Video Article

Detecting Estrogenic Ligands in Personal Care Products using a Yeast Estrogen Screen Optimized for the Undergraduate Teaching Laboratory

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Abstract

The Yeast Estrogen Screen (YES) is used to detect estrogenic ligands in environmental samples and has been broadly applied in studies of endocrine disruption. Estrogenic ligands include both natural and manmade "Environmental Estrogens" (EEs) found in many consumer goods including Personal Care Products (PCPs), plastics, pesticides, and foods. EEs disrupt hormone signaling in humans and other animals, potentially reducing fertility and increasing disease risk. Despite the importance of EEs and other Endocrine Disrupting Chemicals (EDCs) to public health, endocrine disruption is not typically included in undergraduate curricula. This shortcoming is partly due to a lack of relevant laboratory activities that illustrate the principles involved while also being accessible to undergraduate students. This article presents an optimized YES for quantifying ligands in personal care products that bind estrogen receptors alpha (ERα) and/or beta (ERβ). The method incorporates one of the two colorimetric substrates (*ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) or chlorophenol red-β-D-galactopyranoside (CPRG)) that are cleaved by β-galactosidase, a 6-day refrigerated incubation step to facilitate use in undergraduate laboratory courses, an automated application for LacZ calculations, and R code for the associated 4-parameter logistic regression analysis. The protocol has been designed to allow undergraduate students to develop and conduct experiments in which they screen products of their choosing for estrogen mimics. In the process, they learn about endocrine disruption, cell culture, receptor binding, enzyme activity, genetic engineering, statistics, and experimental design. Simultaneously, they also practice fundamental and broadly applicable laboratory skills, such as: calculating concentrations; making solutions; demonstrating sterile technique; serially diluting standards; constructing and interpolating standard curves; identifying variables and controls; collecting, organizing, and analyzing data; constructing and interpreting graphs; and using common laboratory equipment such as micropipettors and spectrophotometers. Thus, implementing this assay encourages students to engage in inquiry-based learning while exploring emerging issues in environmental science and health.

Video Link

The video component of this article can be found at https://www.jove.com/video/55754/

Introduction

The Yeast Estrogen Screen (YES) is widely used to quantify ligands that mimic estradiol (E2 or 17β-estradiol) in a variety of matrices, including water, plant tissues, consumer products, and foods[1,2,3,4]. Collectively, such ligands are termed "Environmental Estrogens (EEs)." The YES was originally developed as a low cost, *in vitro* alternative to *in vivo* tests like the rodent uterotrophic assay[5,6] and the rainbow trout feeding assay[7]. The aim of these tests is to determine if a product contains chemicals that stimulate or block estrogen-dependent mechanisms. Detection of EEs is critical, because they can interfere with normal endogenous estrogen signaling, particularly during fetal development. This interference compromises health by increasing risk of obesity, infertility, cancer, and cognitive loss[8].

Despite the importance of EEs and other EDCs to public health, endocrine disruption is not commonly included in undergraduate curricula. This deficiency is partly due to a dearth of activities that illustrate the principles involved while also being accessible to undergraduate students. Additionally, several variations of the YES protocol exist[9,10,11,12,13], and this diversity makes assay optimization time-consuming for laboratory coordinators not specifically trained in the relevant techniques. Finally, YES assays are usually completed over 1 long day or 2 consecutive days with an O/N incubation. This timing is not compatible with the format of undergraduate laboratory courses, which typically meet once/week for several h.

In response to these needs, this manuscript reports an optimized 96-well YES protocol that includes ethanolic extraction methods for Personal Care Products (PCPs)[3] and a 6-day refrigeration step to accommodate weekly laboratory meetings. Absolute ethanol is a versatile organic solvent that can dissolve a variety of polar and nonpolar solutes. Moreover, it is suitable for undergraduate courses because it is readily available, relatively nontoxic, affordable, and miscible with water; it also evaporates easily without special equipment. However, ethanol is not ideal for extracting strongly hydrophobic endocrine disruptors or many oils and waxes, the latter two being common ingredients in PCPs. Poor extraction efficiency increases the risk of false negative findings. With this constraint in mind, investigators should choose extraction procedures (*e.g.*, ethanolic extraction or solid phase extraction) that address sample characteristics and meet study objectives (research versus undergraduate instruction).

