation of methoxychlor, the chemical purity of a steroid metabolite and unusual chemical artefacts observed with alkylphenolic chemicals) which affect data interpretation are described.

Many of the issues raised will also affect other *in vitro* assays for endocrine activity, and some will be relevant to the interpretation of data from *in vivo* assays. These examples illustrate that considerable care and thought must be applied when interpreting results derived from any single assay. Only by using a suite of assays will we minimise the chances of wrongly labelling chemicals as endocrine disruptors.

INTRODUCTION

A number of chemicals released into the environment are believed to disrupt normal endocrine function in animals, thereby causing reproductive disorders and abnormalities in wildlife (Colborn and Clement, 1992; Colborn *et al.*, 1993). It has also been hypothesised that these chemicals are responsible for effects seen in humans, such as the concurrent increase in reproductive tract abnormalities and putative fall in sperm counts in men (Carlsen *et al.*, 1992; Sharpe and Skakkebaek, 1993), and an increase in breast cancer in women (Raloff, 1993). One major group of endocrine-disrupting chemicals that could be responsible for these reproductive effects are those which mimic natural estrogens (known as xenoestrogens).

Much attention is being devoted to the development of *in vivo* and *in vitro* screening strategies to identify and classify xenoestrogens, in order to determine whether such chemicals pose a hazard to human health. Currently, a number of different *in vivo* and *in vitro* assays are in use, and these have already been used to identify many chemicals, both naturally occurring and of synthetic origin, which have estrogenic activity (Jobling *et al.*, 1995; Soto *et al.*, 1995; Odum *et al.*, 1997). Moreover, much work has been done trying to establish structure-activity relationships (e.g. Waller *et al.*, 1996), but the precise structural requirements for estrogenic activity are not yet fully understood. Therefore, a set of criteria is needed to determine the estrogenic activity of chemicals, to enable those that are capable of endocrine-disruption to be identified.

The most widely used *in vivo* estrogen assay, the rodent uterotrophic assay, relies on the ability of chemicals to stimulate uterine growth (Odum *et al.*, 1997; Shelby *et al.*, 1996). The advantage of this assay is that it incorporates all aspects of the endocrine system, allowing for the absorption, metabolism, distribution and excretion of the chemical, and also for alternative pathways of endocrine disruption. However, even with this 'gold-standard' assay, inconsistencies in results occur, depending on the route of administration and the

response that is monitored (Milligan *et al.*, 1998). Additionally, *in vivo* assays are costly and time consuming, and therefore need to be used in conjunction with one or more reliable *in vitro* tests. The *in vitro* tests currently used range from simple competitive binding assays, relying solely on the chemical's ability to bind to the estrogen receptor (Jobling *et al*., 1995; Shelby *et al*., 1996), to more complex systems where the chemical binds to, and activates, the receptor. These latter assay systems include the proliferation of the human breast cancer cell line (MCF-7) (Soto *et al.*, 1994; Soto *et al*., 1995), vitellogenin gene expression in hepatocyte cultures (Jobling and Sumpter, 1993), and yeast-based assays expressing either rainbow trout (Petit *et al.*, 1997) or human estrogen receptors (Gaido *et al.*, 1997; Routledge and Sumpter, 1996). However, *in vitro* assays do not always reliably predict the outcome of *in vivo* assays, since chemicals can be metabolically activated or inactivated *in vivo*, and may act independently of the receptor. The inconsistent results between different *in vitro* systems (Petit *et al*., 1997; Shelby *et al*., 1996) may also be partially due to the differing metabolic capabilities of the test systems used. Thus, whether a chemical is, or is not, identified as being estrogenic may depend on the actual test system used, and this calls for confirmation of any positive findings using other assays. Thus, suitable test systems do exist, but methods still need to be validated and standardised. An *in vivo* strategy is urgently needed, and indeed the EPA are currently developing a chemical screening and testing program for endocrine effects (Gray, 1998). Additionally, the boundaries within which a biological response leads to a chemical being labelled as 'estrogenic' need to be agreed.

Over the last few years, we have encountered a number of unexpected results that have brought to our attention some of the problems which could arise when interpreting results obtained from a yeast-based *in vitro* assay for estrogenic activity (Routledge and Sumpter, 1996). In a number of cases (reported here), we have investigated these issues further in an attempt to understand them. It is important to realise that many of these issues may also apply to other *in vitro* assays, and may account for some of the inconsistencies reported in the literature.

MATERIALS AND METHOD

CHEMICALS

17 -estradiol (17 -E2; 98% pure), dihydrotestosterone (DHT; 97.5% pure), testosterone (98% pure), and the steroid metabolite estradiol-3-sulfate (approx. 95% pure), were all purchased from Sigma Chemical Company Ltd. (Dorset, UK). A separate preparation of estradiol-3-sulfate was obtained from Steraloids Inc. (Newport, RI).

Different preparations of 4-nonylphenol (NP) were obtained from Acros Organics (supplied by Fisher Scientific Ltd., Leicestershire, UK), Witco (Houston, TX), Fluka (Dorset, UK; 85% pure) and Schenectady International Inc. (Schenectady, NY; 95% pure). 4-*tert*-octylphenol (OP; 98% pure) was also supplied by Schenectady International Inc.

Bisphenol-A (97% pure) and methoxychlor (approx. 95% pure) were purchased from Sigma Chemical Company Ltd. (Sigma). 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE; the diphenol derivative of methoxychlor) was supplied by Cedra Corp. (Austin, TX).

Butyl benzyl phthalate (BBP; 97-99% pure) was purchased from Greyhound Chem Service (Merseyside, UK) and 4-*tert*-butylphenol was purchased from Aldrich (Dorset, UK).

The antiestrogen 4-hydroxytamoxifen (98% pure) was purchased from Sigma, and ICI 182,780 was a gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, Cheshire, UK).

Carboxyfluorescein diacetate (approx. 95% pure) was purchased from Sigma.

Chemicals were 99% pure, unless stated otherwise.

RECOMBINANT YEAST ESTROGENICITY ASSAY

Details of the yeast estrogenicity assay (including details of the medium components) have been previously described (Routledge and Sumpter, 1996). In brief, yeast cells transfected with the human estrogen receptor (ER gene, together with expression plasmids (containing estrogen responsive elements and the *lac*-Z reporter gene encoding the enzyme -galactosidase), were incubated in medium containing test chemical and the chromogenic substrate, chlorophenol red- -D-galactopyranoside (CPRG). Active ligands (which bind to

the receptor) induce -galactosidase (-gal) expression and this causes the CPRG (initially yellow) to change into a red product that can be measured by absorbance.

Standard assay procedure

The standard assay procedure (Routledge and Sumpter, 1996) was used to demonstrate issues related to specificity, purity of test chemicals and metabolic capability, as well as the response of the yeast screen to different stock concentrations of butylphenol, and a chemical artefact referred to as creeping. Stock solutions of chemicals (dissolved in ethanol) were serially diluted in ethanol, and 10 l volumes were transferred to 96-well flat-bottom plates. The ethanol was allowed to evaporate to dryness, after which 200 l of medium, containing CPRG and yeast (at $8x10^5$ cells/ml), was added to each well. The plates were then incubated at 32°C for 3 days, after which absorbance readings were taken at 540 nm. 17 -E2 (serially diluted from $1x10^{-8}$ M to $4.88x10^{-12}$ M) and solvent controls were included in each assay.

All chemicals were tested in duplicate, and each experiment was carried out at least two times. The ED50 for 17 -E2 was $2.2x10^{-10}$ M ± $0.22x10^{-10}$ M (mean ± SE of 17 experiments).

Relative potencies of test chemicals were determined only when dose-response curves were parallel to that of 17 -E2. To do so, the concentration of the test chemical required to produce a half-maximal response (A_{540} between 1.7 and 2.0), was divided by the concentration of 17 -E2 required to produce the same response.

Modified assay procedures

The standard assay procedure was modified in a number of experiments, in order to determine the effects of extended incubation periods, alterations in test chemical administration, and initial cell number on the assay response.

Effect of incubation time. Some plates were incubated for longer than 3 days. These plates were incubated at 32°C for the first 3 days, and were then moved to room temperature

for a further 5 days (a total of 8 days incubation). This reduction in incubation temperature (from 32^{o}C to room temperature) allows the enzyme already in the assay to metabolise the CPRG further and, at the same time, suppresses the gradual increase in the background absorbance of the medium caused by additional constitutive expression of -gal by the yeast.

Mode of addition of test chemical. The vehicle (ethanol) was either allowed to completely evaporate (leaving the test chemical dried in the well) prior to the addition of the medium, or the chemical in ethanol was added directly to the medium. When the ethanol was allowed to evaporate prior to the addition of the medium, the standard assay procedure was followed. However, when the chemical in ethanol was added directly to the medium, 5 l of chemical and 195 l of medium (containing yeast and CPRG) were used, giving a final concentration of 2.5% ethanol.

Different solvents. The estrogenic activities of test chemicals prepared in either ethanol or DMSO were compared. 5 l volumes of the test chemical dissolved in either solvent were added directly to the medium containing yeast and CPRG (195 l), giving final concentrations of 2.5% ethanol and 2.5% DMSO. The solvent methyl tertiary butyl ether (MTBE; BDH Merck Ltd., Dorset, UK) was also assessed, using the standard assay procedure. Appropriate controls (solvent alone) were included.

Initial cell number. The standard assay procedure was followed, but yeast cells were added to the medium (containing CPRG) at different initial densities. Final cell numbers of either $4x10^{6}$ cells/ml (five times more cells than normally used) or $1.6x10^{5}$ cells/ml (five times less cells than normal) were used.

Antiestrogen screen. The antiestrogen screen has been described previously (Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998). In addition to the CPRG and yeast cells, 17 -E2 (the main natural ligand) was added to the medium, at a concentration ($2.5x10^{-10}$ M) which produced a sub-maximal stimulatory response, leading to a measurable elevation in

background absorbance of the medium from 1.0 (yellow) to 2.0 (red). Chemicals that were able to inhibit the activity of the natural ligand, led to a dose-dependent decrease in -gal expression, and this was associated with a concurrent decrease in the rate of change in the colour of the medium.

