

Development of high-throughput yeast-cell-based bioreporter assays for specific monitoring of bisphenol A and chemical testing of endocrine disrupting compounds

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ABSTRACT

Chemicalization of the modern society has become a topic of debate in the past few decades. Especially chemicals that affect the human reproduction and hormonal system, the so-called endocrine disrupting compounds, have raised concern in public and regulatory agencies. There is a growing need for suitable testing methods to screen endocrine disrupting potential of new and old chemicals. While the European Union chemical legislation REACH has increased the need of chemical testing methods, one of its targets is also to decrease the use of animals in these tests. It has been proposed that inexpensive high throughput *in vitro* assays could be used for initial screening of chemicals for further testing with other methods.

In addition to chemical testing, environmental monitoring of endocrine disrupting compounds is important to assess the level of exposure and possible adverse effects of chemicals on humans and wildlife. Chemical analysis methods used in environmental monitoring are sensitive, but they are also laborious, expensive, and require specialized instruments. Consequently, robust biological methods have become valuable tools to measure endocrine disrupting potency of chemicals and environmental samples. For this purpose, several *Saccharomyces cerevisiae* yeast cell-based bioreporters utilizing different nuclear receptors have been developed.

Yeast-cell-based bioreporter assays have several advantages in environmental analytics. In addition to being inexpensive, they are particularly useful in determining the bioavailability of contaminants. Yeast is also very tolerant towards toxicity of different sample matrices. Yeast nuclear receptor bioreporter assays have been used to determine the total hormonal activity of samples containing unknown mixture of chemicals. However, these assays cannot identify the chemicals in the sample, and thus, monitoring of a single chemical has not been possible.

Many cell-based assays have already been adapted to high throughput screening plate formats of 384 and 1536 wells and even higher. However, nearly all yeast nuclear receptor bioreporter assays are still performed in 96-well plates. Consequently, yeast bioreporter assays should be adapted to automated liquid handling and high density well plates to enable screening of large chemical libraries and high number of samples.

In this thesis study, a yeast nuclear receptor bioreporter assay for specific detection of a single chemical, bisphenol A (BPA) was developed. The creation of the BPA-targeted receptor included application of an oligonucleotide-based mutation method and a positive-negative genetic selection method for human estrogen receptor α (publication I). Chemical specificity of the BPA-targeted receptor (BPA-R) bioreporter assay was characterized, and its use demonstrated with chemical mixture and waste water samples (publication II). In addition, the existing battery of yeast bioreporters was adapted to automated liquid handling and high density 384 and 1536 well plates to meet the requirements of high throughput screening (publication III). Finally, a new yeast-based bioreporter utilizing a chimeric human retinoid X receptor was constructed and characterized (publication IV). This bioreporter can be used to measure organotin compounds such as tributyl tin in environmental samples.

TIIVISTELMÄ

Kemikaalit ovat osa modernia maailmaamme. Lähes kaikkien käyttämämme tuotteiden - jopa elintarvikkeiden - valmistuksessa on käytetty erilaisia kemikaaleja takaamaan niiden haluttu koostumus, laatu ja turvallisuus. Kemikaalien turvallisuus ja ihmisten elinympäristön kemikalisoituminen on kuitenkin herättänyt viime vuosikymmeninä huolta niin suuressa yleisössä kuin viranomaisissakin. Erityisesti salakavalalta tuntuvat hormoni-toimintaan vaikuttavat kemikaalit ovat huolestuttaneet ihmisiä. Näiden ns. hormonihäiritsijäkemikaalien on epäilty aiheuttavan mm. lisääntymis- ja terveydellisiä, hermostollisia ja aineenvaihdunnan ongelmia. Tällaisia kemikaaleja ovat esim. torjunta-aineet, dioksiinit, monet palonestoaineet, muoviteollisuuden käyttämät ftalaatit ja bisfenoli A sekä orgaaniset tinayhdisteet.

Euroopan Unionin kemikaaliasetus REACH tähtää kohti turvallisempaa kemikaalien käyttöä. Sen tarkoituksena on laajentaa kemikaalien erilaisten vaikutusten testaamista, saattaa testien teettäminen kemikaalien valmistajien vastuulle sekä parantaa käyttöturvallisuuden tiedonkulkua kemikaalien tuottajilta niiden käyttäjille. Kemikaalien testausvaatimusten lisääntyessä myös uusien testausmenetelmien tarve kasvaa. Eläinkokeiden sijaan REACH pyrkii suosimaan tehokkaampia ja eettisempiä menetelmiä, kuten *in vitro* eli ns. ”koeputkessa” tehtäviä solu- ja kudospohjaisia määrytyksiä.

Hormonihäiritsijäkemikaalien testauksessa sieniin kuuluva tavallinen leivinhoiva (lat. *Saccharomyces cerevisiae*) on osoittautunut hyväksi työkaluksi. Eläinsoluihin verrattuna hiivaa on edullista ja helppoa viljellä ja muokata. Vaikka hiivasoluilta puuttuu eläinsolujen tumareseptoreihin perustuva hormonien viestinvälitysketju, tämä ketju voidaan saada toimimaan myös hiivassa. Monet ihmisen hormonireseptorit, kuten estrogeenireseptori, on onnistuneesti siirretty hiivaan. Kun hiiva altistetaan reseptoriin sitoutuvalla kemikaalilla, reseptori aktivoituu ja tuottaa solussa ulkoisesti havaittavan signaalin. Tätä signaalia mittaamalla voidaan arvioida kemikaalin mahdollista vaikutusta kyseiseen hormonireseptoriin ja sen säätelimiin viestinvälitysketjuihin.

Yksittäisten kemikaalien vaikutusten testaamisen lisäksi hiivasolumenetelmät soveltuvat myös erilaisten tuntemattomien ja puhdistamattomien näytteiden analysointiin. Tällaisia näytteitä ovat esim. ympäristö- ja vesinäytteet tai vaikkapa elintarvikenäytteet. Hiivan avulla voidaan siis seurata ja arvioida ympäristön ja ihmisten mahdollista altistumista hormonaalisesti aktiivisille yhdisteille ja niiden seoksille.

Tässä väitöskirjatyössäni olen vinyt eteenpäin olemassa olevia ja kehittänyt uusia hiivasoluihin perustuvia hormonaalisten kemikaalien testausmenetelmiä. Julkaisussa (I) suunnittelin menetelmän ihmisen estrogeenireseptorin rakenteen muokkaamiseen, ja sovelsin menetelmää kehittääkseni reseptorin, joka aktivoituu vain yhden valitun kemikaalin, bisfenoli A:n, sitoutuessa siihen. Julkaisussa (II) tätä muokattua reseptoria käytettiin spesifisen bisfenoli A:ta mittaavan hiivamenetelmän kehittämiseen. Tätä menetelmää voidaan soveltaa bisfenoli A:n mittaamiseen erilaisissa näytteissä, ja arvioimaan altistusta tälle kemikaalille eri lähteistä. Julkaisussa (III) siirsin olemassa olevien käsin pipetoitavien hiivamenetelmien käytön perinteisestä 96-kuoppalevy-mittauksesta pipetointirobotin avulla 384- ja 1536-kuoppalevyillä tehtäviin määrytyksiin. Menetelmä mahdollistaa tehokkaamman analysoinnin ja laajempien näytemäärien mittaamisen. Julkaisussa (IV) suunnittelin ja olin mukana toteuttamassa uutta hiivamenetelmää, jonka avulla voidaan mitata orgaanisia tinayhdisteitä. Menetelmän avulla voidaan mitata ja arvioida tinayhdisteiden biosaatavuutta ja sedimentin saastuneisuuden astetta esim. laivaväylillä.

ABBREVIATIONS

9cRA	9- <i>cis</i> -retinoic acid
AD	activation domain
atRA	all-trans retinoic acid
BD	binding domain
BPA	bisphenol A
BPA-R	bisphenol A-targeted receptor
DBD	DNA-binding domain
DDT	dichlorodiphenyltrichloroethane
EC ₅₀	half-maximal effective concentration
EDC	endocrine disrupting compound
hAhR	human aryl hydrocarbon receptor
hAR	human androgen receptor
hER	human estrogen receptor
His3	Imidazole glycerol phosphate dehydratase
HRE	hormone receptor responsive element
hRXR	human retinoid X receptor
HTS	high throughput screening
LBD	ligand-binding domain
LOD	limit of detection
NR	nuclear receptor
OE-PCR	overlap extension polymerase chain reaction
PCB	polychlorinated biphenyl
PAH	polycyclic aromatic hydrocarbon
RAR	retinoic acid receptor
TBT	tributyl tin
TPT	triphenyl tin
TRP	N-(5'-phosphoribosyl)anthranilate isomerase
UAS	upstream activating sequence
yEGFP	yeast-enhanced green fluorescent protein

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to by their Roman numerals in the text:

I Johanna Rajasärkkä, Kaisa Hakkila, Marko Virta (2011). Developing a Compound-Specific Receptor for Bisphenol A by Directed Evolution of Human Estrogen Receptor alpha. *Biotechnology and bioengineering*, 108: 2526-2534.

II Johanna Rajasärkkä and Marko Virta. Characterization of a Bisphenol A-Specific Yeast Bioreporter Utilizing the Bisphenol A-Targeted Receptor BPA-R. Manuscript.

III Johanna Rajasärkkä and Marko Virta (2011). Miniaturization of a Panel of High Throughput Yeast-Cell-Based Nuclear Receptor Assays in 384-and 1536-Well Microplates. *Combinatorial chemistry & high throughput screening*, 14: 47-54.

IV Grit Kabiersch, Johanna Rajasärkkä, Marja Tuomela, Annele Hatakka, Marko Virta, Kari Steffen (2013). Bioluminescent yeast assay for detection of organotin compounds. *Analytical Chemistry*, in press.

The author's contribution

The author Johanna Rajasärkkä had following contributions to the research in each publication:

I Johanna Rajasärkkä participated in the planning of the study together with the other authors. She conducted all the experiments except for the flow cytometric sorting. She analyzed the data, wrote the publication and acted as the corresponding author.

II Johanna Rajasärkkä planned the study together with the other author, conducted all the experiments, interpreted the data, and wrote the publication. She acted as the corresponding author.

III Johanna Rajasärkkä planned the study together with the other author, conducted all the experiments, interpreted the data, and wrote the publication. She acted as the corresponding author.

IV Johanna Rajasärkkä planned and constructed the tributyl tin bioreporter strain. She participated in the planning of the characterization study, in the result interpretations, and reviewed the manuscript.

1. INTRODUCTION

1.1. Endocrine disrupting compounds (EDCs)

In the past few decades, the use of different chemicals in everyday life has increased dramatically. After the Second World War chemicals were regarded as indicators of development towards better times. In 1962 Rachel Carson shook this belief with her book *Silent Spring* in which a novel type of toxicity of pesticides such as DDT on wildlife were described for the first time. Later, when more and more evidence on chemicals with special effects on reproduction and development of both humans and wildlife started to gather, a group of scientists established the definition of endocrine disrupting compounds (EDC) in 1991 (Bern et al. 1992). In a book “*Our Stolen Future*” by Theo Colborn et al. (1997) these compounds were brought to public awareness.