The YES relies on recombinant *Saccharomyces cerevisiae* originally created by Dr. Charles Miller at Tulane University. Please see Miller *et al.* (2010) for a complete map of the engineered plasmid[14]. Yeast transformed with these plasmids constitutively express human nuclear ERα or ERβ (also called ESR1 and ESR2, respectively) when grown in media containing galactose (for ERα) or either glucose or galactose (for ERβ). If estrogenic chemicals are present in the media, they bind to the receptors, creating ligand-receptor complexes that activate β-galactosidase (lacZ) expression at a level proportional to the concentration of estrogenic chemicals. Yeast cells are then lysed to release the accumulated β-galactosidase. The lysis buffer contains either ONPG or CPRG, which are cleaved by β-galactosidase to yield yellow or red products, respectively. Colorimetric products can be quantified by measuring absorbance using a microwell plate spectrophotometer. The degree of color change is proportional to the concentration of estrogenic ligands to which the yeast was exposed.

The choice of substrate (CPRG or ONPG) depends on the potential for background absorbance arising from the samples being tested. For example, plant extracts will often add a yellow hue to the media that artificially inflates estrogenicity measures if ONPG (quantified at 405 nm) is used as the substrate for β-galactosidase. With plant extracts, CPRG (quantified at 574 nm) may be a more appropriate colorimetric substrate. CPRG is more expensive than ONPG but is used at one tenth the molarity. This article presents estrogenicities of PCP extracts quantified using both ONPG and CPRG.

Quantifying estrogenicity of environmental samples using both ERα and ERβ is a more comprehensive approach than using only one of these receptors. In animals, these receptors exhibit differential tissue distribution, regulatory activities, and binding affinities for estrogenic and anti-estrogenic ligands[15]. For example, plant-based phytoestrogens typically bind ERβ more strongly[2], whereas synthetic chemicals can show preference for either ERα or ERβ or can bind both receptors equally well[15]. Therefore, binding to one estrogen receptor does not necessarily predict binding to the other.

Although EEs are found in many consumer products (*e.g.*, pesticides, detergents, adhesives, lubricants, plastics, foods, and pharmaceuticals) as well as plants, the presented data were obtained using a selection of PCPs. PCPs are compelling, readily available, budget-friendly, and environmentally relevant for undergraduate students. Students can be invited to bring their favorite PCPs from home to test in the laboratory. They can also search the Skin Deep database developed by the Environmental Working Group[16] to generate hypothesis-driven comparisons of PCPs with high and low toxicity scores. In this way, students can simultaneously develop advanced laboratory skills; engage in self-directed, inquiry-based learning; and explore emerging issues in environmental science and health.

Protocol

1. Making Reagents

1. Prepare glucose and galactose media by mixing 6.7 g yeast nitrogen base, 20 g dextrose or galactose, 20 mg adenine sulfate (added as 10 mL of a 200 mg/100 mL aqueous stock solution), 20 mg uracil (added as 10 mL of a 200 mg/100 mL aqueous stock solution), 60 mg leucine (added as 6 mL of a 1 g/100 mL aqueous stock solution), and 20 mg histidine (added as 2 mL of a 1 g/100 mL aqueous stock solution) in deionized water to a final volume of 1 L. Sterilize by filtration (0.2 µm filter). Media can be stored at 4 °C for several weeks.
2. Prepare estradiol (E2) standards by dissolving powdered E2 in anhydrous ethanol and diluting to the appropriate working concentrations (227.5 nM & 9.75 nM) in 50% ethanol.
   Note: If estradiol is purchased as a vial of 1 mg, add 1 mL ethanol directly to the vial to dissolve estradiol powder. This yields a 3.67 mM stock #1. Dilute 10 µL of stock #1 in 990 µL ethanol to make 1 mL of 36.71 µM stock #2. Then, dilute 10 µL of stock #2 in 990 µL 50% ethanol to make 1 mL of 367.13 nM stock #3. To make working stocks, dilute 619.7 µL stock #3 in 380.3 µL 50% ethanol to make 1 mL of 227.5 nM working stock used to create standard curves with yeast expressing ERα. Dilute 26.56 µL stock #3 in 973.44 µL 50% ethanol to make 1 mL of 9.75 nM working stock used to create standard curves with yeast expressing ERβ. Seal standards with parafilm and store at -20 or -70 °C.
   CAUTION: E2 is a suspected carcinogen and reproductive toxicant. It is harmful if inhaled, swallowed, or absorbed through the skin. E2 may cause harm to breast-feeding children and fetuses. E2 is very toxic to aquatic life. Use appropriate personal protection (gloves, fume hood, dust mask) and avoid exposure during pregnancy and lactation. Dispose E2 as hazardous waste and do not release into the environment.
3. Prepare LacZ buffer by mixing 8.52 g Na_2HPO_4, 5.52 g $NaH_2PO_4•H_2O$, 95.21 mg $MgCl_2$, 745.5 mg KCl, 2 g N-lauroylsarcosine sodium salt, and either ONPG (400 mg) or CPRG (77.7 mg) in deionized water to a final volume of 1 L. Store LacZ buffer in 20 mL aliquots at -20 °C.
   NOTE: A 20 mL aliquot is sufficient for one 96-well plate.
4. Prepare sodium carbonate by mixing 105.9 g sodium carbonate in deionized water to a final volume of 1 L. Store at RT.
5. Prepare 1 M dithiothreitol (DTT) in water. Freeze 25 µL aliquots of DTT at -20 °C.
   CAUTION: DTT is an acute skin and eye irritant. Use appropriate personal protection equipment (gloves, fume hood, dust mask) to avoid skin and eye contact, inhalation and ingestion.
   NOTE: Each aliquot is sufficient for one 96-well plate.