Fluorescent staining of yeast cells

Yeast cells were stained using a method described previously (Breeuwer *et al.*, 1994). Carboxyfluorescein diacetate (cFDA) is a nonfluorescent compound which is hydrolysed by nonspecific esterases inside cells to produce a fluorescent product (carboxyfluorescein; cF) which is retained by viable cells, but which is lost by cells with damaged membranes.

An overnight culture of recombinant yeast cells was prepared, as described by Routledge and Sumpter (1996). Washed cells were resuspended in McIlvaine buffer (100 mM citric acid and 200 mM disodium hydrogen phosphate dihydrate, pH 4.0), and cFDA (43 mM in acetone) was added to a final concentration of 43 M. The cells were incubated for 15 min at 40^{o}C, and then placed on ice.

Slides were prepared with DPX mountant (BDH Merck Ltd.), and examined using a Leica epifluoresence compound microscope (Model DMRB), equipped with a 100x oil immersion objective and a fluorescein filter set. Photographs were taken with Kodak 1600 ASA film, using a Leica Wild MPS48/52 camera system.

RESULTS

Specificity. The specificity of the screen was assessed by the ability of androgens to stimulate the synthesis of -gal in the yeast. The data presented in Fig. 1 indicate that the steroidal androgen DHT is more potent than bisphenol-A (a so-called xenoestrogen), in the yeast estrogenicity screen. DHT and bisphenol-A had potencies approximately 4000 and 10,000 times less than that of E2, respectively. DHT was also purchased from Riedel-de Haën (supplied by Philip Harris, Staffordshire, UK), and this sample had the same potency as the Sigma DHT (data not shown). DHT and bisphenol-A, like 17 -E2 (the main natural ligand), produced dose-response curves which occurred over the whole absorbance range of the assay, i.e., full dose-response curves. Testosterone and BBP (Fig. 1A and 1B, respectively) were both less potent than bisphenol-A, and induced responses of 39% and 22% of the maximum response obtained with E2. Any such curves that fail to reach the maximum response obtained with 17 -E2 will be referred to as sub-maximal responses or sub-maximal dose-response curves.

Purity of test chemical. Two samples of estradiol-3-sulfate were obtained, at different times, from Sigma. The first sample was 300 times less potent than E2 (Fig. 2A). The second sample was much less potent than the first, being approximately 30,000 times less potent than E2. The estradiol-3-sulfate purchased from Steraloids was approximately 10 times less potent than E2. Steraloids were unable to supply a different batch to test, but a second aliquot of the same batch was purchased, and this was found to have a similar potency to the first (data not shown).

All four stocks of NP had similar potencies (Fig. 2B), being approximately 4000 times less potent than E2.

Effect of incubation time. With increased incubation time, the E2 dose-response curve shifted to the left, and hence the assay became more sensitive (Fig. 3). The concentration of E2 required to produce half the maximal response reduced from $2 \times 10^{-}$

10^{-10} M to $7x10^{-11}$ M. The dose-response curves of E2 and NP shifted the same distance to the left with time, so that the relative potency of NP remained the same irrespective of when the readings were taken; that is, NP was approximately 4000 times less potent than E2, after both 3 and 8 days incubation. BBP produced sub-maximal dose-response curves throughout the incubation periods, although after 8 days the gradient of the dose-response curve was much steeper (Fig. 3). By day 8, the absorbance values of the blank wells had increased from 1.0 to about 1.2, due to constitutive expression of -gal.

Mode of addition of test chemical. Figure 4 shows that the assay was more sensitive to both E2 and NP when the chemicals (dissolved in ethanol) were added directly to the medium containing the yeast, as opposed to allowing the ethanol to evaporate prior to addition of the medium containing the yeast to the wells. For example, when E2 (serially diluted in ethanol) was allowed to evaporate prior to addition of the seeded assay medium, a concentration of approximately $2x10^{-10}$ M produced a half-maximal response, whereas the same response was achieved with $9x10^{-11}$ M when the E2 in ethanol was added directly to the same assay medium. Because the increased sensitivity was much the same with both chemicals, the potency of NP relative to E2 was similar (approximately 4000 times less potent), whichever approach was adopted. A BBP solution (in ethanol) produced a slightly steeper dose-response curve when it was added directly to the medium (Fig. 4), although this response was still sub-maximal.

Different solvents. The assay was slightly more sensitive when DMSO was used as the solvent. For example, slightly less 17 -E2 was required to produce a half maximal response when it was dissolved in DMSO, than when it was dissolved in ethanol (Fig. 5). Despite this, the potency of NP ranged from 3000 to 4000 times less than that of E2, irrespective of the solvent used. The BBP dose-response curve was much steeper when DMSO was used as the solvent, although it still produced a sub-maximal dose-response curve compared with the maximal response obtained with E2.

DMSO also had an effect on the rate of colour change of the background (the baseline). Using ethanol as the solvent, the background colour had an absorbance value of 1.0 (yellow) after 3 days incubation (Fig. 5). Using DMSO as the solvent, the background was a similar colour after 3 days incubation, giving an absorbance of around 1.0 (Fig. 5). However, whereas the background colour of the medium subsequently changed only slowly in the presence of ethanol, in the presence of DMSO it changed rapidly (not shown), such that by the next day (after 4 days incubation) it had a background absorbance of 2.0 (and was light red).

The solvent MTBE was also tested. This solvent dissolved the plastic plates, and hence reliable plate readings could not be taken, as absorbance values were affected. When the E2, bisphenol-A and BBP stock solutions were prepared in MTBE, serially diluted in MTBE, and allowed to evaporate to dryness before adding the medium, no colour change was seen (that is, no signs of estrogenicity were observed). When stock solutions of chemicals dissolved in MTBE were serially diluted in ethanol (and this allowed to evaporate to dryness before adding the medium containing the yeast), a colour change (yellow to red) was seen once the MTBE had been diluted to 50% by the ethanol (i.e. from the second well onwards).

Initial cell number. Increasing the initial number of yeast cells present in the wells made the assay more sensitive (Fig 6), as indicated by a shift of the E2 curve to the left as the cell number increased. As the curve for E2 moved to the left, so did the dose-response curve for bisphenol-A. Thus, altering the number of yeast cells had no effect on the potency of bisphenol-A relative to E2; in all three cases bisphenol-A was approximately 10,000 times less potent than E2. Higher initial yeast cell numbers not only increased the gradient of the BBP dose-response curve, but also resulted in a higher baseline.

The pesticide o,p'-DDT also produces sub-maximal dose-response curves (Routledge and Sumpter, 1996). The o,p'-DDT (MTM, Lancashire, UK) response was examined using

different incubation times, and the effect of adding the chemical (made up and diluted in ethanol) to the assay plate and allowing it to evaporate to dryness prior to adding the assay medium was compared to the response when the same chemical was added directly to the assay medium. The responses were similar to those obtained with BBP, in that the slope was steeper, and a higher response was obtained, when the plates were incubated for longer, and when the chemical in solvent was added directly to the medium (data not shown).

Metabolic capability. Both methoxychlor (the parent compound) and its main metabolite (HPTE) were active in the yeast estrogenicity assay. After 3 days incubation, HPTE produced a full dose-response curve, whereas that for methoxychlor was sub-maximal (Fig. 7). At this time, HPTE and methoxychlor were approximately 8000 times and 80,000 times less potent than E2, respectively. After 4 days incubation, both HPTE and methoxychlor gave full dose-response curves (Fig. 7). The potencies of HPTE and methoxychlor relative to 17 -E2, did not alter with incubation time. Additionally, methoxychlor was obtained from Greyhound Chem Service, and this sample was also active when tested in the assay (data not shown).

Fluorescent staining of yeast cells. The yeast cells were able to metabolise cFDA into the fluorescent product cF (Fig. 8).

Antiestrogen screen. Hydroxytamoxifen behaved as a partial agonist in the estrogen screen, producing a dose-dependent increase in the absorbance of the medium which reached a maximal absorbance of approximately 1.75 at between 10^{-7} M to 10^{-6} M (Fig 9A(i)). At higher concentrations, between 10^{-6} M and 10^{-5} M, there was a reduction in the response seen, which did not coincide with a fall in the turbidity of the medium (i.e. the chemical was not toxic). In the antiestrogen screen, hydroxytamoxifen was able to inhibit the response to E2 (Fig. 9A(ii)), resulting in a dose-dependent decrease in -gal production at concentrations of hydroxytamoxifen greater than 2.4×10^{-8} M. Hydroxytamoxifen was toxic to the yeast at concentrations in excess of 10^{-5} M.

In contrast, the antiestrogen ICI 182,780 produced a full agonistic dose-response curve in the estrogen screen (Fig. 9B(i)), and was approximately 30,000 times less potent than E2. In the antiestrogen screen, ICI 182,780 was unable to inhibit the response induced by E2 (Fig. 9B(ii)), but instead enhanced the response of E2 in a dose-dependent manner; that is, it acted additively with E2.

Response to different stock concentrations of butylphenol. The same preparation of butylphenol produced dose-response curves with different potencies, depending only on the concentration that the initial stock solution was made up at (Fig. 10). For example, when the initial stock solution was made up at $1x10^{-2}$ M (serially diluted from $5x10^{-4}$ to $2.4 x10^{-7}$ M in the assay), no response was seen. However, when the initial stock solution was made up at $4x10^{-2}$ M (serially diluted from $2x10^{-3}$ to $9.77x10^{-7}$ M in the assay), a full dose-response curve was obtained, with the chemical being approximately one million times less potent than E2. Intermediate concentrations of stock solutions ($2x10^{-2}$ M and $3x10^{-2}$ M) produced intermediate responses of increasing potency, respectively.