A more recent definition for EDCs by The International Programme on Chemical Safety describe them as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse effects in an intact organism, or its progeny, or (sub)populations.” (Damstra et al. 2002). EDCs have been suspected to cause adverse effects such as cancers, embryotoxicity, effects in immune and neuronal systems, and impaired reproduction and development in wildlife and humans (Damstra et al. 2002, Sharpe & Irvine 2004). In recent years some of these chemicals have also been suggested to play a role in the increasing rates of obesity and metabolic syndrome in western world (reviewed by Grün and Blumberg 2007).

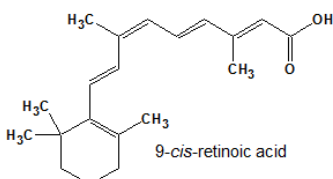
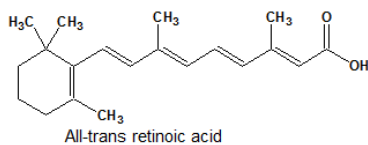
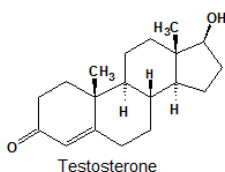
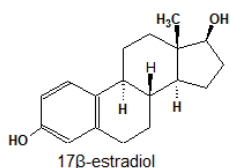
EDCs have been suggested to disrupt the normal functioning of the endocrine system in vertebrates primarily via binding to nuclear hormone receptors (NRs). Indeed, EDCs share some structural and chemical similarities with natural steroid hormones and other small-molecule NR ligands. Well-known EDCs are, for example, dioxins, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), some flame retardants, parabens used in cosmetics, organotins such as tributyl tin, and plastic ingredients like phthalates and bisphenol A. Some natural ligands of NRs and suspected EDCs are shown in Figure 1.

Bisphenol A (BPA) is a well-known xenoestrogen. It is a high production volume chemical with over 1 million tonnes annual production in EU (European Union 2010). It is used in numerous products such as polycarbonate plastics, epoxy resins, dental sealants, food and beverage packaging, and thermal paper. BPA-containing resins have also been used to line water pipes to cut down costs of renovations.

Biomonitoring studies have shown that humans are constantly exposed to BPA (Vandenberg et al. 2010). Although the effects of current BPA exposure levels are still controversial there is growing evidence on adverse effects on both humans and wildlife (Crain et al. 2007, Flint et al. 2012, Vandenberg et al. 2009, Grün and Blumberg 2007). The main exposure routes of BPA for humans occur via food and drink, while

environmental BPA emissions are in water phase (European Union 2010). BPA is indeed one of the most frequently detected contaminant in water samples (Bono-Blay et al. 2012, Loos et al. 2010, Vethaak et al. 2005, Fromme et al. 2002). In order to assess the exposure of humans and wildlife to BPA more biomonitoring studies are needed (vom Saal et al. 2007).

Natural ligands of nuclear receptors



Suspected endocrine disruptors

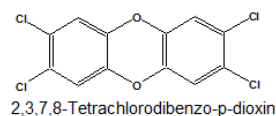
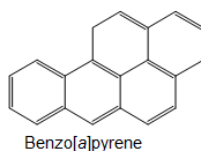
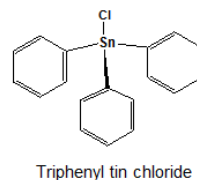
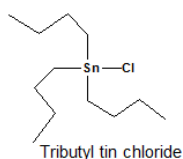
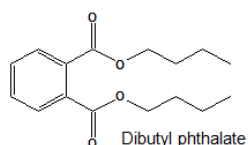
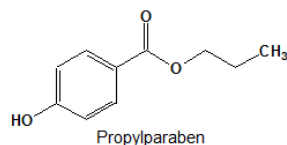
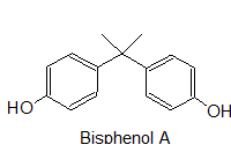


Figure 1. Structures of some natural ligands of nuclear receptors and suspected endocrine disruptors.

Another example of well characterized EDCs is a group of organic compounds of tin (Sn). These chemicals include, for example tributyl tin (TBT) and triphenyl tin (TPT) (Figure 1.). TBT is used as an industrial biocide, and until its world-wide ban by International Maritime Organization in 2008, it was widely used in antifouling paints in ship hulls. Both compounds are persistent and bioaccumulating. Organotin compounds can cause, for example, imposex in marine organisms, deterioration of the immune system, and impaired reproduction, development, and bone cell differentiation (Nishikawa et al. 2004, Titley-O'Neal et al. 2011, Gumy et al. 2008, Sekizawa 1999, Yonezawa et al. 2007). Organotin compounds are also suspected to be environmental obesogens (Kirchner et al. 2010, Grün & Blumberg 2007). While humans are mostly exposed via (sea)food, most of the environmental organotin compounds are found in sediments at or close to harbours and shipping channels where their degradation is

slow (Sekizawa 1999, Antizar-Ladislao 2008). Concentrations as high as $\mu\text{g g}^{-1}$ have been detected (Antizar-Ladislao 2008), also in Finland (Helminen & Peltonen 2009).

In 1990's it became evident that vast majority of chemicals used European Union (EU) lacked proper safety data, and the current legislation was unable to ensure public safety against risks of chemicals (European Commission "White paper" 2001). In order to change this situation, and to manage the risks of chemicals in European markets, EU launched the new legislation REACH (Registration, Evaluation, Authorization and Restriction of CHemicals). Along REACH, a new era in toxicity testing evoked.

While the existing EU chemical legislation REACH does not require testing endocrine disruptive property as an endpoint, the European Commission has addressed that there is a need to ensure a proper testing strategy and risk assessment for these chemicals. It is clear that there is no comprehensive testing method for endocrine disrupting activity of chemicals that could be solely used. Rather, a combination of different testing methods should be used. OECD has published a framework report for testing endocrine disrupting activity of chemicals (OECD 2012). A battery of both *in vitro* and *in vivo* methods was suggested. Since one of the aims of REACH is to reduce the need of animal tests, robust cell-based assays could be suitable first level testing methods.

In addition to general endocrine disruption activity measurements, there is a need for exposure assessment on some existing suspected EDCs, such as bisphenol A (BPA). Although chemical analysis methods, such as liquid chromatography (Wille et al. 2012) are sensitive, they usually require extensive sample purification, specialized instrumentation, and high level technical expertise. They are also poorly suited for high throughput screening of a large number of samples. Cell-based bioreporter assays, however, are cost-efficient and well suitable for high-throughput screening of both environmental and pure chemical samples (Bovee & Pikkemaat 2009, Leskinen et al. 2005 & 2008). Bioreporters can also measure bioavailability (Hynninen & Virta 2010), and the mixture effect of chemicals or total hormonal activity of complex samples (Leskinen et al. 2005).

1.2. Yeast-based nuclear receptor bioreporters for EDCs

Yeast-based nuclear receptor (NR) bioreporter assays have been reviewed before in literature (Rajasärkkä & Virta 2013, Bovee & Pikkemaat 2009, Svobodová & Cajthaml 2010). The following chapters present a short overview of the currently available assays and their suitability to high throughput screening (HTS).

1.2.1. Yeast as a nuclear receptor activity study organism

The common baker's yeast *Saccharomyces cerevisiae* is a thoroughly studied and well characterized unicellular eukaryotic organism. It is easy to cultivate and there are numerous tools available for its genetic manipulation. Due to its similarity with mammalian cells, yeast has become a popular organism in studying NR functions and ligand affinities.

NRs are a superfamily of receptors specific to animals. In human genome 48 NRs have been found so far. They are divided into three subclasses: type I for steroid hormone receptors (e.g. estrogen receptor), type II for other receptors (e.g. thyroid receptor), and type III for orphan receptors with no known natural ligand (Jacobs et al. 2003). NRs function in cells as transcription factors: ligand-bound receptor forms a dimer with another receptor, translocates to the cell nucleus, and binds on response elements on promoters of genes controlled by the receptor. NRs control numerous signaling pathways of, for example, growth, reproduction, and development.

Many mammalian and yeast-cell-based assays for studying NR activity have been developed. Although yeast cells have no endogenous NR signaling pathways, functional NRs have been successfully expressed in yeast (Metzger et al. 1988, Schena & Yamamoto 1988, Mak et al. 1989, Purvis et al. 1991). The lack of NR-dependent signaling system in yeast is an advantage since there is no risk of cross-talk between the studied and endogenous receptors, whereas this can be the case in mammalian cell assays (Bovee et al. 2007, Miller et al. 2010).

However, yeast and mammalian cell assays have some differences. One of these is the ability to distinguish between agonistic, i.e. "receptor activating", and antagonistic, i.e. "receptor repressing" ligands. In natural system in mammalian cells the distinction is based on the ligand-activated conformational change of the receptor. Depending on the conformation of the NR, and other NRs and cofactors in particular cell type, the receptor either activates or represses transcription. Since yeast has no NR cofactor proteins, NR-binding ligands show mostly agonistic activity. It has been proposed that the higher insensitivity of yeast to antagonists could be an advantage in screening hormonal compound because of lower rate of false negatives (Connolly et al. 2011). Yet, recent studies have shown that yeast-based NR assays can detect partial or full antagonism of chemicals (Michelini et al. 2005, Leskinen et al. 2005, Liu et al. 1999, Bovee et al. 2007, Bovee et al. 2010, Kolle et al. 2010). In testing antagonistic effect, response of the chemical is measured in the presence of externally added native hormone that normally activates the receptor. Thus, the negative effect to transcription can be detected (Bovee et al. 2010). The differential response of yeast NR bioreporters to antagonists should, however, be considered when testing chemical mixtures and complex samples: in principle it is possible that yeast assays would show higher activity than mammalian cell assays.

Differences in metabolic activation or inactivation of a chemical can also result in variability between mammalian and yeast cell assays (Beresford et al. 2000, Charles et

al. 2000, Hoogenboom et al. 2001). It is possible, however, to incorporate an enzyme-based metabolic activation step into yeast NR assays (Morohoshi et al. 2005, Rijk et al. 2008, S. Wang et al. 2010). For example, bovine liver enzyme preparation fraction S9 is able to convert steroid pro-hormones into active hormones. Using this method the predictive value of the bioassay in assessing the potential activity of compounds *in vivo* can be increased. In addition to metabolic effects, other indirect effects that do not involve receptor binding, such as enhanced receptor degradation (Wijayarathne & McDonnell 2001), are naturally not detected in yeast.

Mammalian cell NR assays are also usually more sensitive than yeast NR assays (Schultis & Metzger 2004, Murk et al. 2002, Svobodová & Cajthaml 2010). It has been proposed that yeast cells use transporters to pump out some of the potential NR ligands, reducing sensitivity of the assay (Liu et al. 1999, Dudley et al. 2000). By removing such transporter from the genome of yeast, sensitivity of a yeast assay can be improved (Balsiger et al. 2010). In addition, low permeability of potential NR ligands through yeast cell wall can limit sensitivity (Lyttle et al. 1992). However, this effect does not seem to harm most of the yeast assays even in short (4-12 hours) incubation time (Bovee et al. 2004). It has also been shown that the accuracy of NR yeast assay is nearly comparable with mammalian cell assay (Kolle et al. 2010).