2. Preparing Samples and Extraction Controls

1. Select samples to test.

NOTE: This article presents data for fifteen PCPs, including soap, hair cream, shampoo, sunscreen, lip balm, foundation, shaving cream, nail polish, and lotion.

2. Combine 1 g of sample with 10 mL of anhydrous ethanol in a 15 mL conical tube. Prepare a negative extraction control consisting of 11 mL of ethanol in a 15 mL conical tube. Prepare replicates as needed.
   NOTE: For the presented experimental design, three aliquots of all 15 PCPs and triplicate extraction control solutions were prepared, yielding a total of 48 samples, each of which was analyzed in triplicate using the protocol in section 4. A single plate can accommodate up to 23 samples in triplicates. Anhydrous ethanol is used in this step because it evaporates readily. Use negative extraction controls to verify that estrogens are not leaching into samples from the conical tubes.
3. Homogenize tube contents for 30 min by a combination of manual shaking and vortexing or by shaking tubes on a multi-tube vortexer.
4. Centrifuge conical tubes at the maximal allowable speed in a bucket centrifuge for 10 min. Decant the supernatant from each tube through a 40 µm cell strainer into a 50 mL conical tube. Empty contents of each 50 mL tube into a glass scintillation vial.
5. Leave vials open in a ventilated hood for one week to allow ethanol to evaporate completely. Alternatively, dry samples in a ventilated hood under nitrogen or with a rotary evaporator. To avoid degradation of light sensitive constituents, protect drying samples from light (*e.g.*, place opaque fabric over ventilated hood door).
6. Reconstitute samples in 1.0 mL of 50% ethanol and vortex to homogenize.

3. Culturing and Subculturing Yeast[14]

1. Maintain active yeast cultures (recombinant W303a strain of *S. cerevisiae*[14]) by incubating yeast in filter-sterilized glucose media at 30 °C. Subculture yeast weekly in fresh filter-sterilized glucose media.
2. Two nights (about 42 h) prior to preparing the YES plates, subculture yeast by adding 0.1 mL of active yeast cultures to 10 mL of filter-sterilized glucose media using sterile technique. Incubate at 30 °C for two nights. Shaking is not required if yeast are grown as shallow cultures in sterile 250 mL Erlenmeyer flasks.
   NOTE: Yeast can also be stored on refrigerated agar plates for several months. For longer term storage (years), combine O/N cultures with 15 - 50% sterile glycerol, vortex, and freeze at -70 °C. To prepare sterile glycerol, use a syringe to transfer 300 µL glycerol into a cryovial and autoclave with the cap loosened. Then add 1,200 µL O/N yeast culture using sterile technique.

4. Preparing YES Plates (Day 1 of the Assay)

1. In a sterile beaker and using sterile technique, dilute yeast from step 3.2 to an optical density of 0.065 ±0.005 at 610 nm (OD_{610}) in filter-sterilized galactose media.
   1. Confirm optical density by pipetting 120 µL of each yeast sample in triplicate along with triplicate galactose blanks into a clear polystyrene plate (plate does not need to be sterile). Use a plate spectrophotometer to verify that the diluted yeast culture has a net OD_{610} of 0.065 ±0.005 Subtract OD610 readings of galactose blanks from yeast OD_{610} readings to determine net OD_{610} of the yeast. Prepare a final volume of at least 30 mL diluted yeast for each 96-well plate.
      NOTE: Spectrophotometer filters measuring absorbance between 595 and 610 nm can be used for this measurement. An approximate 1:6 v:v ratio of yeast:media typically yields an OD_{610} close to 0.065, so start by adding 4.5 mL yeast to 30 mL media and add more yeast or media as appropriate to achieve a netOD_{610} between 0.060 and 0.070.