Chemical artefact: creeping. Fig. 11 shows the results of a serial dilution of OP from $4.8x10^{-4}$ to $2.4x10^{-7}$ M (row D), with blank rows (medium only) on either side (rows A-C and E-H). The OP caused an estrogenic response; the highest concentrations of OP were toxic (resulting in clear yellow wells), but below these concentrations there was a dose-dependent stimulation of -gal. However, the blank rows on either side of the OP dose-response curve (which contained seeded assay medium alone) were also positive, indicating that the chemical was not restricted to the wells it was placed in, but instead moved freely across the plate. This response decreased with increasing distance away from the row of wells containing OP. Although the estrogenic response 'crept' across the plate to affect blank rows, there was no 'creeping' of the toxic response.

DISCUSSION

This paper illustrates, through a series of examples, potential issues often encountered using *in vitro* screens which may affect data interpretation. In order to fully understand the reason why certain chemicals behave differently under varying assay conditions, analytical chemistry would be needed to assess the solubility and stability of test compounds in the assay medium, and their metabolic fate in each case. However, in addition to the high cost of carrying out these assessments, there are some serious practical problems associated with this type of work. The chemicals tested in the yeast screen are present in small volumes (200 l) and at very low concentrations. Therefore, it would be necessary to scale up the experiments to provide enough test chemical to conduct the analysis. Additionally, such analytical work is exacerbated by the fact that many test chemicals are often complex mixtures which partition between the yeast and medium. Nevertheless, despite the absence of analytical chemistry, it is important to be aware of the issues described in this manuscript, as they may affect the response of active chemicals, but more importantly could lead to the mislabelling of certain chemicals as endocrine disruptors.

The yeast assay was sensitive enough to consistently detect an increase in -gal production at E2 concentrations above $1x10^{-11}$ M. This compares with the detection limit of $3x10^{-11}$ M E2 reported for the MCF-7 cell proliferation assay (Sonnenschein *et al.*, 1995).

The yeast estrogenicity assay reported here also has certain advantages over other yeast-based assays, in that the colour development of the medium can be easily monitored over a period of time. This can be done because the yeast secrete the -gal directly into the medium, where it causes a progressive concentration-dependent colour change. Longer incubation periods led to a shift in the dose-response curves to the left, making the assay even more sensitive with time. In other yeast-based assays reported to date (Arnold *et al.*, 1996; Coldham *et al.*, 1997; Gaido *et al.*, 1997; Petit *et al.*, 1997), the -gal is retained inside

the yeast, and hence the yeast cells need to be broken open to release the enzyme, which is then measured using a traditional enzyme assay.

The yeast estrogenicity assay was previously shown to be highly specific to a range of steroids and steroid metabolites (Routledge and Sumpter, 1996; Routledge and Sumpter, 1997). However, when the steroidal androgens DHT and testosterone were tested in this yeast-based study at higher concentrations than previously tested, they also produced responses of similar potency and magnitude to those of many established xenoestrogens. In support of this finding, testosterone and 11-ketotestosterone (11-KT) have also been shown to induce vitellogenin synthesis in rainbow trout hepatocyte cultures (Pelissero *et al.*, 1993), a response considered to be very estrogen-specific. Since 11-KT is not thought to be aromatizable, Pelissero *et al*. (1993) concluded that this androgen was probably interacting directly with the estrogen receptor. This conjecture was further supported by the fact that vitellogenin induction by 11-KT could be blocked with tamoxifen (an established antiestrogen). Additionally, testosterone stimulated the proliferation of MCF-7 cells, although in this case it was probably the result of metabolic conversion of testosterone to E2 by the aromatase enzyme (Kudoh *et al.*, 1996). Moreover, androgens have been found to produce positive responses in other yeast-based estrogen assays (Coldham *et al*., 1997; Gaido *et al*., 1997). The positive estrogenic responses seen here with DHT and testosterone may have been due to metabolic activation (to an estrogen) and/or direct interaction with the estrogen receptor, since both steroidal androgens are structurally similar to E2. Although the androgens are estrogenic at high doses, their activity is unlikely to be of physiological importance, because the concentrations of androgen required to produce a direct estrogenic response would not occur under natural conditions in the body (Gaido *et al*., 1997). Nevertheless, this finding does pose the question as to whether it is right to label chemicals such as bisphenol-A, o,p'-DDT and some phthalates 'estrogenic', when they react with the estrogen receptor with less affinity than an established androgen.

The problem of impurities causing false positives has been discussed by Harris *et al*. (1997), who found ditridecyl phthalate to be contaminated with an isomer of bisphenol-A; the latter causing the former to appear weakly estrogenic. Here, we show that estradiol-3-sulfate produced three quite different dose-response curves. Since the samples were prepared in the same manner just prior to use, the variable responses are likely to be due to actual differences in the nature of the initial chemical purchased; that is, they are dependent on the source/origin of the sample. Impurities were thought to be the reason for the estradiol-3-sulfate testing positive, when it was tested previously (Routledge and Sumpter, 1996). One supplier, Steraloids, suggested that their estradiol-3-sulfate had probably come into contact with moisture, resulting in hydrolytic cleavage of the sulfate group to produce E2 (personal communication). Thus, the purity or composition of a chemical (which may also vary depending on storage conditions) are crucial for accurate interpretation of the results, and any assay that covers a wide concentration range is likely to be sensitive to this issue. Additionally, as the three estradiol-3-sulfate samples we tested differed in potency by over 3 orders of magnitude, it is highly likely that impurities were responsible for their measured activity, rather than intrinsic activity by the chemical itself (it may have some, but this must be very slight, as one sample was over 10,000 times less potent than E2). Because the yeast-based assay can cover a very wide potency range (at least one million-fold), even a slight contamination by a weak estrogen can significantly affect the results (e.g. Harris *et al.*, 1997). Similarly, minimal medium and medium components prepared in glassware contaminated with an estrogenic chemical will lead to elevated background expression, and contaminated chemical stocks will give false positives.

This situation, where a supposedly fairly pure (~95%) and homogeneous chemical, estradiol-3-sulfate, shows very variable potency, contrasts with our results obtained with different preparations of NP. Commercially available alkylphenolic compounds are a complex mixture of isomers and oligomers; for example, recent chemical analysis of 4-NP identified 22 isomers (Wheeler *et al.*, 1997). Despite this, the potencies of NP from 4 different suppliers were very similar. However, because of the complex nature of alkylphenols, differences in potencies between batch and suppliers will always be a possibility.

In addition to the effects of chemical purity/composition on *in vitro* data and its interpretation, we investigated the effect of alterations in assay methodology on the response to certain xenoestrogens. None of the four parameters examined (incubation time, whether the solvent was allowed to evaporate or not, the type of solvent, and initial yeast cell number) had any appreciable effect on the relative potencies of NP and bisphenol-A. However, altering these criteria did affect both the gradient and the magnitude of the dose-response curve produced by BBP and o,p'-DDT. The fact that BBP produces a sub-maximal dose-response curve has been interpreted as indicating partial agonism (Sonnenschein *et al*., 1995). However, the sub-maximal response may be due to a number of factors, including bioavailability, metabolism and binding affinity of the chemical to the estrogen receptor (Arnold *et al*., 1996). Phthalates usually produce sub-maximal (less than 50%) responses in *in vitro* assays, which may be considered too small to warrant labelling phthalates as estrogenic (Meek *et al.*, 1996) . However, the BBP dose-response curve was virtually maximal in the yeast estrogenicity assay, following an extended incubation period (Harris *et al*., 1997). Harris *et al*. (1997) proposed that the partial agonistic behaviour of BBP was in part a consequence of its poor solubility, and/or the result of reduced bioavailability due to adsorption of the chemical to the plastic plates. However, in the MCF-7 cell proliferation assay (where chemicals dissolved in solvent are added directly to the medium), BBP still produced a sub-maximal response (Harris *et al*., 1997; Sonnenschein *et al*., 1995). Similarly, although addition of BBP directly to the medium in the yeast estrogenicity assay produced a response of higher magnitude, this was still sub-maximal. BBP was reported to be inactive in a different yeast-based assay (Gaido *et al*., 1997), and it was suggested that BBP needed be metabolically activated. However, this is unlikely to be the case, since the primary metabolites of BBP have been shown to be inactive (Harris *et al*., 1997). Our results indicate that bioavailability/solubility issues were only partly responsible for the reduced response to BBP, and that other factors, such as receptor binding affinity, also play a part. The increase in the magnitude of the response when BBP was dissolved in DMSO (rather than ethanol) was partly a consequence of the slightly increased sensitivity of the

assay with DMSO. It is also likely that DMSO altered cell wall permeability, resulting in easier uptake of BBP into the yeast.

The solvent MTBE interacted with the plastic plates, and was therefore unsuitable as a solvent. This solvent totally inhibited the responses usually produced by 17 -E2, bisphenol-A and BBP. MTBE induces endocrine alterations in mice, but the responses seen were thought not to be mediated through the estrogen receptor (Moser *et al.*, 1998). It is possible that when the multi-well plate was dissolved by the solvent, either a chemical was released from the plastic plate that completely blocked the response of the estrogenic chemicals, or that the test chemical became locked within the plastic, preventing it from interacting with the estrogen receptor. Whatever the explanation, the results show that the choice of solvent is important; different solvents produce different effects.

The gradients of the BBP and o,p'-DDT dose-response curves were altered by the assay conditions tested, and there may be other factors, such as plate layout, incubation temperature, purity/condition of chemical stocks, and the growth stage of the yeast, all of which could also lead to an increase in the height of the response. Other chemicals that produce sub-maximal responses may also behave similarly to BBP and o,p'-DDT, and produce different responses depending on the assay conditions used. This cautions against using terms such as 'partial agonists', because a chemical that is a 'partial agonist' under one set of conditions may be a so-called full agonist under different conditions.

Increasing the initial number of yeast cells added to each well caused an increase in the development rate of the assay, but did not affect the relative potency of bisphenol-A. However, increasing the initial number of yeast cells in the assay resulted in a steeper dose-response curve for BBP. In contrast, with fewer cells the BBP dose-response curve had a very shallow gradient, although this did steepen with increasing incubation time (data not shown). If an assay had been carried out with relatively low cell number initially, and without an extended incubation period, the estrogenic activity of BBP may have gone undetected.