Yeast assays have some advantages over mammalian cell assays. Yeast is a fast-growing, easy-to-cultivate organism, and it does not require serum for growth. Whereas typical mammalian MCF7 and HeLa cell line assays require several days of growth in a steroid-free serum and a 16 hour long incubation with the test chemicals (Balaguer et al. 1999), yeast grows overnight and the assays can be performed during one working day (Leskinen et al. 2005). Yeast assays are more robust and cost-efficient, and no specialized performers or instruments are needed. In addition, yeast is generally more tolerant towards toxicity of non-purified complex samples compared to mammalian cells. Yeast assays have already been used to screen for hormonal activity in complex samples (Bovee et al. 2008, Bovee et al. 2009b, Inoue et al. 2011) and pure chemicals (Kamata et al. 2008). Thus, yeast assays are a valuable addition to the battery of NR-dependent screening methods.

1.2.2. Principle of yeast-based nuclear receptor bioreporter assays

The ability of yeast to reproduce the NR signaling pathway is remarkable since yeast does not have mammalian cell-like NRs. However, some yeast receptors with similarly functioning domains and cofactors have been discovered, suggesting a common ancestor for these receptors (Phelps et al. 2006, Näär and Thakur 2009).

Yeast NR assays detect either ligand-dependent NR-mediated transcriptional activation of a reporter gene (Figure 2A), or ligand-dependent interaction of the receptor with a cofactor protein (the yeast two-hybrid method, Figure 2B). In transcription activation assay (Figure 2A) the ligand-bound NR binds the hormone

receptor responsive elements (HRE) inserted on the promoter of the reporter gene. Then transcriptional activation of the reporter takes place via recruitment of various transcription cofactors of yeast origin, for example chromatin remodeling complexes or histone modification factors (mechanisms of NR functioning in yeast reviewed by Kennedy 2002).

In yeast two-hybrid method (Figure 2B) the ligand binding domain of the receptor is coupled to activation (AD) or DNA-binding domain (BD) of the Gal4 transcription factor. In this method, the ligand-bound receptor interacts with a chosen cofactor protein, and the complex activates the expression of a reporter protein by binding to Gal4-specific upstream activating sequence (UAS) on its promoter.

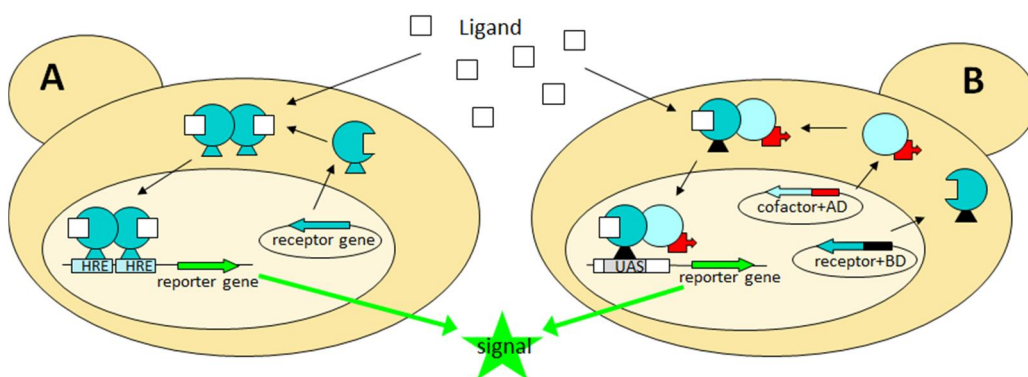


Figure 2. Principle of yeast-based NR bioreporter assays. A: Transcription activation assay; B: Yeast two-hybrid assay. HRE = hormone receptor responsive element, UAS = upstream activating sequence, AD = activation domain, BD = binding domain.

The expression of a reporter protein is coupled to the ligand-dependent activation of a NR. Thus, the reporter protein produces a quantifiable signal that is dependent on the compound concentration. By comparing to the dose-response curve, the activity of an unknown sample can be translated into an equivalent concentration of a corresponding reference compound, such as 17 β -estradiol for estrogen receptor.

Majority of the current yeast-based NR assays use β -galactosidase enzyme as a reporter. β -galactosidase catalyses the breakdown of a chromogenic substrate, and the absorbance resulting from the color change is measured. Despite its popularity, β -galactosidase has some drawbacks. To produce better signal, cell lysis is usually needed (Schultis & Metzger 2004). Bioluminescent or fluorescent substrates for β -galactosidases can also be used, although they require either an extra incubation time or cell lysis (Balsiger et al. 2010, Berg et al. 2000).

Luciferases are enzymes that produce bioluminescence by oxidation of a substrate. Luciferases are favored in cell-based high throughput format assays because of low natural background and high specific activity (Fan & Wood 2007). Yeast-based NR

assays with luciferase enzyme from firefly as a reporter do not require cell lysis but they do need addition of substrate (Leskinen et al. 2005 & 2008). Luciferase enzyme from bacteria together with genes required for the biosynthesis of bioluminescence reaction substrate (the lux-operon) has also been used as reporter in yeast (Sanseverino et al. 2005, Eldridge et al. 2007). Although no substrate addition is needed, the signal is low compared to firefly luciferase.

Fluorescent proteins have been extensively used in imaging structures and functions of live cells (reviewed by Chudakov et al. 2010). An astonishing variety of fluorescent proteins with different palette of excitation and emission wavelengths exist today. The application repertoire is also wide, for example, imaging of tissue and cell architecture, protein and cell organelle localization, protein interaction, and promoter activity. Green fluorescent protein (GFP) is probably the best characterized and used of the fluorescent proteins. It has also been used as a reporter protein in yeast NR assays. A codon-optimised yeast-enhanced GFP (yEGFP) (Cormack et al. 1997) is used in several yeast NR assays (Bovee et al. 2004, 2007; & 2011, Chatterjee et al. 2008). The greatest benefit of yEGFP and other fluorescent proteins is that no substrate addition is needed. However, because of high natural background of green fluorescence, long ligand exposure times are usually needed with yEGFP to obtain a sufficiently high signal-to-background ratio (Bovee et al. 2007).

Growth reporter proteins are another class of reporters. They are metabolically essential enzymes that have been removed from the genome of the yeast that is used as a host strain in the reporter assay. For example, imidazole glycerol phosphate dehydratase (His3) synthesizes amino acid histidine. If no histidine is added externally, these strains are able to grow only in the presence of a receptor-activating ligand. Because these methods are based on cell growth, they are suitable for HTS, although cell growth is somewhat time-consuming compared to other reporters. Growth reporter proteins have been mainly used as a genetic selection tool for NR mutant libraries in yeast (Chen & Zhao 2005, Miller & Whelan 1998, Schwimmer et al. 2004).

Test chemicals and solvents can exhibit non-NR-dependent effects such as toxicity or growth promotion, which can cause false negative or positive results of the NR assay. A popular approach to correct these effects is to measure cell density after incubation with the chemical (Sohoni & Sumpter 1998, Bovee et al. 2004). Although this is a practical approach, it only reflects general toxicity on yeast but not possible inhibitory effect on the reporter protein. The toxic effects on yeast metabolism and reporter protein can be corrected by using a control strain constitutively expressing the reporter protein in parallel with the NR yeast strain (Michelini et al. 2005, Leskinen et al. 2005, Eldridge et al. 2007). In this method, the only change in reporter signal is measured.

1.2.3. Current yeast-based nuclear receptor bioreporter assays

Yeast cell NR-based bioreporter assays are useful in detection of endocrine disrupting potential of chemicals as well as environmental samples. Yeast NR assays are especially suitable for robust initial screening of chemicals and samples for further verification. However, because yeast NR assays do not have intrinsic NR signaling system, conclusions about toxicity on a whole organism-level cannot be drawn.

To date, there is a large selection of different yeast NR bioreporter assays available. Many of the assays are also capable of detecting antagonistic activity of chemicals. Some yeast NR bioreporter assays have also been internationally validated (Bovee et al. 2009a & 2009b).

The first and most studied yeast NR bioreporter assays were developed using the human estrogen receptor. Estrogen receptor (ER) is a steroid hormone sensing nuclear receptor that regulates genes involved in growth, development, differentiation, and activity of tissues. The natural ligands for ER are 17 β -estradiol, estrone, and estriol. In human, two subtypes of ER are known: ER α and ER β .

The first yeast ER bioreporter assay was the YES (Yeast Estrogen Screen) assay utilizing β -galactosidase reporter (Routledge & Sumpter 1996). Nowadays there are ER assays with firefly luciferase (Leskinen et al. 2005 & 2008), yEGFP (Bovee et al. 2004), and even bacterial luciferase-luciferin system (Sanseverino et al. 2005) as reporter proteins (Table 1).

Yeast ER assays are relatively sensitive with detection range starting from sub-nanomolar concentrations of 17 β -estradiol (Table 1). Several other chemicals have also been shown to activate the ER in yeast. These chemicals include, for example, natural steroid hormones, phytoestrogens, diethyl stilbestrol, bisphenol A, alkyl phenolic compounds, plasticizers, parabens, PCB compounds, pesticides, and pharmaceuticals (Sohoni & Sumpter 1998, Leskinen et al. 2005, Schultis & Metzger 2004, Bovee et al. 2004, Murk et al. 2002, Routledge & Sumpter 1997, Sanseverino et al. 2009, Nishikawa et al. 1999, Shiraishi et al. 2003). Several of these chemicals have also been shown to be active ligands of ER in mammalian cell assays (reviewed by Giesy et al. 2002).

As mentioned above, yeast-based ER bioreporter assays usually detect ER agonists, but assays that are able to detect ER antagonists have also been developed (Table 1). Chemicals such as tamoxifen, hydroxytamoxifen, and ICI 182780 have shown partial or full antagonism in some yeast assays, whereas in others they are detected as agonists only (Sohoni & Sumpter 1998, Leskinen et al. 2005, Kolle et al. 2010, Bovee et al. 2008, Legler et al. 2002). Yeast ER assays have been used to screen for ER antagonists, even at a comparable accuracy with a mammalian cell assay (Bovee et al. 2010, Kolle et al. 2010).

Table 1. Yeast-based nuclear receptor bioreporter assays.