2. Using sterile technique, add this diluted yeast suspension to the wells of a sterile, polypropylene, 96-well microplate according to the plate layout (**Figure 1**). During pipetting, keep the source yeast suspended by continuously swirling the container or mixing with the pipette. For this step, it is helpful to use a repeating pipettor with a sterile syringe tip.
   Note: Polypropylene plates are sterilized by autoclaving two stacked plates wrapped in foil. The top plate serves as a lid for the sample plate and can be washed, re-autoclaved, and reused.
3. Add 120 µL filter-sterilized galactose media to 3 additional wells (H10 - 12) according to the plate layout (**Figure 1**). These wells serve as media controls to account for absorbance contributed by the media alone.
4. Construct a standard curve in each plate by serially diluting triplicates of E2 working standards (227.5 nM for ERα, 9.75 nM for ERβ) in wells containing the yeast suspension (A1 - G3) according to the plate layout (**Figure 1**). Begin by by adding 5 µL of E2 standards to wells A1 through A3. Mix microwell contents by pipetting, and then serially dilute this suspension by moving 205 µL from wells A1 - 3 into wells B1 - 3. Repeat the mixing and transfer of 205 µL through row G.
   Note: After the serial dilution is completed, discard 205 µL from wells G1 - 3. At the end of this step, all standard wells should contain 120 µL. The serial dilution will yield the final E2 concentrations listed in **Table 1**. For the serial dilution steps, it is helpful to use a multichannel pipettor with sterile tips.
5. Add 5 µL of vehicle (50% ethanol) to vehicle control wells containing the yeast suspension (H1 - 3) according to the plate layout (**Figure 1**).
   NOTE: Vehicle control wells are used to quantify background absorbance associated with yeast cells and media. Additionally, cytotoxicity or growth promoting effects of test samples can be detected by comparing the OD_{610} values of test wells with those of vehicle control wells.
6. Add 5 µL triplicates of samples and negative extraction controls to wells containing the yeast suspension according to the plate layout (**Figure 1**).
   Note: Make a note of which samples or negative extraction controls are added to each well. To keep track of which wells have samples and which do not, start with a fresh box of tips and use tips from the same locations in the box as the locations of the corresponding wells in the plate. One 96-well plate can accommodate up to 23 samples in triplicate.
7. Mix the contents of the sample, extraction control, and vehicle control wells by pipetting. Adjust volumes of these wells to 120 µL by removing 205 µL from each as described in the legend for **Figure 1**.
8. Seal the plate(s) with a sterile, adhesive, porous film. Label plates and incubate for 17 h at 30 °C. The plate does not need to be shaken during incubation

5. Processing YES Plates (Day 2 of the Assay)

1. After the 17 h incubation at 30 °C, remove plates from the incubator. If plates will be processed immediately, proceed to step 5.1.1. If plates will be processed at a later date, proceed to step 5.1.2.
    1. Use a multichannel pipettor to mix the contents of each row of wells, and then transfer 50 µL of yeast suspension from each well to the corresponding well of a clear, polystyrene, 96-well microplate.
    Note: Polystyrene plates do not need to be sterile but should have a lid. Use new pipet tips for each row of wells to avoid cross-contaminating wells, although if pipetting across triplicates with an 8-tip multichannel pipette, tips only need to be changed between triplicate sets.
        1. Label the new plate. Proceed to step 5.2.

    2. To store plates before processing, wrap them in plastic wrap. Refrigerate wrapped plates at 4 °C for 6 d. Afterward, remove the plates from the refrigerator, unwrap them, and transfer the contents of all wells by pipetting as in step 5.1.1. Proceed to step 5.2.