Some results relevant to the metabolic competence of this yeast estrogenicity assay have been reported previously (Odum *et al*., 1997). Methoxychlor (a proestrogen) is metabolically converted to the active estrogenic product HPTE (Bulger *et al.*, 1978). Shelby *et al*. (1996) found that methoxychlor was inactive in two *in vitro* assays, whereas HPTE was active in both their assays. This finding suggested that their *in vitro* assays lacked the ability to demethylate methoxychlor. Any assay that lacks the ability to metabolise chemicals will miss certain proestrogens, leading to false negative results. Our yeast estrogenicity assay has intrinsic metabolic capabilities, as illustrated by the fact that methoxychlor (and its metabolite HPTE) were both active, and that the yeast was able to metabolise cFDA into the fluorescent cF. A longer incubation time was required for methoxychlor to produce a full dose-response curve (compared to HPTE), which is consistent with its metabolic conversion to HPTE. For this reason, metabolites (when known and available) should be tested alongside the parent chemical, in order to determine the role of metabolism in biological activity.

A major criticism of yeast-based estrogen assays has been their reported inability to differentiate between estrogen agonists and antagonists (e.g. Sonnenschein and Soto, 1998). However, tamoxifen and hydroxytamoxifen (type I antiestrogens) were previously shown to inhibit estradiol-induced expression of -gal in this yeast estrogenicity screen (Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998). Hydroxytamoxifen on its own behaved as a weak partial agonist, producing a stimulatory response which was less than half the magnitude of that produced by 17 -E2, at a concentration 3 to 4 orders more than that of 17 -E2. The shape of the hydroxytamoxifen dose-response curve is consistent with a model in which tamoxifen preferentially binds to (and inhibits) the ligand-binding domain of the estrogen receptor at low concentrations, but allows constitutive expression of the ligand-independent activation function (AF-1). As the concentration of tamoxifen increases (and the ligand-binding domain becomes saturated), it is proposed that the antiestrogen interacts with a second domain on the receptor (which is not recognised by estradiol), which completely abolishes the activity of the receptor protein (Hedden *et al.*, 1995). In contrast,

the type II (or 'pure') antiestrogen, ICI 182, 780, was unable to inhibit the same estradiol-induced response, but instead produced a purely agonistic response in the yeast screen. Both types of antiestrogen (I and II) are known to compete with estradiol for binding to the estrogen receptor, but their actions appear to differ thereafter. ICI 182, 780 has been shown to exhibit antiestrogenic properties in *in vitro* and *in vivo* mammalian studies (Zacharewski, 1997), but yeast may not contain the full complement of appropriate repressor proteins necessary for antagonism in this case (Gaido *et al*., 1997). Nevertheless, although the yeast-based estrogen assay used in this study cannot detect all antiestrogens, it obviously can detect some.

The estrogenic effect of OP was seen to move across the plate and contaminate adjacent rows of blank wells; an artefact we refer to as 'creeping'. Thus, the potency of the chemical is likely to be underestimated (the actual concentration in the test wells will be less than the nominal concentration, because some of the chemical has 'crept' out). In the absence of blank rows (containing yeast but no test chemical), this artefact may not have been discovered. It is possible that with other test systems (where responses cannot be visualised by the naked eye), cross-contamination of test chemicals might unknowingly occur, leading to mislabelling of chemicals as endocrine disruptors. To avoid false positives, we tested each alkylphenolic chemical on a separate plate. Alternatively, such errors can be avoided by altering the position of chemicals on a plate when retesting (anomalous results might suggest creeping). To date, we have found only alkylphenols and some PCBs to creep, but there may be other chemicals that behave in this manner. Our results suggest that OP appears to contain at least two isomers; one that is estrogenic and able to creep across multi-well plates, and a second that is toxic and unable to creep; that is, the chemical's estrogenic component creeps, but its toxic component does not.

Butylphenol produced different responses depending on the concentration of the stock solution, unlike all other chemicals that have been tested using this yeast estrogenicity assay. Indeed, the chemical would have been thought to be non estrogenic, if it had been tested

using only the lowest concentration of stock solution. When the lowest stock concentration of butylphenol was serially diluted in the assay, it remained non estrogenic, whereas higher concentrations, when diluted, were estrogenic. Dr. C Green (Witco; personal communication) believes that some alkylphenols can exist in a pseudo liquid crystal state under certain conditions, and that factors (such as temperature) could affect the 3-dimensional configuration of these chemicals. Therefore, it is plausible that depending on the position of the alkyl group (either above or below the plane of the phenolic ring), they may be either estrogenic or non estrogenic. This might suggest that when the stock solutions were made up, the configuration that the butylphenol molecules adopted was dependent on the initial temperature (or some other factor associated with concentration), and remained stable thereafter.

The forthcoming screening of thousands of chemicals, together with the increasing widespread use of many different *in vitro* assays for different endocrine activities, has led us to report on our experiences gained from using a yeast-based estrogenicity assay for the last 4 years. The potency estimates obtained with the assay are similar to those observed in various other *in vitro* assays. However, 'problems' have been encountered that probably apply to all *in vitro* assays; certainly issues such as purity, effect of different assay conditions, and 'creeping' will. No single assay can be expected to be 'the best' to assess estrogenicity, and any response seen in *in vitro* assays needs to be confirmed *in vivo*. Only by using a suite of assays in this way will it be possible to minimise the chances of wrongly labelling chemicals as endocrine disruptors.

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LEGENDS

Figure 1. *Specificity.* Response of the yeast estrogenicity screen to (A) the steroidal androgens DHT (—◈—) and testosterone (—◁—), and (B) the xenoestrogens bisphenol-A (—◇—) and BBP (—○—). Each data point is the mean of duplicate values, and the data is representative of the experiments carried out.

Figure 2. *Purity of chemicals from different sources.* (A) Yeast cells were incubated with two samples of estradiol-3-sulphate obtained from Sigma: first sample (—○—) and second sample (—△—), and a single sample obtained from Steraloids (—◇—). (B) NP from four sources was tested for estrogenicity: Acros Organics (—⊞—), Witco (—▽—), Fluka (—◑—) and Schenectady (—✳—).

Figure 3. *Effect of incubation time.* Absorbance readings were taken after 3 days incubation (open symbols) and 8 days incubation (filled symbols). Yeast cells were incubated with the chemicals NP (—△—) and BBP (—○—).

Figure 4. *Mode of addition of test chemical.* The test chemicals (NP (—△—) and BBP (—○—)) were prepared in ethanol, which was either allowed to evaporate to dryness before addition of the medium (open symbols), or was added directly to the medium (filled symbols).

Figure 5. *Different solvents.* The test chemicals (NP (—△—) and BBP (—○—)) were prepared in either ethanol (open symbols) or DMSO (filled symbols). The chemicals were added directly to the medium.

Figure 6. *Initial cell number.* Different numbers of yeast cells were added to the medium; 1.6×10^5 cells/ml (open symbols) and 4×10^6 cells/ml (filled symbols). The estrogenic responses of bisphenol-A (—◇—) and BBP (—○—) were measured.

Figure 7. *Metabolic capability.* The estrogenic responses to methoxychlor (—✣—) and its metabolite HPTE (—▷—) were measured after 3 days incubation (open symbols), and 4 days incubation (filled symbols) of the yeast cells.

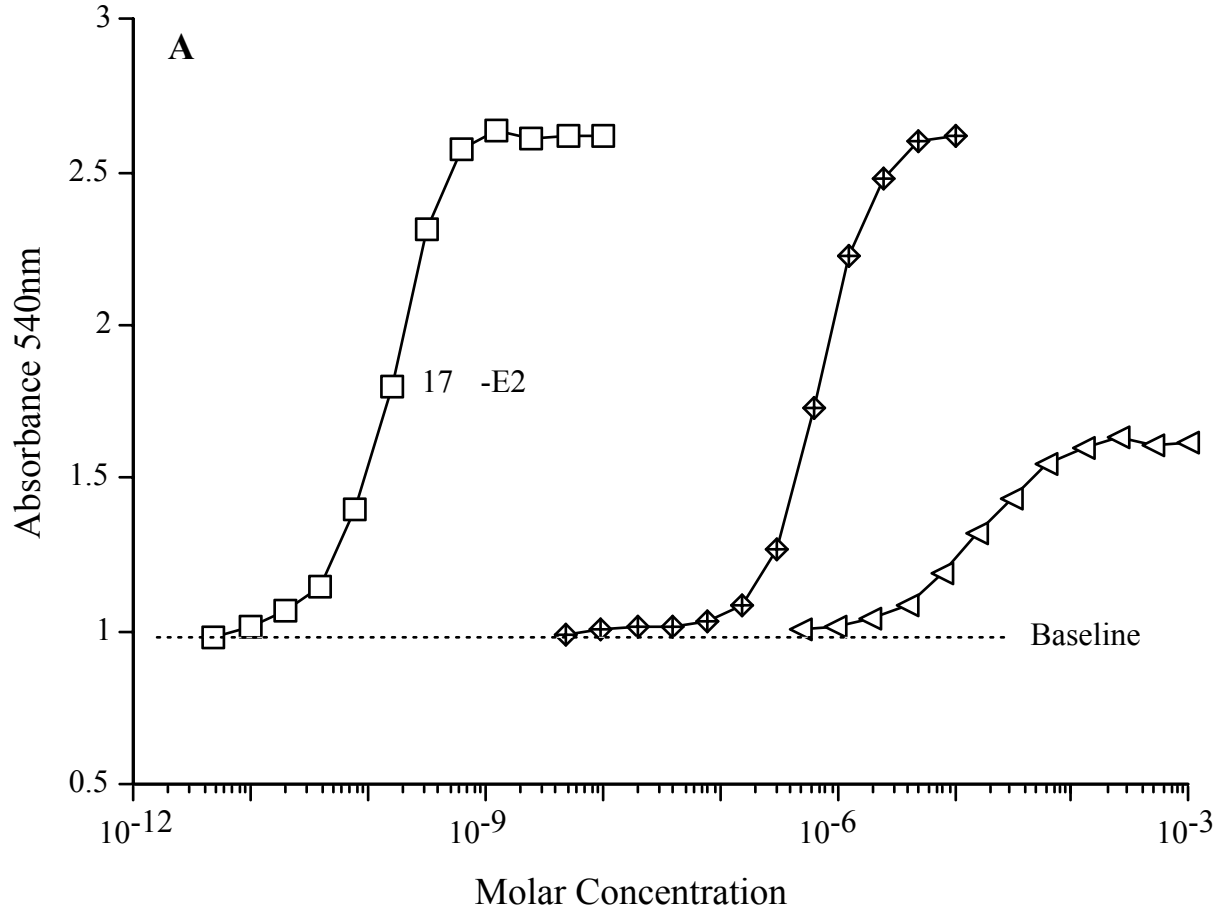
Figure 8. *Fluorescent staining of yeast cells.* Yeast cells were loaded with cFDA which was metabolised by the cells to the fluorescent cF.