Yeast NR assay	Reporter	Incubation time	Reference compound	Detection limit	EC ₅₀	Antagonism detection	Reference
hER α transactivation	β -galactosidase	3-4 days		-	0.22 nM	yes	Beresford et al. 2000
Yeast two-hybrid hER α	β -galactosidase	4 h		0.1 nM	-	nd	Nishikawa et al. 1999
hER α transactivation	bacterial luciferase	6 h	17 β -estradiol	0.045 nM	0.24 nM	nd	Sanseverino et al. 2005
hER α transactivation	firefly luciferase	2.5 h		0.03 nM	0.5 nM	partial	Leskinen et al. 2005
hER β transactivation	firefly luciferase	2.5 h		0.1 nM	0.5 nM	partial	Leskinen et al. 2005
hER α transactivation	yEGFP	4 h		-	0.4 nM	yes	Bovee et al. 2004
hAR transactivation	β -galactosidase	40 h		-	~1 nM	yes	Sohoni & Sumpter 1998
hAR transactivation	β -galactosidase	16 h		0.1 nM	4 nM	yes	Chatterjee et al. 2007
Yeast two-hybrid hAR	β -galactosidase	2 h	dihydro testosterone	-	13 nM	yes	Li et al. 2008a&b
hAR transactivation	bacterial luciferase	3-4 h		2.5 nM	9.7 nM	nd	Eldridge et al. 2007
hAR transactivation	firefly luciferase	2.5 h		0.5 nM	5.5 nM	yes	Leskinen et al. 2005
hAR transactivation	yEGFP	24 h		3 nM	33 nM	yes	Bovee et al. 2007& 2009b
hPR transactivation	β -galactosidase	4 h	progesterone	0.1 nM	0.5 nM	yes	Gaido et al. 1997
hPR transactivation	yEGFP	24 h		-	1 nM	yes	Chatterjee et al. 2008
hAhR transactivation	firefly luciferase	3.5 h		3.3 nM	190 nM	nd	Leskinen et al. 2008
hAhR transactivation	β -galactosidase	18 h	benzo[a]-pyrene	-	40 nM	nd	Miller 1997
hAhR+hER transactivation	β -galactosidase	4-6 days		-	180 nM	nd	Kawanishi et al. 2008
hRXR&RAR transactivation	β -galactosidase	16 h	9- <i>cis</i> -retinoid acid	-	50-150 nM	nd	Allegretto et al. 1993
Yeast two-hybrid hRXR	β -galactosidase	4 h		-	> 100 nM	nd	Nishikawa et al. 2004
Yeast two-hybrid hRXR	β -galactosidase	2 h		-	150 nM	yes	Li et al. 2008b

nd: not determined

ER yeast bioreporter assays have also been used to measure estrogenic activity of environmental samples, such as waste water and environmental water (Balsiger et al. 2010, Rutishauser et al. 2004, Salste et al. 2007, Inoue et al. 2011, Murk et al. 2002, Fine et al. 2006, Pinto et al. 2005). Also sediment extracts, moisturizer lotions, and diverse biological samples have been successfully analyzed (Grund et al. 2011, Leskinen et al. 2005, Bovee, et al. 2009a, Garritano et al. 2006, Becue et al. 2011).

Androgen receptor (AR) regulates genes responsible for male reproduction and development. The natural ligands of AR are testosterone and dihydrotestosterone. Xenobiotic compounds binding to AR act mainly in antagonistic fashion. Such chemicals include, for example, fungicide vinclozolin and DDT. Some estrogenic compounds also work as anti-androgens in yeast assay (Sohoni & Sumpter 1998). Many AR ligands act as antagonists in yeast assays while only few exhibit agonistic effects (Sohoni & Sumpter 1998, Bovee et al. 2007 & 2008, Kolle et al. 2010). Thus, detection of antagonism is especially important when AR-binding compounds are screened.

Examples of yeast AR bioreporter assays are presented in Table 1. The yeast-based AR assays have been used to screen for (anti)androgenic chemicals such as steroid hormones, plant-derived compounds, flame retardants, pesticides, and phenolic compounds (Bovee et al. 2010, Kolle et al. 2010, Sanseverino et al. 2009, Li et al. 2008a). Complex samples such as hair samples, dietary supplements, calf urine and feed, waste water, and pulp and paper mill effluents have also been measured (Becue et al. 2011, Rijk et al. 2009, Bovee et al. 2009b, Michelini et al. 2005, Chatterjee et al. 2007).

Progesterone is another steroid hormone that binds to nuclear receptor and regulates reproduction, and preparation and maintenance of uterus in pregnancy (reviewed by Li X. et al. 2004). Together with estrogen hormone, progesterone regulates cell proliferation and differentiation in reproductive tissues. The few chemicals tested for PR so far have mainly showed anti-progesterone-like, i.e. antagonistic, activity. It is, thus, important to screen antagonistic effect in addition to agonistic effect in PR assays as well. Examples of PR yeast bioreporter assays are presented in Table 1. Because high progesterone concentrations seem to inhibit yeast growth (Gaido et al. 1997), the ligand exposure times have to be kept as short as possible. The PR yeast bioreporter assays have been used to analyze, for example, doping compounds, pesticides, nonylphenol, and endosulfan, and waste waters (Death 2004, Chatterjee et al. 2008, Li et al. 2008b & 2011).

Aryl hydrocarbon receptor (AhR) (reviewed by Safe 2001) is a mammalian nuclear receptor. It is associated with activation of the drug and xenobiotic metabolizing enzymes, for example, cytochrome P450 1A1 mono-oxygenase. AhR signaling pathway can also cross-talk with other NR pathways, for example ER, causing estrogenic and anti-estrogenic effects (Ohtake et al. 2003, Kawanishi et al. 2008). AhR is also known as the dioxin receptor. While the natural ligand of AhR yet remains

unconfirmed, this receptor has been shown to bind and be activated by dioxin-like coplanar aromatic substances such as 2,3,7,8-tetrachloro-dibenzop-dioxin (TCDD) (reviewed by Safe 2001, and Janosek et al. 2006). Other well-known AhR ligands are benzo[a]pyrene, PCBs, and PAHs. In addition, compounds like tryptophan, indole, indole acetic acid, tryptamine, and indole-3-carbinol have been detected to activate the receptor (Miller 1997).

Only few AhR yeast bioreporter assays have been developed so far (Table 1). A yeast bioreporter assay with both ER and AhR has been developed to measure AhR transactivation potency on ER signaling pathway (Kawanishi et al. 2008). The AhR yeast bioreporters have been used to measure water samples, sediment samples, and different chemicals (Kawanishi et al. 2008, Leskinen et al. 2008, Kamata et al. 2009, Allinson et al. 2011).

Natural retinoids, such as vitamin A, act via the nuclear receptors retinoid acid receptor (RAR) and retinoic X receptor RXR (review by Inoue et al. 2010, and Janosek et al. 2006). They control many functions including embryonic development, apoptosis, vision, bone development, and nervous and immune systems. RAR and RXR can form heterodimers with each other, and RXR also with other NRs, for example, thyroid hormone receptor, peroxisome proliferator-activated receptors, vitamin D receptor, estrogen receptor, and liver X receptor (Aranda & Pascual 2001, Nakanishi et al. 2005). This enables numerous different regulatory modes for the receptors, depending on the cellular environment.

Of the natural retinoids, all-trans-retinoic acid (atRA) and 9-*cis*-retinoic acid (9cRA) (Figure 1) bind to RAR, whereas only 9cRA seems to bind to RXR. Perhaps the best-known exogenous compound binding to RXR are organotin compounds such as tributyl tin (TBT) and triphenyl tin (TPT) (Figure 1) (Nishikawa et al. 2004, Nakanishi et al. 2005). The most bioactive forms are tri-substituted forms, while di- and mono-substituted are less active.

The functioning of RARs and RXRs in yeast was demonstrated by Hall et al. (1993) and Heery et al. (1993 & 1994). However, only few bioreporters utilizing RAR or RXR have been developed so far (Table 1). These bioreporters have been used to determine RAR and RXR agonism and antagonism of several chemicals, such as pesticides, parabens, phenols, phthalates, but also waste water (Kamata et al. 2008, Li et al. 2008b, Inoue et al. 2011).

Yeast-based assays have been developed for other nuclear receptors in addition to the ones discussed above. These include, for example thyroid hormone, and glucocorticoid and mineral corticoid receptors (Nishikawa et al. 1999, Miller et al. 2010). Together, the different yeast NR bioreporter assays make an extensive battery for testing endocrine disrupting activity of chemicals and samples.

1.3. High-throughput screening of EDCs

High throughput screening (HTS) refers to a methodology in which large libraries of compounds and environmental samples can be effectively analyzed for their biological activity (Clemons et al. 2009). This requires automation, miniaturization of assay formats, and efficient data analysis (Wölcke & Ullmann 2001, Mayr & Bojanic 2009).

Miniaturization of an assay aims to reduce assay volume and increase screening capacity. However, miniaturization might encounter challenges, such as effects caused by altered reagent stability due to changed surface:volume ratio, evaporation, and adsorption. Good data quality should also be retained. In order to accomplish this, an ideal HTS assay should have both high signal-to-background ratio and low standard deviation (Zhang 1999). It is also important to ensure as low variability in cell number as possible in the low volumes used (Wölcke & Ullmann 2001). This is accomplished by proper suspension of cells and accurate liquid handling.

In future, miniaturized cell-based assays are predicted to become more and more important as primary screening tools (Wölcke & Ullmann 2001). Already now 384-well plate format is routinely used in cell-based assays (Mayr & Bojanic 2009), and an increasing number of 1536-well format assays and even higher are being developed (Clemons et al. 2009).

Several mammalian-cell-based NR assays have already been miniaturized even up to 3456-well plate format (Shi et al. 2005, Peekhaus et al. 2003, Chin et al. 2003, Wilkinson et al. 2008). Almost all yeast-based NR bioreporter assays, however, are still performed in 96-well plate formats, and only very few have been miniaturized into 384- and 1536-well plate formats (Berg et al. 2000). It is certain that in future there is a growing need for miniaturized yeast-based NR assays.

Yeast-based NR bioreporter assays have several properties that make them well suitable for HTS. They are robust, inexpensive, and generally easy and fast to perform. They have potential to be applied to high density well plate formats and automated assays. The current yeast NR assays use many different reporter proteins. An ideal reporter for high-throughput screening purposes should require minimal handling. Additional steps like centrifugation, extra incubations, and addition of substrate or cell-disrupting agents are poorly suited to HTS because they increase the risk of cross-contamination and assay duration. The modern yeast-based NR assays require relatively short incubation times, even as short as 2.5 hours (Leskinen et al. 2005). Yeast cell cultivation is also fast compared to mammalian cell cultivations. By combining the different yeast assays for different nuclear receptors (Table 1) into HTS assay batteries it is possible to screen for several nuclear receptor-mediated effects of chemicals.

1.4. Bioreporters for specific detection of chemicals

NR bioreporters are extremely useful in determining the total hormonal activity of a complex sample. However, no conclusions about the identity of chemicals in the sample can be done. In environmental analytics, the lack of chemical specificity of the bioreporters has been regarded as their main drawback of (Bovee & Pikkemaat 2009).

The identity of specific chemicals in the sample can be determined using chemical analysis methods. Although these methods may be more sensitive than cell-based bioreporters, they are usually more expensive than cellular assays, and they require specialized instruments and users, and heavy sample preparations. Bioreporters are easy to use, they are suitable to HTS of large numbers of samples, and they usually require minimal sample handling. Bioreporters are also considered a good method to assess the bioavailable fraction of contaminants in the environment (Liao et al. 2006). Bioreporters for specific detection of chemicals could have use in cost-efficient monitoring of contaminants.

1.4.1. Modification of nuclear receptor ligand-binding properties

Properties and functions of proteins can be changed towards desired way by using directed evolution (Jäckel et al. 2008). In directed evolution, DNA sequence of a protein is mutated randomly, and mutants with desired functions are selected/screened from the population of different mutants. The process of mutagenesis and selection/screening can also be repeated several times using mutants with most desirable properties. In this text, selection refers to process where the whole population of mutants is tested against a chosen property, whereas screening means testing individual mutants.