2. Add 20 µL of 1 M DTT to 20 mL of thawed, room-temperature LacZ buffer for a final concentration of 1 mM DTT. Mix LacZ buffer well. If using a multi-channel pipette, dispense into a reagent reservoir.
CAUTION: Wear gloves and work with DTT in a hood, if possible
3. Using either a multichannel pipette or repeating pipettor, add 200 µL of LacZ buffer containing DTT and either ONPG or CPRG to all wells of the polystyrene plate, and immediately measure and record the OD_{610} of all wells using a plate spectrophotometer. Repeat as needed for additional plates.
Note: The OD_{610} readings are used in the denominator of the LacZ calculation (step 6.1). For this reason, obtain the readings immediately after pipetting and before cells settle or are lysed by LacZ buffer. If OD_{610} values for sample wells are noticeably higher or lower than OD_{610} values for vehicle control wells, the sample extracts are either growth-promoting or cytotoxic to yeast, respectively. The LacZ calculation will correct for small differences in cell densities across wells, but if differences exceed 30% in either direction, then dilute the reconstituted sample (from step 2.6) in additional 50% ethanol and retest the sample by returning to step 3.2[12].
4. Cover plates with a lid. Incubate plates containing yeast that express ERα[14] at 30 °C for 40 min (for ONPG) or 3 h (for CPRG). Incubate plates containing yeast that express ERβ[14] at 30 °C for 70 min (for ONPG) or 4 h (for CPRG).
Note: When working with CPRG, incubation times are flexible; incubating for 2 - 4 h yields suitable results with both receptors, with longer incubations increasing assay sensitivity.
5. After incubating plates, use a multichannel pipette or repeating pipettor to add 100 µL of sodium carbonate to each well. Sodium carbonate raises the pH and halts the β-galactosidase reaction.
6. Measure and record the OD_{405} (for ONPG) or OD_{574} (for CPRG) of all wells using a plate spectrophotometer.

6. Calculating LacZ Values

NOTE: LacZ values quantify the degree of color change for each sample and offer a normalized method of comparing values among separate assays by accounting for several variables (*e.g.*, yeast optical density, media optical density, and incubation time if this differs among wells on the same plate)[12].

1. Calculate means of triplicate OD_{610} values for media (galactose) control wells (H10-12), and calculate means of triplicate OD_{405} or OD_{574} values for vehicle (50% ethanol) controls (H1-3). Use these means and the incubation time (t) in hours for the plate to change color (step 5.5) to calculate LacZ values (corresponding to the degree of color change in each well) for all standard and sample wells using the following equations:

$$\text{LacZ value (for wells containing ONPG)} = \frac{\left(OD_{405} - \text{MeanOD}_{405^{vehicle\ control}}\right) \bullet 1{,}000}{\left(OD_{610} - \text{MeanOD}_{610^{media\ control}}\right) \bullet 50\ \mu l \bullet t}$$

$$\text{LacZ value (for wells containing CPRG)} = \frac{\left(OD_{574} - \text{MeanOD}_{574^{vehicle\ control}}\right) \bullet 1{,}000}{\left(OD_{610} - \text{MeanOD}_{610^{media\ control}}\right) \bullet 50\ \mu l \bullet t}$$

    1. Alternatively, use the automated application in **Appendix 1** to calculate LacZ values for standards and samples.
    Note: The plate layout used with the application must be identical to the plate layout presented in **Figure 1**. If some sample wells were not used, retain absorbance readings from the empty wells as place holders in the absorbance dataset being pasted into the **Appendix 1** LacZ application. This preserves the spatial layout of the plate in the application and ensures that media control wells are in their required location. Additionally, the application will not execute if there are empty cells.

2. Calculate means of triplicate LacZ values for each E2 standard and each sample. Report mean LacZ values as $\mu L^{-1}h^{-1}$. Log-transform the concentrations of each E2 standard, and generate a spreadsheet document formatted as in the template.csv file (**Appendix 2**).

7. Interpolating Sample Estradiol Equivalents (EEQs) Using 4-Parameter Logistic Regression

NOTE: EEQs relate sample LacZ values (color change) to the LacZ values of the standard curve created with E2. EEQs thus determine how much sample is required to elicit the same color change response as a known concentration of E2.

1. To calculate EEQs for test samples, first plot the standard curve. Fit mean LacZ values for E2 standards (y-axis) and the corresponding log-transformed E2 concentrations (x-axis) to a four-parameter logistic regression model.
2. Use the model to interpolate EEQs for test sample LacZ values. Conduct logistic regression calculations of EEQs using statistical software as outlined in **Appendix 2**.