Figure 9. *Antiestrogen screen.* (A) Hydroxytamoxifen (—◇—) was tested (in the absence of 17 -E2) for estrogenic activity (i), and in the presence of 17 -E2 for antiestrogenic activity (ii). When testing for antiestrogenic activity, 17 -E2 was added to all wells to raise the background absorbance to approx. 2.0, whilst hydroxytamoxifen was serially diluted at the concentrations shown.

(B) Similarly, ICI 182,780 (—△—) was test for (i) estrogenic and (ii) antiestrogenic activity.

Figure 10. *Response to different stock solution concentrations of butylphenol.* The yeast cells were incubated with butylphenol, the stock solutions from which the serial dilutions were done were prepared at different concentrations: $1x10^{-2}$ M (—◇—), $2x10^{-2}$ M (—○—), $3x10^{-2}$ M (—△—), and $4x10^{-2}$ M (—⊞—). Toxic concentrations are not shown.

Figure 11. *Chemical artefact: creeping.* The chemical OP was serially diluted before being transferred to row D. All rows either side (A-C and E-H) were blank rows containing seeded medium only.


A
Absorbance 540nm
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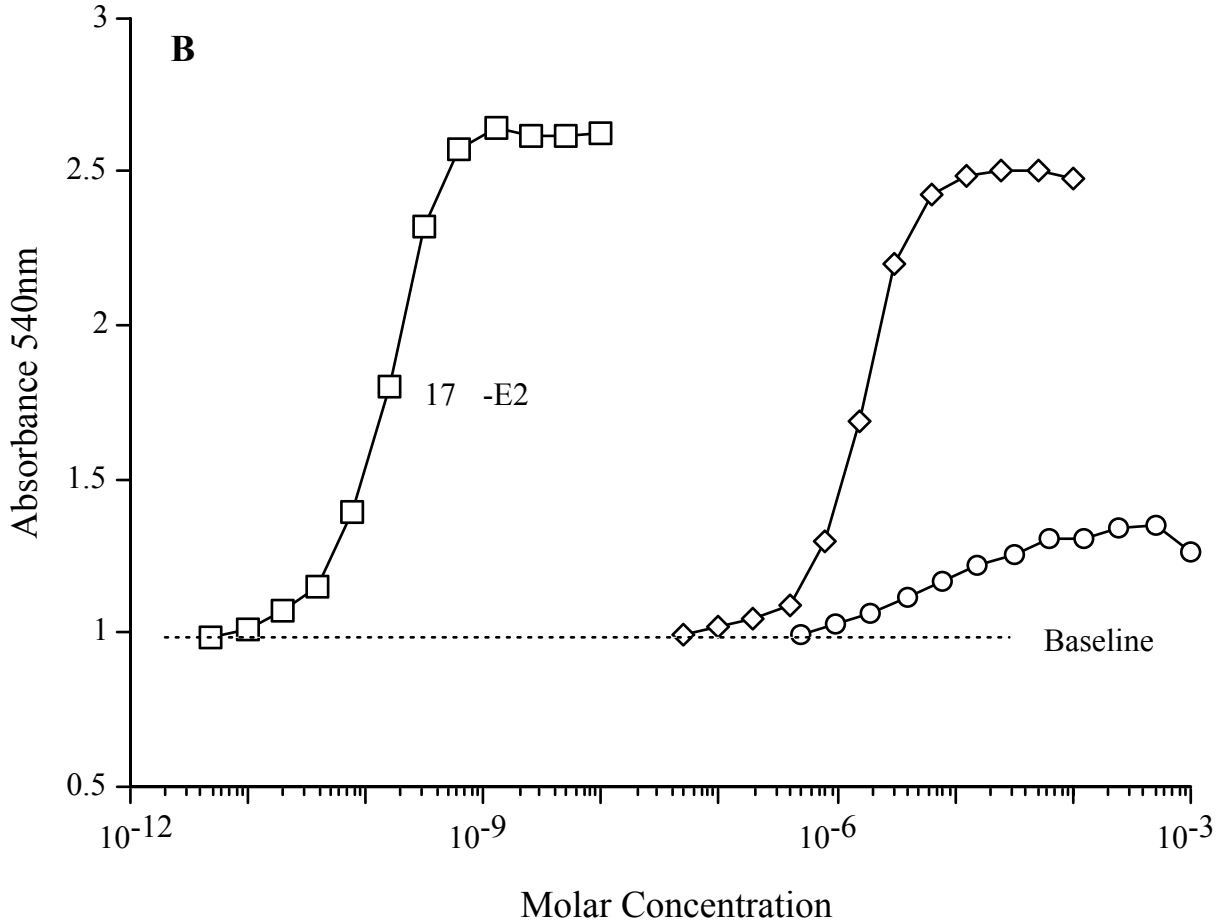
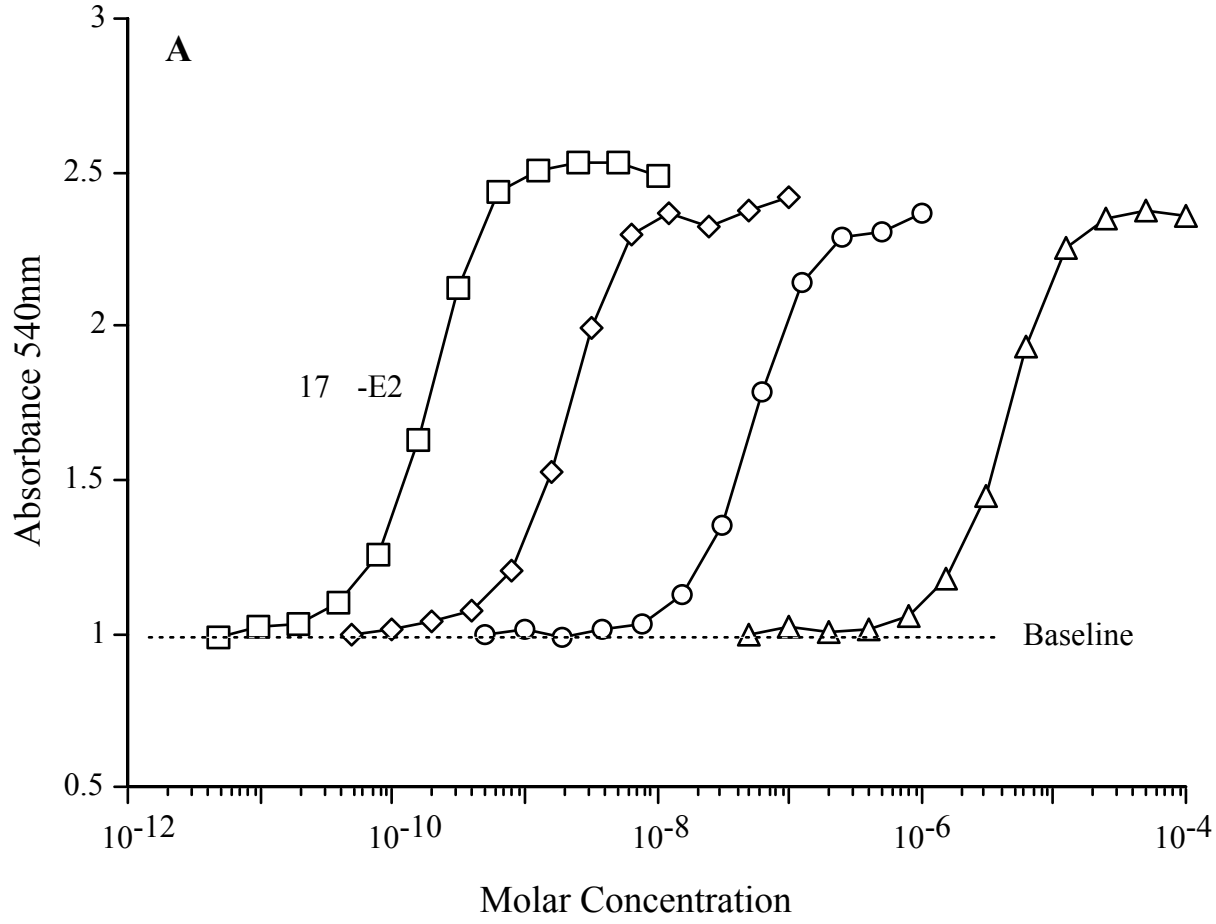
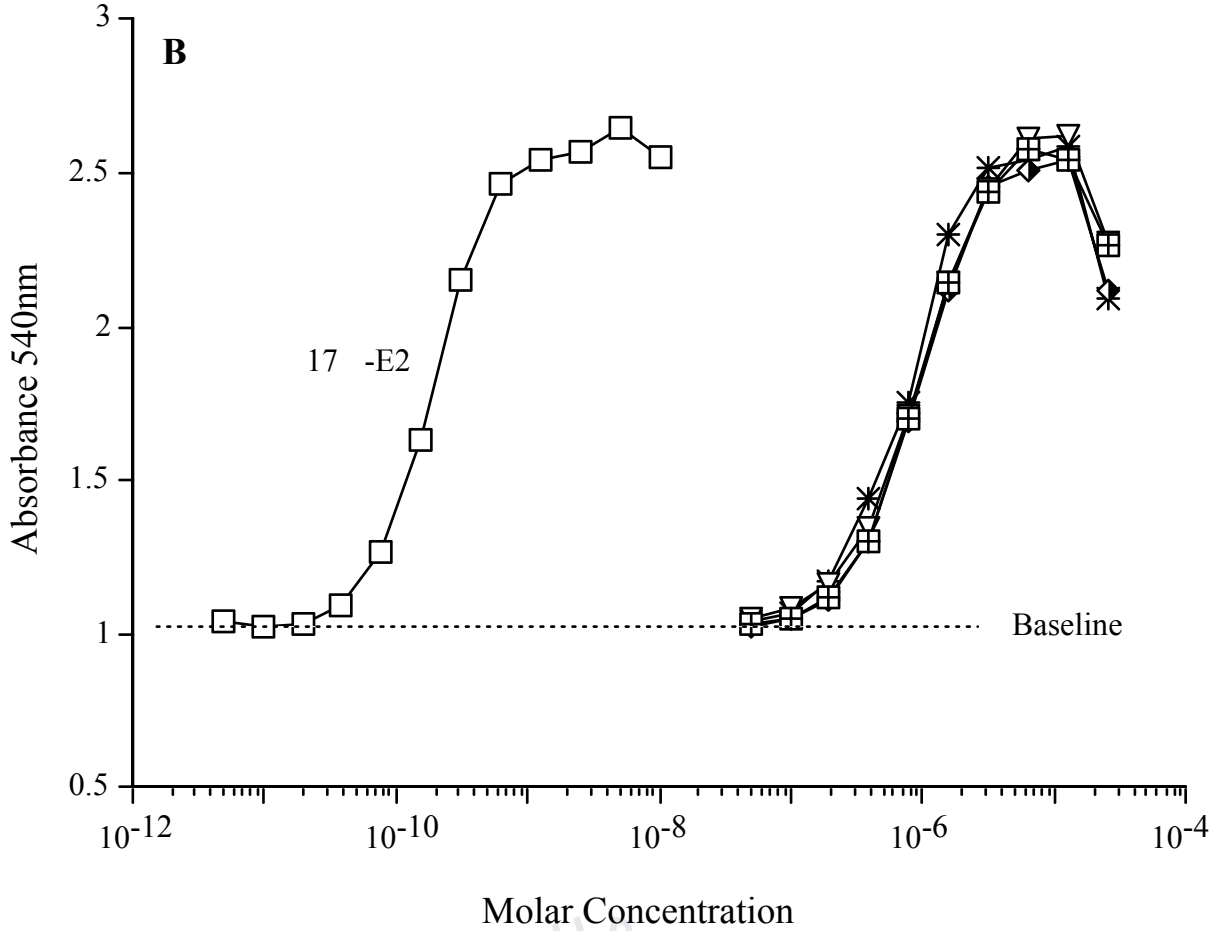
2
1.5
1
0.5
17 -E2
Baseline
10-12
10-9
10-6
10-3
Molar Concentration