NRs have been an easy target for directed evolution because of the easiness to link the changes in ligand-induced activation to screening and selection. For example, estrogen receptor and retinoid X receptor have been mutated to enhance the potency of weak or even completely novel ligands (Islam et al. 2009, Chen & Zhao 2005, Chen et al. 2004, Chockalingam et al. 2005, Schwimmer et al. 2004). In addition, hER has been modified to bind testosterone and even progesterone and corticosterone, which have no detectable affinity to the wild type receptor (Chen & Zhao 2005).

Mutations can be generated randomly on a whole or on a limited sequence or sequences of a gene, or by designing specific mutations on selected residues, or even combining several different approaches. Mutations in NRs have been generated using error-prone PCR (Miller & Whelan 1998, Chen & Zhao 2005), and oligonucleotide-based mutagenesis in which specific (Chen et al. 2004) or random (Schwimmer et al. 2004, Chockalingam et al. 2005) mutations are targeted on chosen amino acids. Combinations of methods have also been popular (Islam et al. 2009, Chockalingam et al. 2005, Chen et al. 2004). Several rounds of mutagenesis is usually needed to

produce NRs with desired ligand-binding properties (Islam et al. 2009, Chockalingam et al. 2005, Chen & Zhao 2005).

Random mutations can be generated by using oligonucleotides, in which the mutagenized areas are synthesized using only certain bases. For example, using a codon sequence NNS, in which N=any base and S=G or C, enables insertion of codons for all 20 possible amino acids. Another approach is to use “doped” oligonucleotides, i.e. in incorporation of certain bases, there are other bases in lower proportion compared to the original base. In this manner, relatively long stretches of sequence can be randomly mutated even at a relatively high mutation frequency compared to error-prone PCR. Another advantage of this method is to create mutations in only those stretches of sequence that are of particular interest. This method has been used in a study where the metal binding properties of a bacterial metal-binding protein MerR were directed towards chosen metals (Hakkila et al. 2011).

The number of possible mutant variants generated by a mutation method defines the theoretical diversity of a mutant library. However, transformation efficiency limits the diversity by limiting the true size of the library. In addition, possibility to go through the whole existing library to find desired mutants depends on the efficiency of the selection/screening methods. Indeed, there is a need for NR mutant library selection/screening methods able to cover libraries with more than 4×10^5 mutants (Chen & Zhao 2005).

The screening methods used for nearly all yeast NR mutant libraries are based on chemical complementation, i.e. growth-based reporter proteins (Islam et al. 2009, Chen & Zhao 2005, Chen et al. 2004, Chockalingam et al. 2005, Schwimmer et al. 2004). Although relatively efficient, these methods have not been designed to actively implement screening of reduced affinity towards an undesired ligand. Only one method has so far introduced this kind of negative screening (Shaffer et al. 2012).

Flow cytometry is an efficient method to screen for certain type of cells even in a very large population (Daugherty et al. 2000). For example, the fluorescence-activated cell sorter can handle as many as 10^5 clones per second (Aharoni et al. 2005). Flow cytometry is based on counting and separating cells according to differential size and biomarker expression. Even thousands of cells per second can be analyzed by forcing cells into a narrow fluid stream. This high throughput screening tool has already been used for bacterial metal-binding receptor mutant library (Hakkila et al. 2011).

In order to develop chemical-specific NRs for the use of environmental analytics, there is a need for a methodology combining a simple but powerful mutation method with an efficient mutant library screening method which is able to combine positive and negative selection of target ligands.

2. AIMS OF THE STUDY

The aims of this thesis study were as follows:

1. To develop a chemical-specific yeast-cell-based bioreporter for monitoring of bisphenol A in complex environmental samples.
2. To generate a method to select a mutant receptor library for both positive and negative ligand-binding affinities, and to test the efficiency of the used mutation and selection methods using human estrogen receptor α .
3. To adapt the existing yeast-based bioreporter assays for high throughput screening (HTS) by optimizing their use in 384 and 1536 well plates.
4. To develop a new yeast-based bioreporter for organotin compounds by utilizing human retinoid X receptor.

3. SUMMARY OF MATERIALS AND METHODS

3.1. Yeast strains

The yeast strains and constructs used in this study are mainly based on the previous work by Pii Leskinen (Leskinen et al. 2003, 2005 & 2008). All *Saccharomyces cerevisiae* yeast bioreporters described in this thesis have been constructed in the parent strain BMA64-1A (Baudin-Baillieu et al. 1997).

The yeast strains used in this study are presented in Table 2. For all bioreporter assays, the yeasts were cultivated in selective synthetic minimal medium (see detailed description in publications indicated in Table 2).

Table 2. Yeast strains used in this study.

Yeast strain	Expressed receptor	Reporter protein(s)	References
BMAERE/BPA-R	BPA-R	firefly luciferase	II
BMAEREyEGFPHis+	(mutant ER library)	yEGFP-His3 fusion	I
BMAEREIuc/RXR-ER	hRXR+hER α DBD	firefly luciferase	IV
BMAEREIuc/ER α	hER α	firefly luciferase	Leskinen et al., 2005
BMAEREIuc/ER β	hER β	firefly luciferase	Leskinen et al., 2005
BMAAREIuc/AR	hAR	firefly luciferase	Leskinen et al., 2005
BMAXREIuc/AhR	hAhR	firefly luciferase	Leskinen et al., 2008
BMA64/luc	none	firefly luciferase	Leskinen et al., 2005

The bisphenol A-specific receptor (BPA-R) yeast bioreporter (**II**) was constructed using the BMAEREIuc reporter yeast strain (Leskinen et al. 2005) in which firefly luciferase reporter protein is under the control of estrogen receptor-inducible promoter. In the BPA-R strain, the wild type estrogen receptor of the parental BMAEREIuc/ER α was replaced by P4E C8 mutant (**I**) of human estrogen receptor α .

The yeast strain BMAEREyEGFPHis+ used for mutant library selection (**I**) was constructed using the plasmid YIpEREIuc (Leskinen et al. 2003) as a backbone. The firefly luciferase reporter gene was replaced with a fusion of two reporter genes: the yeast-enhanced green fluorescent protein (yEGFP), and imidazole glycerol phosphate dehydratase (His3) genes, yielding the plasmid YIpEREyEGFPHis+ (Figure 3). The resulting reporter construct produced a fusion protein of yEGFP-His3 in estrogen receptor-inducible manner. The linker connecting the yEGFP and His3 reporter proteins consisted of a flexible triplicate glycine-serine amino acid sequence (Figure 3).

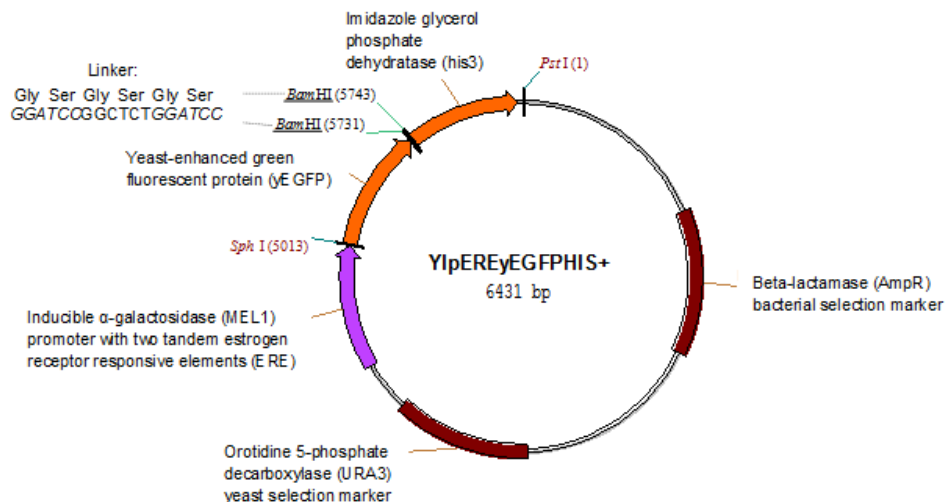


Figure 3. Plasmid map of YIpEREyEGFPHis+.

The BMAEREIuc yeast strain (Leskinen et al. 2005) was used to construct the hRXR-ER organotin yeast bioreporter strain (IV). The DNA binding domain (DBD) of human retinoid X receptor (hRXR) (amino acids 133-208) was replaced with the DBD of human estrogen receptor α (hER α) (amino acids 176-282). The hybrid receptor was designed according to the sequences of hRXR α (mRNA accession number NM_002957) and hER α (mRNA accession number NM_000125). The resulting chimeric receptor gene was optimized for yeast expression and synthesized by GenScript Corporation (USA). The hybrid gene was cut from carrier plasmid pUC57 with *Bam*HI/*Sal*I digestion and ligated to the corresponding sites on plasmid pESC-TRP (Stratagene, USA) yielding plasmid pESC-RXR-ER (Figure 4.). The hybrid receptor was expressed constitutively under strong galactose-inducible promoter of the pESC-plasmid.

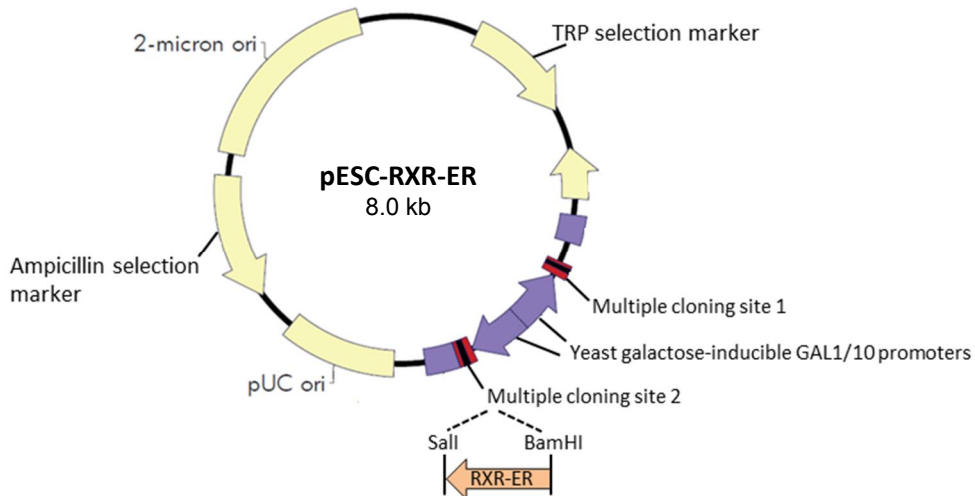


Figure 4. Plasmid map of pESC-RXR-ER. TRP = N-(5'-phosphoribosyl)anthranilate isomerase, ori = origin of replication.

3.2. Yeast bioreporter assay protocols

The miniaturized yeast bioreporter assay protocols used in this study are shown in Table 3. The 384-well format assay protocol was used in publications **I**, **II**, **III**, and **IV**. The 1536-well format assay protocol was used in publication **III**. The original 96-well format assay protocols have been published previously (Leskinen et al. 2005 & 2008).