B
Absorbance 540nm
3
2.5
2
1.5
1
0.5
17 -E2
Baseline
10-12
10-9
10-6
10-3
Molar Concentration


A
Absorbance 540nm
3
2.5
2
1.5
1
0.5
17 -E2
Baseline
10-12
10-10
10-8
10-6
10-4
Molar Concentration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Absorbance 540nm
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2.5
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17 -E2
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10-12
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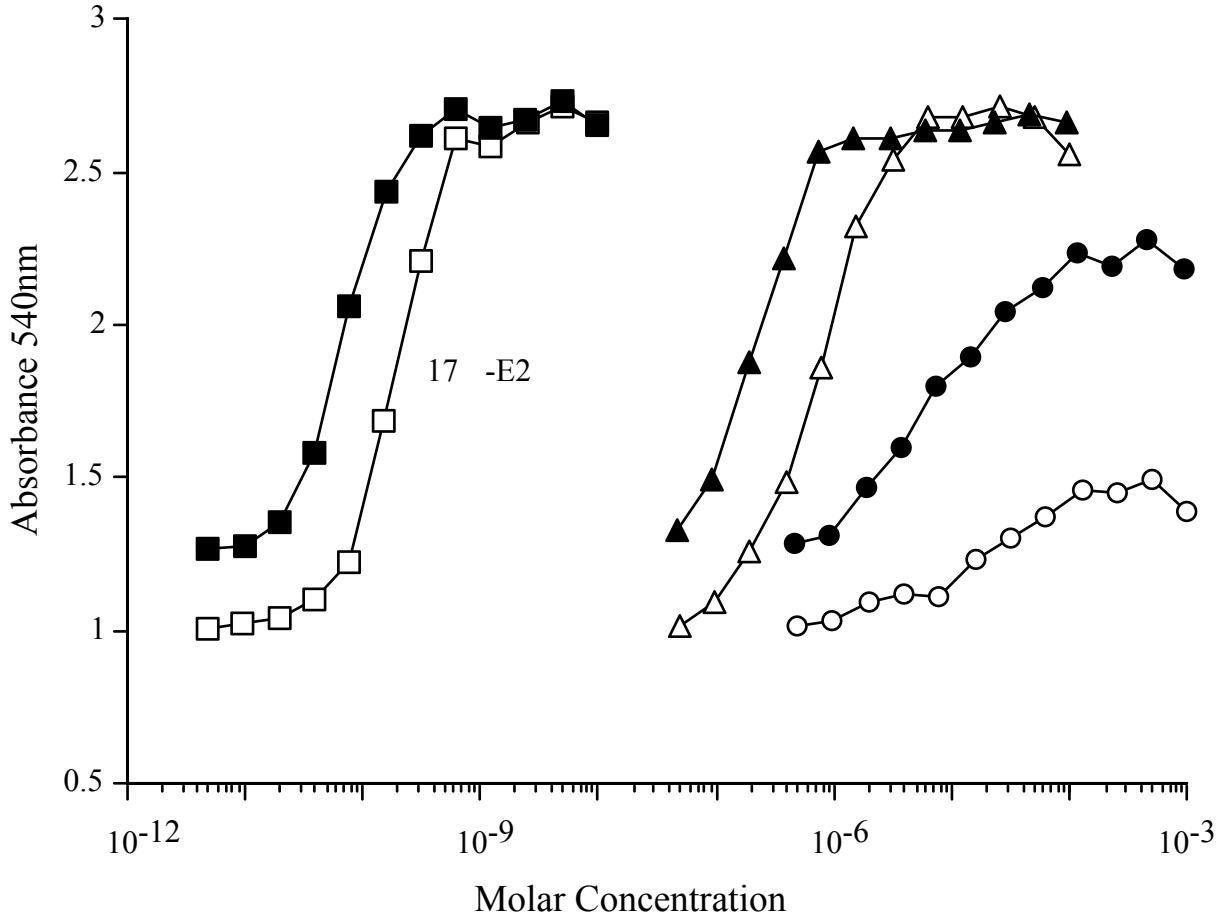
Molar Concentration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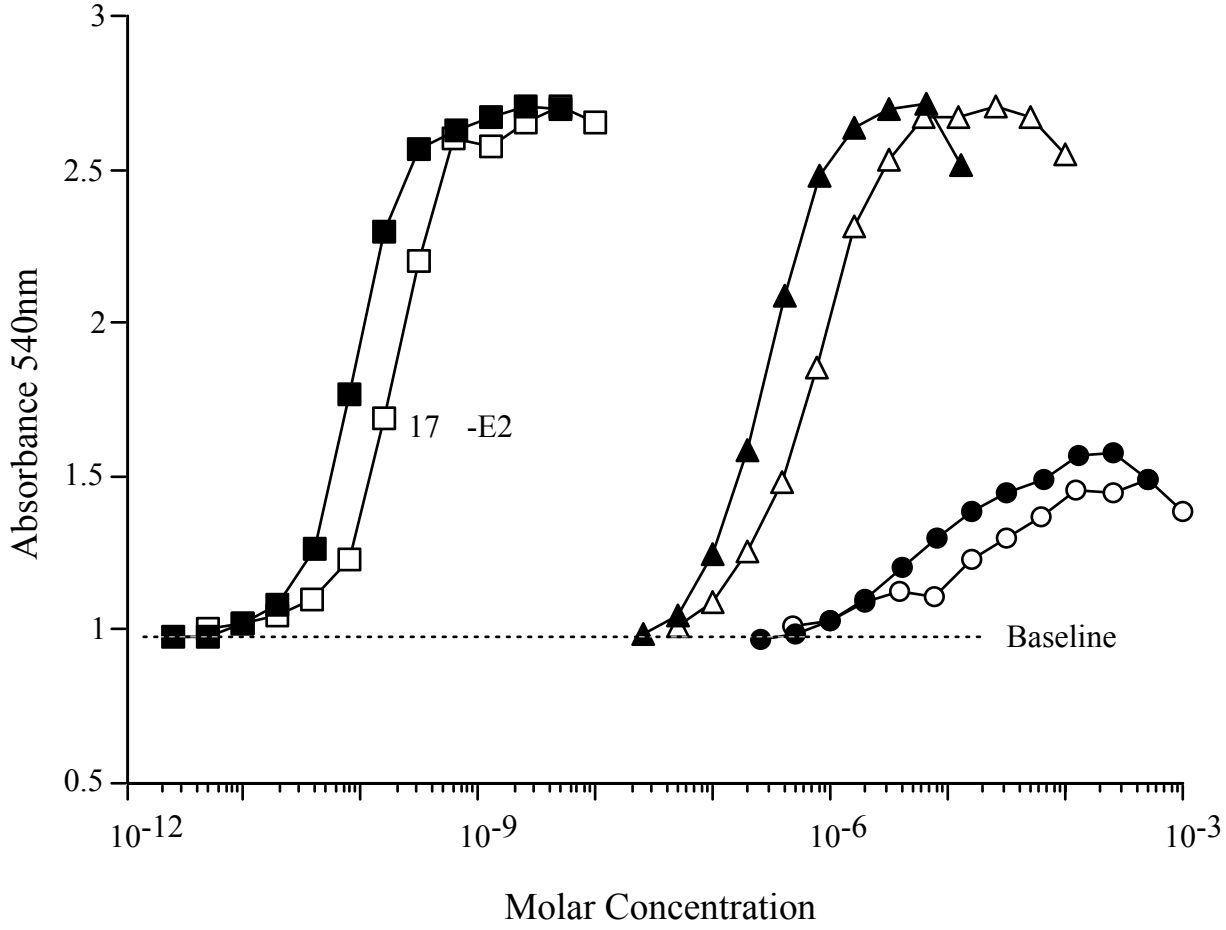
Absorbance 540nm
17 -E2