Conditions were optimized for the miniaturized assays using the hER α bioreporter by measuring the 17 β -estradiol standard curve with varying D-luciferin substrate concentrations (0.1 mM-2 mM) and incubation times (1-4.5 h).

Liquid handling of all well plates was done using Biomek NX^P Laboratory Automation Workstation (Beckman Coulter, USA) fitted with disposable pipette tips. All well plates were imaged using Victor3 1420 Multilabel Counter (PerkinElmer/Wallac, Finland).

The BPA-R and RXR-ER yeast bioreporter assay characterizations were performed using the 384-well plate assay protocol (Table 3), except for the RXR-ER bioreporter sediment analyses which were performed using the 96-well plate assay protocol.

Table 3. Yeast bioreporter assay protocols.

<p>1. Inoculate an overnight culture at SD medium. Culture at 30°C in an incubating shaker.</p> <p>2. Dilute the culture to get to optical density 0.4. at 600 nM (OD₆₀₀). Culture at 30°C in a incubating shaker until the OD₆₀₀ reaches 0.6-0.7.</p>		
<p>1536-well plate assay protocol (III)</p>	<p>384-well plate assay protocol (III)</p>	<p>96-well plate assay protocol (Leskinen et al. 2005 & 2008)</p>
<p>3. Pipette 4 µL sample onto well plate.</p> <p>4. Mix D-luciferin substrate stock solution (10 mM) with the yeast culture into final concentration of 0.5 mM.</p> <p>5. Add 4 µL yeast+substrate mixture onto well plate.</p> <p>6. Shake briefly, cover with a lid and incubate at 30°C for 4.5 h.</p>	<p>3. Pipette 5 µL sample in well plate.</p> <p>4. Mix D-luciferin substrate stock solution (10 mM) with the yeast culture into final concentration of 0.5 mM.</p> <p>5. Add 45 µL yeast+substrate mixture onto well plate.</p> <p>6. Shake briefly, cover with a lid and incubate at 30°C for 3 h.</p>	<p>3. Pipette 10 µL sample in well plate.</p> <p>4. Add 90 µL yeast culture.</p> <p>5. Shake briefly, cover with a lid and incubate at 30°C for 2.5-3.5 h.</p> <p>6. Add 100 µL of 1 mM D-luciferin substrate and shake briefly.</p>
<p>7. Measure luminescence signal with a plate reader set in luminescence mode for 1 s well⁻¹.</p>		

3.3. Mutagenesis of hER α and selection of the mutant receptor library (I)

The human estrogen receptor α was mutated in three regions of the ligand binding domain (LBD). The regions were 13, 11, and 12 amino acids in length located on helices 3, 8, and 11 of the LBD, respectively.

Mutations were generated using randomized oligonucleotides. Three “doped” PCR oligos, one for each region to be mutated, were designed so that in synthesis reaction of the randomized regions 79% of the original bases and 7% each of the other three bases were used. All primers were synthesized by Sigma–Aldrich. The mutated LBDs were constructed from four separately generated PRC-products using OE-PCR (An et al. 2005).

Mutant receptor library was generated in the BMAEREyEGFP_{HIS+} selection yeast strain by co-transforming the mutated LBDs together with the linearized expression plasmid pG1/ER(G) (Liu & Picard 1998) using the high-efficiency lithium acetate method (Gietz & Woods 2002). The expression plasmid regenerated in the yeast cells

by homologous recombination between the matching regions at the ends of the plasmid backbone and the mutated LBD. Ten parallel transformations were performed.

A genetic selection system comprising of positive selection against BPA affinity and negative selection against 17 β -estradiol (E2) was used. The positive selection was performed on histidine-free agar plates containing 5 mg l⁻¹ BPA. Cells were grown at 30°C for 2 days, after which they were harvested and pooled. Next, cells were selected for negative potency towards E2. Library cells were grown in liquid culture in the presence of 10 nM E2, after which they were subjected to flow cytometric sorting. The sorting was performed at the Turku Centre for Biotechnology Cell Imaging Core (Turku, Finland) using BD FACSVantage flow cytometer. Cells with low fluorescent signal were collected, after which they were plated again on histidine-free agar plates containing 5 mg l⁻¹ BPA.

Cells growing on the agar plates were harvested, and mutant receptor plasmids were recovered and transformed into the luminescent reporter yeast strain BMAERELuc. Individual transformants were picked and screened for luminescence induction upon exposure on BPA and E2 on 384-well plates using the protocol described in Table 3. Plasmids from the transformants with desired properties were recovered and the mutant receptor genes sequenced.

The ligand-binding properties of the desired mutant receptors were further tested with other estrogenic chemicals using the 384-well plate protocol described in Table 3.

3.4. Waste water sample treatment (II)

Waste water effluent and influent samples of 1 l volume were collected from the Viikinmäki waste water treatment plant in Helsinki, Finland on December 7th and 13th 2011. The samples were divided into 30 ml aliquots, and three samples were spiked with either 0.2 mg l⁻¹ (0.88 μ M) BPA, 0.8 nM E2, or both.

The samples were frozen in -80 °C after which they were freeze dried for about 40 h into near dryness using Edwards Super Modulyo freeze drier (Severn Science, Bristol, UK). The freeze dried samples were re-suspended into 3 ml of Milli-Q water in order to obtain a total of 10-fold concentration.

In addition, three influent samples (volume 3 ml) were spiked after freeze drying using the same theoretical concentrations (2 mg l⁻¹ BPA and 8 nM E2) as the concentrated samples spiked before freeze drying were expected to have.

4. SUMMARY OF RESULTS AND DISCUSSION

4.1. Modification of hER α ligand specificity (I)

Human estrogen receptor α (hER α) ligand binding properties were modified in this study. The aim was to create a novel receptor with increased potency towards bisphenol A, which is a weak ligand of hER α , and at the same time to decrease the potency of this receptor towards natural ligand, 17 β -estradiol (E2).

Our approach in creating mutations in the hER α ligand binding domain (LBD) was to target a reasonably high frequency of random mutations on selected regions. The purpose of high frequency of mutations was to test whether in this manner only one round of mutagenesis would be sufficient to generate desirable mutants. In other NR mutation studies, several rounds of mutations and screening have been performed (Chockalingam et al. 2005, Islam et al. 2009, Schwimmer et al. 2004).

The chosen mutation areas were located on three different α -helices that have been shown to line the ligand binding cavity in the LBD, and in which some amino acid residues are in contact with the native ligand (Brzozowski et al. 1997). A total of 108 bases on the hER α LBD were subjected to a 21% chance of mutation each, yielding an expected average of 22-23 base mutations per receptor. In theory such a high number of mutations require a fairly large mutant receptor library in order to include as many mutant variants as possible. However, creating such a comprehensive library is difficult. This was also the case in our study, mainly due to rather low transformation efficiency (9×10^5 colony forming units / μ g vector DNA), resulting in a library with approximately 6.8×10^6 mutant hER α variants.

Exploring a large library requires an efficient method to select for mutants with desired properties. In this study, the library was selected for two kinds of affinities: positive potency for BPA, and decreased potency for E2. The selection and screening protocol is illustrated in Figure 5. Library size after each step is shown.

The positive and negative selections were performed using the genetic selection yeast strain BMAEREyEGFP HIS^+ which had a fusion reporter protein comprising of yeast enhanced green fluorescent protein (yEGFP) and histidine auxotrophy-complementing His3-enzyme. The synthesis of the reporter protein was induced by a ligand-bound receptor, allowing cell growth in histidine-free medium and producing fluorescence.

In the first selection step, positive growth-based selection was used to enrich the library with BPA-inducible mutants. However, it was anticipated that this step would also enrich the proportion of constitutive mutants. It has been shown that a truncated hER lacking the LBD functions in a constitutive manner in yeast cells (Metzger et al. 1995, White et al. 1988). Thus, a stop-codon formation in a mutant receptor was expected to yield a constitutive phenotype. Flow cytometric sorting of cells with low

fluorescence was used to decrease the proportion of both E2-inducible and constitutive mutants in the library.

After the flow cytometric selection step, the GFP-expressing genetic selection yeast strain was replaced with the luminescence reporter yeast strain BMAERE_{luc} (Leskinen et al. 2005). Individual mutants were picked from plasmid transformation agar plates and screened in high throughput 384-well plates. From a total of 470 individual tested mutants, 9 mutants with desired properties were identified.

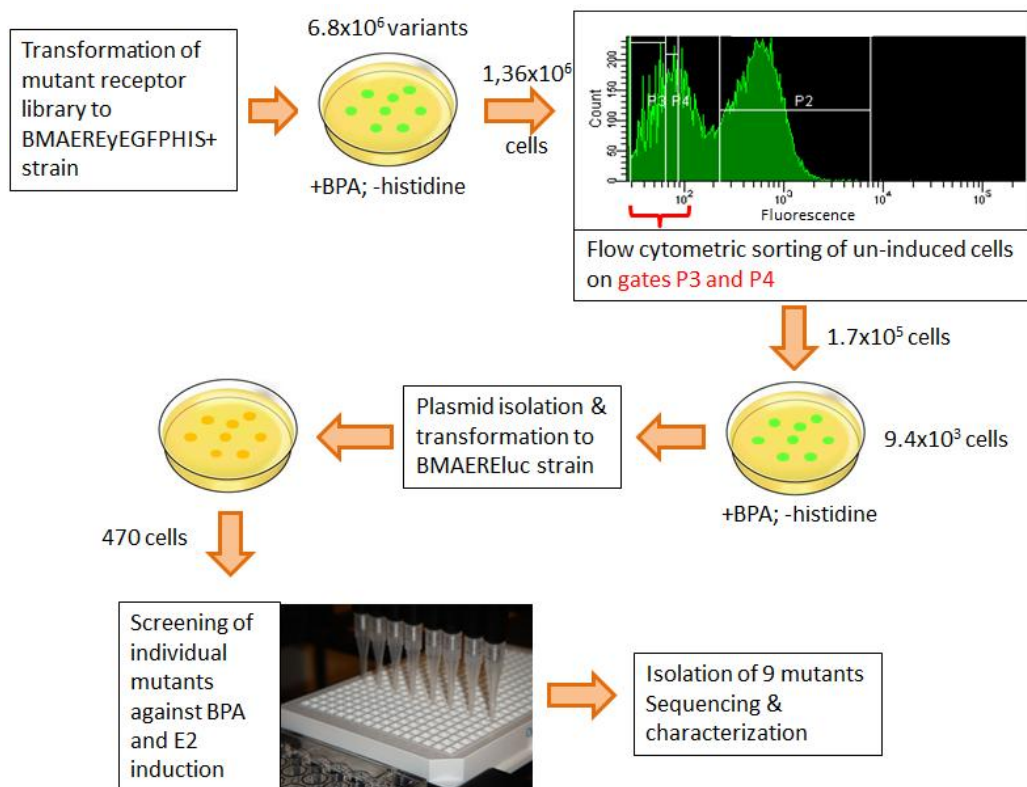


Figure 5. Screening protocol for the hER α mutant library.

Although the flow cytometric selection was used to discard the mutants with high fluorescent signal, nearly 84% of mutants screened in the final selection step were identified as constitutive phenotype. However, without the negative selection step, finding the desired mutants would probably have required screening a far greater number of mutants. Of the total of 1.36×10^6 cells analyzed in the flow cytometer, about 13% were sorted from gates P3 and P4 into test tube and plated. If assumed that the discarded 87% contained only undesirable mutants, and that they would have grown on BPA agar plates at similar efficacy as the sorted cells, then $100/13 = 7.7$ times more individual cells (i.e. about 3,600) should have been screened in order to recover the 9

mutants with desired properties. Now only 470 individual mutants needed to be screened.

The 9 mutants that exhibited the desired properties (induced with BPA but not with E2) had 7-15 base mutations contributing to 4-9 mutations on amino acid level. No mutations were detected on region 1, and two of the mutants had only 1-2 mutations on region 2, whereas the rest of the mutations were concentrated on mutation region 3. Three randomly chosen, non-selected mutants were sequenced to confirm that the mutation frequency was similar on all regions. These mutants contained mutations on regions 1 and 2 as well region 3 with base mutation frequencies ranging from 11-31% on all three regions.

Amino acids in mutation region 3 (helix 11) form contacts with the D-ring of E2 (Brzozowski et al. 1997). Although D-ring of E2 molecule has a hydroxyl group just like BPA, the D-ring is a non-aromatic 5-carbon ring whereas the rings in BPA molecule are aromatic phenol rings (Figure 1). The other two regions, in turn, form contacts with the A-ring, which is a phenol ring. Since the structural differences between the molecules are mainly located on the D-ring-end of E2, it is logical that mutations enhancing BPA binding and reducing E2 binding are located on LBD region contacting this end of E2.

Of the identified mutants, 8 out of 9 showed higher potency towards BPA compared to the wild type hER α , the most sensitive mutant P3D E6 being about 7-fold more sensitive in respect of LOD (60 nM for mutant P3D E6 and 420 nM for hER α). Only mutant P4B E2 responded to E2 in the highest tested concentrations of 90 nM. When tested with other estrogenic compounds (estrone, estriol, diethyl stilbestrol, nonylphenol, hydroquinone, and β -sitosterol), the mutants showed no or very poor sensitivity compared to the wild type hER α . Mutant P4E C8 seemed to be the most specific of the mutants since it had lowest responses to these other estrogenic compounds. Thus, the developed mutation and screening method successfully produced mutants with increased potency towards BPA and decreased potency towards E2.

The most sensitive and specific mutants P3D E6 and P4E C8 could be improved for bioreporter purposes by increasing specificity and sensitivity in order to lower responses to other chemicals and to allow detection of even lower concentrations of BPA. This could be done by further mutagenesis of the receptor, and possibly also by using lower concentration of BPA on the selection agar plates. Screening of individual mutants could in turn be facilitated by running the sorted cells again in the flow cytometer after some growing, thus further removing constitutive mutants.

4.2. Bisphenol A-specific yeast bioreporter (II)

Using the mutated human estrogen receptor P4E C8 (created in publication I) the first yeast-cell-based bioreporter assay targeted for the detection of a single chemical, bisphenol A (BPA), was developed. The bisphenol A-specific receptor (BPA-R) yeast bioreporter assay was characterized and tested using mixtures of BPA and the native hormone 17 β -estradiol (E2). In addition, a chemical cocktail containing some of the highest reported concentrations of BPA, E2, estrone, estriol, ethinyl estradiol, nonylphenol, and propyl paraben in waste water was also analyzed. BPA-R bioreporter was also tested for real-life analytics with waste water samples spiked with BPA, E2 and both chemicals.

The BPA-R bioreporter showed high specificity towards BPA in the tested concentrations (ranging from 525 μ M to 9 nM of BPA, and from 1 μ M to 1 pM E2, Figure 6.). Compared to the wild type hER α bioreporter, the BPA-R bioreporter was over 4-fold more sensitive towards BPA. E2 induced BPA-R bioreporter only in the highest tested concentration of E2 over its detection limit. This E2 concentration of 1 μ M (i.e. 190 μ g l⁻¹) is 1.7 \times 10⁵-times higher compared to the detection limit of hER α bioreporter (6 pM, i.e. 1.6 μ g l⁻¹). Although E2 was no longer a strong inducer of BPA-R, it did have a clear inhibitory effect in concentrations starting at 62.5 nM (17 μ g l⁻¹) when E2 was tested with a constant BPA concentration of 3 μ M. However, both inducing and inhibiting E2 concentrations are clearly higher than the highest measured E2 concentrations in influent waste water (150 ng l⁻¹, Vethaak et al. 2005, L. Wang et al. 2012). Thus, even concentrated samples can be measured without risk of E2 interfering the BPA-R bioreporter response.

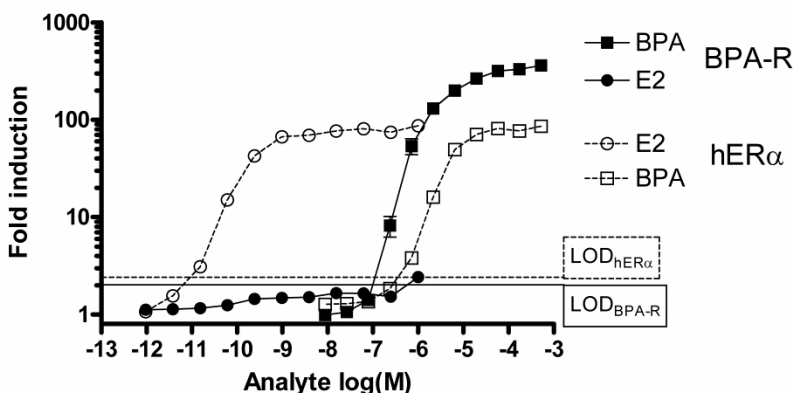


Figure 6. Dose-response curves of BPA-R and hER α bioreporters with bisphenol A (BPA) and 17 β -estradiol (E2). Concentrations are given as the total analyte concentrations in the mixture with yeast cells (1 part sample +9 parts yeast culture). Values represent the mean \pm standard error of the mean of five independent experiments, each comprising four parallel data points. LOD: limit of detection.

To mimic a situation where a highly contaminated water sample is concentrated 5-fold, a chemical cocktail with estrogenic chemicals was prepared using a 5-fold concentrations of some of the highest reported concentrations of E2 ($5 \times 200 \text{ ng l}^{-1}$), BPA ($85 \text{ } \mu\text{g l}^{-1}$), and other estrogenic chemicals: ethinyl estradiol ($5 \times 830 \text{ ng l}^{-1}$), estrone ($5 \times 360 \text{ ng l}^{-1}$), estriol ($5 \times 180 \text{ ng l}^{-1}$), nonylphenol ($5 \times 40 \text{ } \mu\text{g l}^{-1}$), and propyl paraben ($5 \times 2.8 \text{ } \mu\text{g l}^{-1}$). BPA-R bioreporter exhibited similar induction with BPA-containing cocktail and sample with only BPA, whereas it did not respond to cocktail without BPA. The hER α bioreporter showed similar high induction with both cocktail samples, but BPA alone was below detection limit. Thus, BPA-R bioreporter succeeded in detecting BPA in a chemical cocktail with high concentrations of other potential environmental estrogenic chemicals.

The LOD of BPA-R bioreporter for BPA was still rather high, 107 nM (i.e. $24 \text{ } \mu\text{g l}^{-1}$). In many environmental samples such as landfill leachates, sewage sludge, compost water, and some surface waters close to local discharge spots, BPA concentration from micro- to milligrams per liter have been reported (Fromme et al. 2002, Wang G. et al. 2012, Yamamoto et al. 2001, Svenson et al. 2009, Kamata et al. 2011, Yamada et al. 1999, Belfroid et al. 2002, Kolpin et al. 2002). However, in most environmental and waste water samples only low BPA concentrations ranging from nanograms up to few micrograms per liter have been measured (Bono-Blay et al. 2012, Vethaak et al. 2005, Fernandez et al. 2009, Deblonde et al. 2011, Melcer & Klečka 2011, Wang G: et al. 2012, Arditsoglou & Voutsas 2008, Terasaki et al. 2007). Thus, it is probable that when measuring water samples, some sample concentration is needed to reach the LOD of the BPA-R bioreporter.

To show the applicability of the BPA-R bioreporter, influent and effluent waste water samples were spiked with BPA and E2, and measured with the BPA-R and hER α bioreporters. The BPA-R bioreporter detected only BPA in spiked waste water samples, and the response on the sample spiked with both BPA and E2 was similar within variations to the sample spiked with BPA only. The BPA measurements of the hER α bioreporter, in turn, detected an excess of BPA due to background estrogenicity in the waste water.

In this study, the detected concentrations of BPA and E2 were 30-50% of the expected spiked concentrations. Possible reasons for these rather low detection efficiencies were assessed. Bioreporter assays have been reported to suffer from matrix effect, i.e. reduced recovery, when measuring waste water samples (Salste et al. 2007, Balsiger et al. 2010, Mispagel et al. 2009). Chemical analysis methods have also been reported to exhibit some matrix effect, although the effect is lower (Wille et al. 2012).

Another factor that could affect detection efficiencies is sample preparation. Freeze drying was used in this study to concentrate waste water samples. Freeze drying has been considered a good option for water sample concentration because of minimal sample activity loss (Salste et al. 2007). In this study, however, freeze drying reduced

the detected E2 concentrations of hER α bioreporter by 37%, and BPA detection of BPA-R by 31%. It has been suggested that hydrophobic compounds, such as steroid hormones, could get adsorbed to the walls of plastic containers during freeze drying (Balsiger et al. 2010). Anyhow, freeze drying was considered a reasonably good method for concentrating waste water samples.

As a general conclusion, factors influencing the detection efficiency such as sample concentration or bioavailability, should be taken into account. The effects should be tested, preferably separately for each compound, by, for example, using control samples spiked with a known concentration of each compound of interest.

BPA-R bioreporter had several other advantages compared to hER α bioreporter, such as lower background, higher maximal luminescence level, and lower variation. The BPA-R yeast, however, was growing somewhat slower compared to the wild type hER α bioreporter. This made parallel analysis using both bioreporters and the toxicity control stain challenging because of difficulties in synchronizing their growth. The slow growth of BPA-R bioreporter could be due to inefficient mutated codons in the mutant receptor P4E C8 (I). Optimization of these codons for more efficient expression in yeast could improve growth rate by facilitating the production of the receptor.

4.3. High-throughput yeast bioreporter assays (III)

The yeast bioreporter assays utilizing receptors hER α , hER β , hAR, and hAhR were miniaturized into high throughput 384 and 1536-well plate formats. Performance of the miniaturized bioreporter assays versus the original 96-well plate assays was evaluated by comparing the detection limits (LODs) obtained with the reference compound of each bioreporter. In addition to LODs, the assay quality was evaluated in each format by using Z-factor. Z-factor is a measure of the quality and suitability of an assay to high throughput screening (HTS) (Zhang 1999). It shows how well a signal is separated from background by taking into account the background and maximal signal levels (i.e. the dynamic range of an assay) and the data variability (i.e. the standard deviation). According to Zhang et al. (1999), an assay with a Z-factor of 0.5-1 (1 is maximum) is excellent for HTS.