10-12
10-9
10-6
10-3
Molar Concentration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Absorbance 540nm
17 -E2
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10-12
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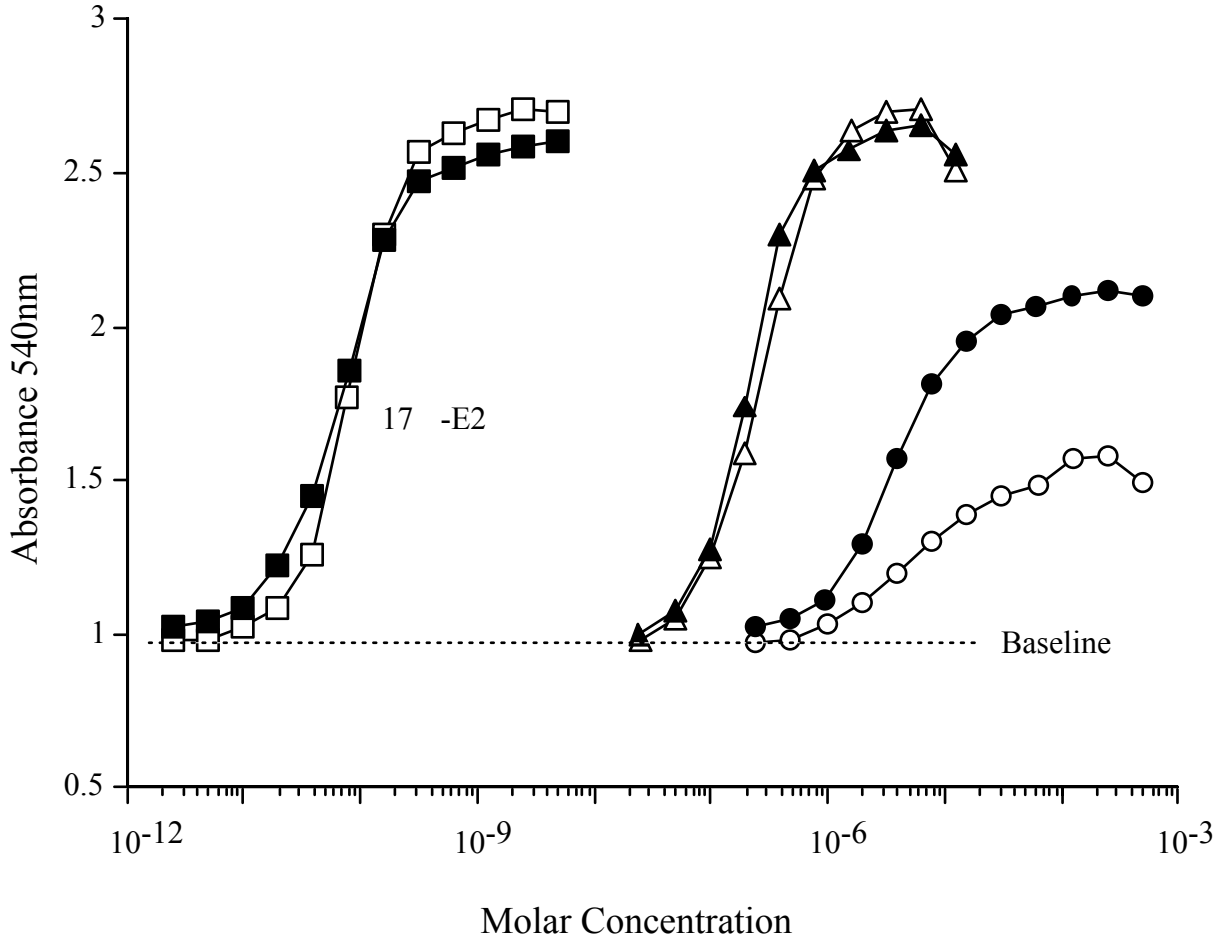
Molar Concentration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Baseline
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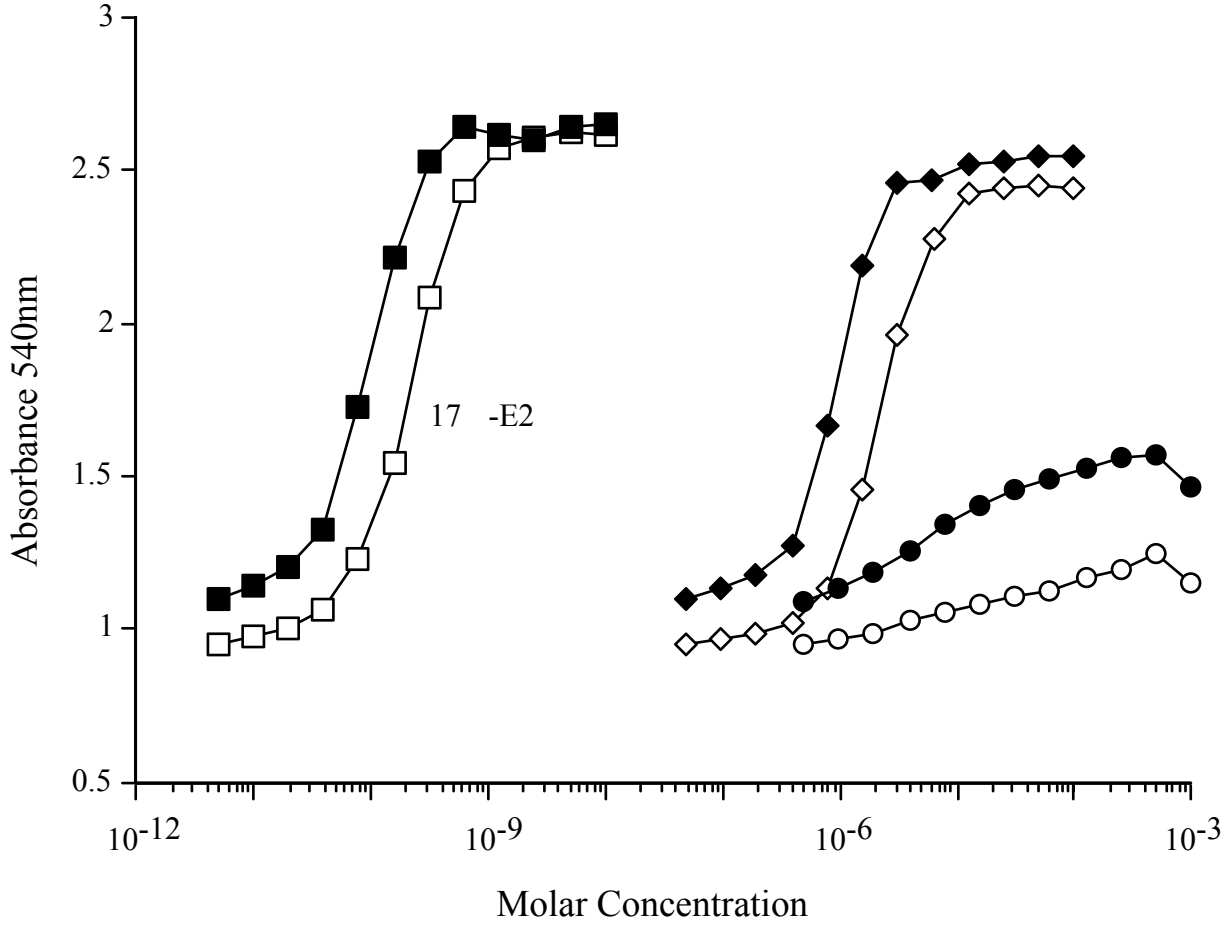
Molar Concentration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Absorbance 540nm
17 -E2
10-12
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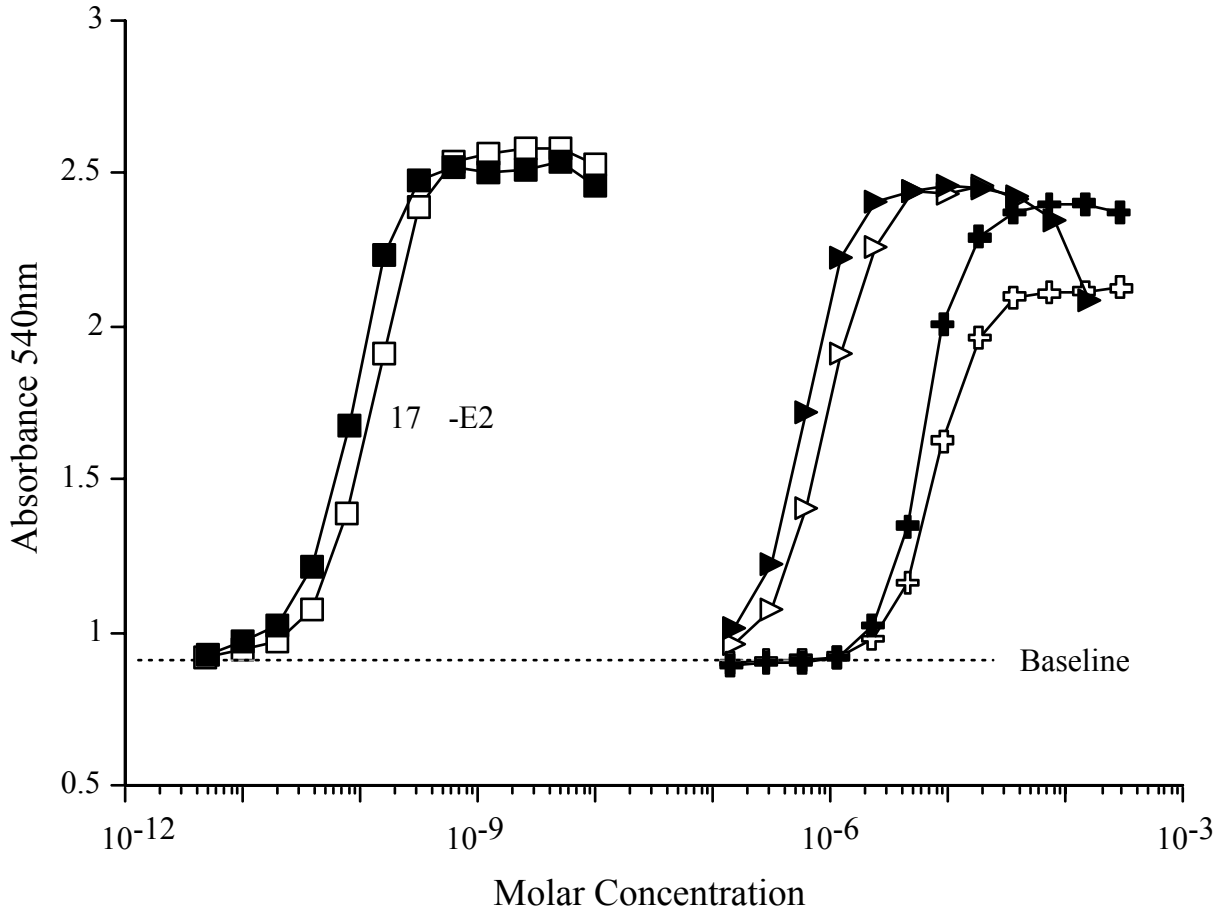
10-6
10-3
Molar Concentration