All bioreporter assays had a Z-factor of 0.5 or higher, except for the hER β in 1536-well plate format in which the Z-factor was below 0. The reason for the low z-factor of hER β bioreporter in 1536-well plate assay was the low maximal signal level and high variation. Overall, the variation was somewhat higher in the miniaturized assay formats, probably due to variation of cell number in the low volumes. It seems that assays that have high signal-to-background ratio in 96-well plates are best suitable for miniaturization to low volume 384 and 1536 well plate formats since they have highest probability to retain a good Z-factor.

Miniaturization had varying effect on the LODs of the bioreporters. In many cases the lowest LODs were in the miniaturized formats whereas in others the 96-well format was the most sensitive. In another study with yeast-based NR bioreporter, the tested chemicals showed always highest potencies in 96-well plate format, especially at low compound concentrations (Berg et al. 2000). In our study (III), however, most of the bioreporters appeared to benefit somewhat of miniaturization in respect to LODs.

The 384 and 1536-well formats required some adjustments to the yeast bioreporter assays. Because the luciferase substrate D-luciferin is light and oxygen-sensitive, the substrate was previously dispensed on the plates just before measurement by the plate reader. However, standard plate readers are usually restricted to dispensing only well plates of up to 96 wells. For this reason the D-luciferin substrate was dispensed to the 384 and 1536-well plates as a mixture with the yeast culture already before the incubation step. Since all previously published cell-based assays using luminescent reporter have added the substrate after incubation (Leskinen et al. 2005, Almeida et al. 2008, Maffia 1999), the survival of the luminescence signal during incubation was assessed using the constitutively luminescent BMA64/luc control strain in the presence of different D-luciferin concentrations. Of the tested concentrations, 0.5 mM D-luciferin was considered most optimal since the signal did not decline during the 4.5 hour measurement, and the variation of the signal was low.

Incubation time was optimized for the miniaturized assays using the hER α bioreporter. The most suitable tested incubation time was only 30 min longer for the 384-well format assays compared to the 96-well format assays, whereas the 1536-well format assay was found to benefit from longer incubation of up to 4.5 hours. Probably in this format, cell number is critical to obtain a high enough signal.

In conclusion, miniaturization of the yeast bioreporter assays was successful according to the magic triangle of HTS (Mayr & Bojanic 2009): (i) need of reagents and consumables became lower, (ii) the assay time was shortened due to automated liquid handling and exclusion of substrate addition step, and (iii) good data quality was retained. The yeast assays in our research are nowadays routinely performed in 384-well formats.

The variability of the miniaturized assays could be lowered by further optimizing the automated pipetting techniques. Since the signal intensity of luminescent yeasts is not as high as, for example, those of luminescent bacteria, the number of yeast cells should be kept as high as possible in miniaturized formats. This could be achieved by longer pre-growth, concentration yeast suspension, or using readily suspended freeze-dried yeast. Freeze-dried yeasts could also simplify the assay and shorten the time needed for cultivations.

4.4. Tributyl tin yeast bioreporter (IV)

In this study, a yeast bioreporter for measuring organotin compounds was developed and characterized. The bioreporter is the first retinoid X receptor-based yeast bioreporter characterized for environmental analysis of organotin compounds. None of the other published RXR yeast bioreporters has been used for detection of organotin compounds in the environment. A bacterial bioreporter has been developed for this purpose (Durand et al. 2003), however, it does not utilize NRs, and the mechanism of induction is unknown. In addition, TBT, which is together with TPT the most biologically active organotin compound was less potent than dibutyl tin (DBT), while TPT showed no potency at all. It is possible that different forms of organotin compounds have different impact on bacteria than on eukaryotic organisms.

In this study, DNA-binding domain (DBD) of human retinoid X receptor α (RXR) was replaced with the DBD of hER α to enable the use of the reporter strain BMAEReluc which has firefly luciferase reporter gene under control of estrogen receptor-responsive elements (ERE). Heery et al. (1993) have constructed a similar chimeric receptor with hER DBD but using mouse RXR γ . However, this receptor was not applied for environmental studies.

The responses of the RXR-ER bioreporter on different organotin and retinoic acid compounds after 4 hours incubation are shown in Figure 7. Tributyl tin (TBT) and triphenyl tin (TPT) were found to be the most potent of the tested chemicals, with LODs of 88 nM and 170 nM, respectively. The LOD of the RXR natural ligand 9-*cis*-retinoic acid (9cRA) was even slightly higher, 260 nM. TBT has also previously been reported to be more potent than 9cRA in RXR yeast bioreporter (Nishikawa et al. 2004).

Nishikawa et al. (2004) developed a RXR-based bioreporter that used β -galactosidase as a reporter. Compared to our RXR-ER bioreporter, this previous one was more sensitive towards TBT. However, due to the need for cell lysis for β -galactosidase assay, this bioreporter is less suitable for HTS formats.

All-trans retinoic acid (atRA) activated the receptor in this study, although other studies (Heery et al. 1993 and 1994, Hall et al. 1993, Li et al. 2008b) have not detected any activity of this compound on the RXR receptor. Concentrations of atRA tested in this study, however, were higher compared to other studies (Heery et al. 1994, Li et al. 2008b).

The dose-response curve of RXR-ER bioreporter towards TBT reached plateau at about 1 μ M (Figure 7). At 10 μ M the dose-response curve started falling even though the inductions were corrected for toxicity using the constitutive control strain BMA64/luc.

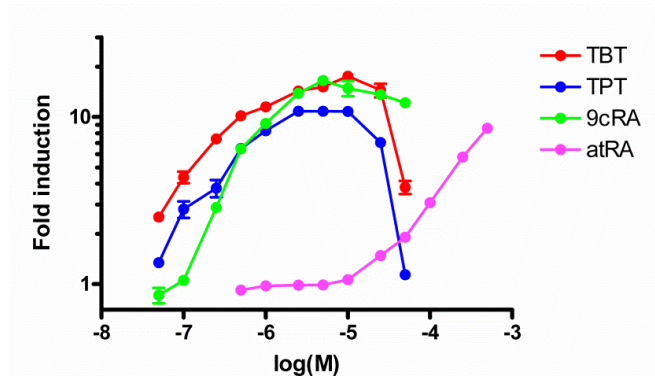


Figure 7. RXR-ER bioreporter dose-response curves of organotin and retinoic acid compounds. Values represent the mean \pm standard deviation of three independent experiments, each comprising four parallel data points.

The control strain showed some concentration-dependent toxicity, expressed as correction factor, with TBT and TPT (Figure 8). Surprisingly, the control strain showed very little toxicity in the highest tested concentration of 50 μ M (Figure 8). This suggests that yeast might have some form of resistance mechanism towards organotins that is induced at high concentrations. Such would be, for example, an efflux pump, which would lower the concentration of organotins in the cell and thus lower the response of the bioreporter. An efflux-pump-based resistance mechanism has been shown to lower the sensitivity of heavy metal bacterial bioreporters (Hynninen et al. 2010). Removing some efflux pumps from the RXR-ER bioreporter could thus improve its sensitivity.

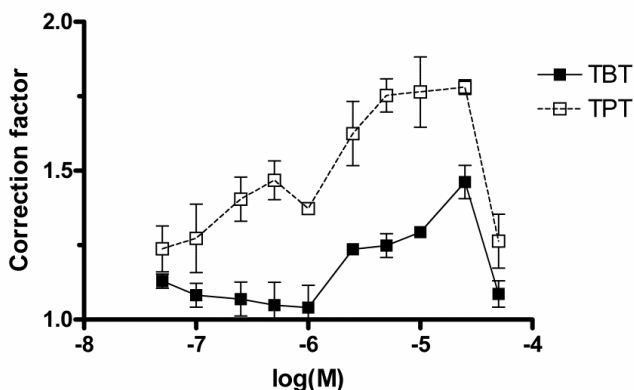


Figure 8. Correction factors obtained by the control yeast strain BMA64/luc with tributyl tin (TBT) and triphenyl tin (TPT). Correction factor = signal of blank sample / signal of chemical sample. Values represent the mean \pm standard deviation of three independent experiments, each comprising four parallel data points.

TBT has been suspected to be toxic also in bacterial bioreporters. (Durand et al. 2003). The bacterial bioreporter seems to be more sensitive towards TBT than yeast since the dose-response curve started falling already at concentrations higher than 1 μM .

The RXR-ER bioreporter was applied in 384-well plate format. In addition, sediments spiked with TBT were measured in 96-well plate format. Since the detection limit in sediment measurements was at μg TBT per g of sediment, direct measurement of sediments require relatively high rate of organotin contamination. The success of organotin measurement in natural sediment samples depends also on factors such as pH and the organic matter content of the sediment. For example, organic matter might reduce bioavailability of organotins (Rüdel 2003).

5. CONCLUSIONS AND FUTURE PROSPECTS

Yeast-based nuclear receptor bioreporters are useful tools for cost-efficient chemical testing and environmental monitoring of endocrine disrupting compounds. They are easy to handle and they require no special laboratory conditions, instruments or experienced users.

In this thesis study the battery of existing yeast nuclear receptor bioassays using firefly luciferase reporter was expanded with two new bioreporters: the bisphenol A-targeted BPA-R and organotin RXR-ER bioreporter strains.

The BPA-R bioreporter enables specific analysis of BPA in environmental and other complex samples. It is well suitable for high throughput screening of large number of samples. BPA exposure and possible sources are a continuous topic of studies even today. The BPA-R bioreporter offers a new tool for assessing exposure of humans and wildlife as well as monitoring possible sources and environmental levels. Possible application could include, for example measuring BPA in food, and environmental or waste water samples, or monitoring BPA leakage from water pipes lined with epoxy resin. BPA-R bioreporter has been planned to be used in direct analysis of BPA in thermal paper. Another interesting application could be to monitor environmental BPA sources, such as landfill effluents.

On the course creating the BPA-R bioreporter, a mutation and selection method for hER α was applied. Using this method, the ligand-binding properties of hER α was modified by only one round of mutation, and the library was efficiently selected against both positive and negative ligand activities. Possible further applications of this method could be improving the sensitivities of existing bioreporters BPA-R and RXR-ER, which still have somewhat high detection limits. In addition, receptors with targeted affinities to other interesting chemicals could be accomplished, creating more tools for bioreporter-based specific detection of chemicals.

Although popular and promising, the yeast NR bioreporter assays have suffered from lack of development towards truly high throughput screening formats. In this thesis study the applicability of yeast NR bioassays for robotic liquid handling and high density 384 and 1536-well plates were evaluated. Nearly all assays were readily applied to both formats, reducing the need of material, sample, and reagents as well as assay time. At present, all our yeast assays are routinely performed in 384-well plate format, enabling analysis of high number of samples. Furthermore, all assays can be performed during only one working day.

Future improvement in the yeast bioreporter assays could be minimizing need for cultivations by using freeze dried cells. The existing yeast bioreporter assays could also be evaluated by subjecting them to international validation in measuring large numbers of some complex samples, or screening a whole compound library.

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Johanna Rajasärkkä

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