3
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2
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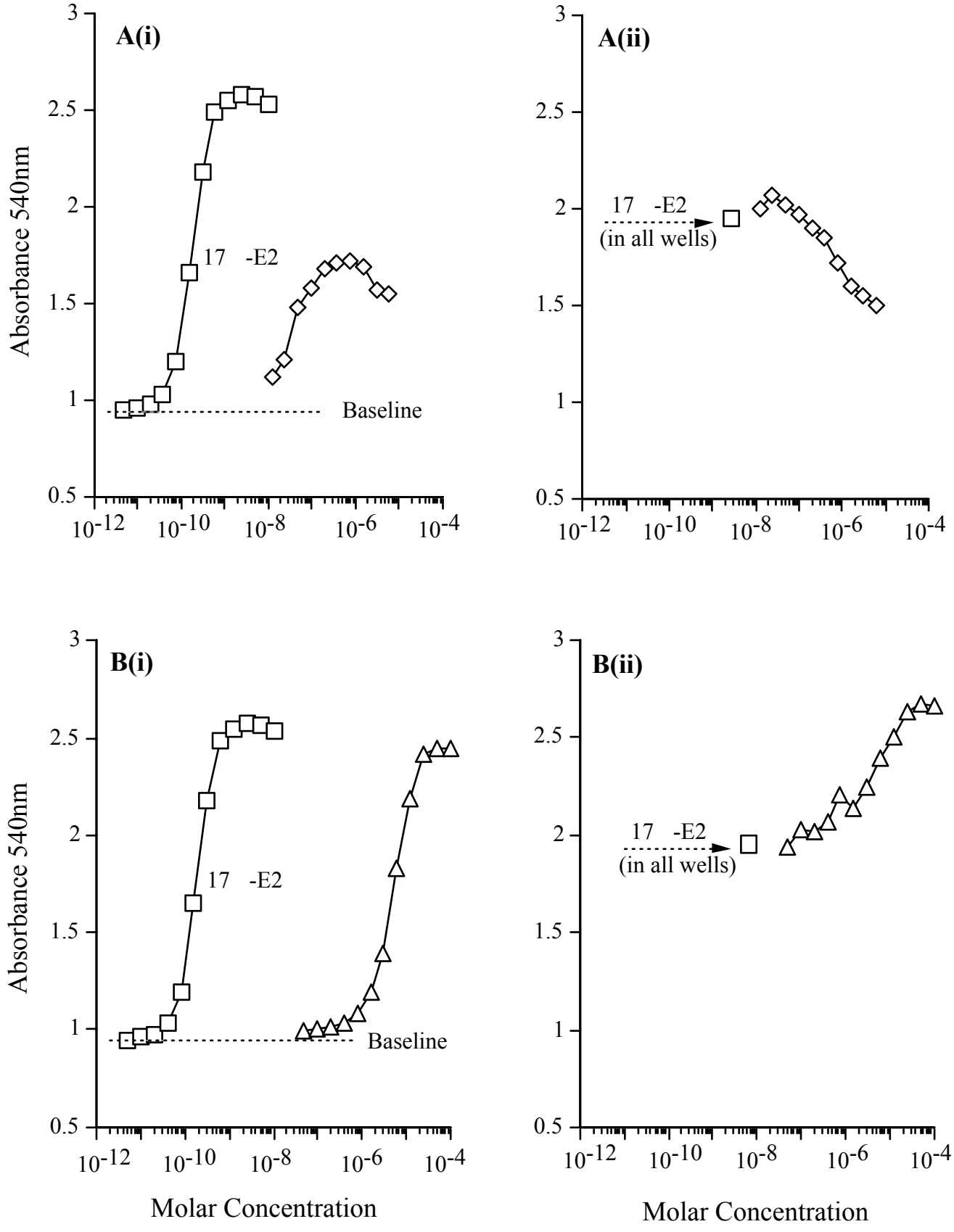
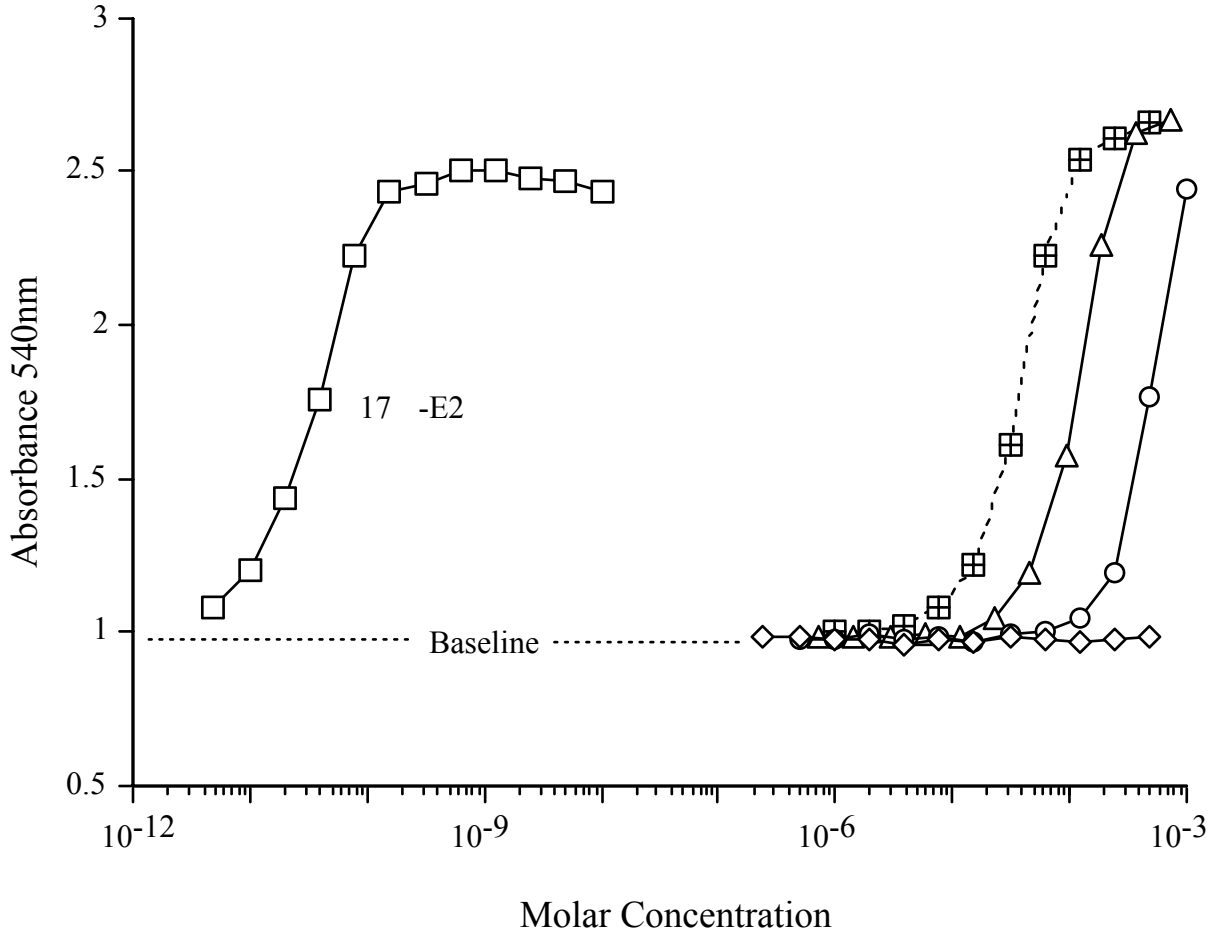
0.5
Absorbance 540nm
17 -E2
Baseline
10-12
10-9
10-6
10-3
Molar Concentration


A(i)
A(ii)
B(i)
B(ii)
Absorbance 540nm
Molar Concentration
17 -E2
(in all wells)
Baseline
3
2.5
2
1.5
1
0.5
10-12
10-10
10-8
10-6
10-4


3
2.5
2
1.5
1
0.5
Absorbance 540nm
17 -E2
Baseline
10-12
10-9
10-6
10-3
Molar Concentration