8. Standardizing EEQs (ng/mL) to PCP Sample Mass

1. To relate EEQs to the amount of PCP sample added to each well in step 4.5, multiply EEQ (ng/mL) by the total volume plated into the sample wells (325 µL) and then divide by the volume of extracted sample added to each well (5 µL).
   NOTE: These values represent EEQ per mL of extracted sample. If samples were prepared using 1 g of PCP, these values also represent EEQ per gram of PCP (ng/g). Expressed in this way, EEQ values (ng/g) can be compared across different PCPs. EEQ (ng/g) values for the personal care products tested in this study are shown in **Table 2**, and sample data collected throughout the entire protocol are included in **Appendix 3**.

Representative Results

The estrogenicities of triplicate samples of 15 PCPs were evaluated using this YES protocol. As noted by Miller *et al.* (2010), assays with yeast expressing ERβ were more than an order of magnitude more sensitive than assays with yeast expressing ERα (**Figure 2**)[14]. Therefore, estrogenic activity was more often detected with ERβ-expressing yeast (**Table 2**). Eight PCPs exhibited estrogenic activity with ERβ and 5 PCPs exhibited estrogenic activity with ERα. Hair cream, sunscreen, lotion, and lip balm samples were estrogenic with both receptors, whereas foundation, shaving cream, and nail polish were only estrogenic with ERβ at the tested concentrations. Seven other PCPs (4 shampoos, 2 soaps, and 1 lip balm) were not estrogenic with either receptor at the tested concentrations.

In addition to receptor sensitivity differences, EEQs for each sample differed depending on the colorimetric substrate (ONPG or CPRG) used in the assay (**Table 2**). In all but one sample, EEQs determined using ONPG were higher than those determined using CPRG. Moreover, variation among extraction replicates was lowest for EEQs measured with ERβ and CPRG and highest for EEQs determined with ERα and ONPG (**Table 2**).

In addition to comparing colorimetric substrates, 1 aim of the present study was to test incubation duration times that are compatible with the schedule of an undergraduate laboratory course. The typical YES assay requires a 17 h incubation followed immediately by incubation in LacZ buffer for 40 min - 4 h, a schedule that is impossible to implement in a teaching laboratory constrained by a single weekly session. To facilitate use of this assay in teaching, the assay was modified by introducing a 6 d refrigeration period (step 5.1.2) after the 17 h incubation. Standard curves from refrigerated plates were comparable to those from non-refrigerated plates (**Figures 2 & 3**), except that the standard errors of parameters estimated using four-parameter logistic regression models were all smaller for refrigerated plates than for nonrefrigerated plates (**Table 3**). Thus, refrigerating plates for 6 d reduces error and improves the accuracy of EEQ estimates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	320 µl yeast + 5 µl E2	320 µl yeast + 5 µl E2	320 µl yeast + 5 µl E2	320 µl yeast + 5 µl A	320 µl yeast + 5 µl A	320 µl yeast + 5 µl A	320 µl yeast + 5 µl I	320 µl yeast + 5 µl I	320 µl yeast + 5 µl I	320 µl yeast + 5 µl Q	320 µl yeast + 5 µl Q	320 µl yeast + 5 µl Q
B	120 µl yeast	120 µl yeast	120 µl yeast	320 µl yeast + 5 µl B	320 µl yeast + 5 µl B	320 µl yeast + 5 µl B	320 µl yeast + 5 µl J	320 µl yeast + 5 µl J	320 µl yeast + 5 µl J	320 µl yeast + 5 µl R	320 µl yeast + 5 µl R	320 µl yeast + 5 µl R
C	120 µl yeast	120 µl yeast	120 µl yeast	320 µl yeast + 5 µl C	320 µl yeast + 5 µl C	320 µl yeast + 5 µl C	320 µl yeast + 5 µl K	320 µl yeast + 5 µl K	320 µl yeast + 5 µl K	320 µl yeast + 5 µl S	320 µl yeast + 5 µl S	320 µl yeast + 5 µl S
D	120 µl yeast	120 µl yeast	120 µl yeast	320 µl yeast + 5 µl D	320 µl yeast + 5 µl D	320 µl yeast + 5 µl D	320 µl yeast + 5 µl L	320 µl yeast + 5 µl L	320 µl yeast + 5 µl L	320 µl yeast + 5 µl T	320 µl yeast + 5 µl T	320 µl yeast + 5 µl T
E	120 µl yeast	120 µl yeast	120 µl yeast	320 µl yeast + 5 µl E	320 µl yeast + 5 µl E	320 µl yeast + 5 µl E	320 µl yeast + 5 µl M	320 µl yeast + 5 µl M	320 µl yeast + 5 µl M	320 µl yeast + 5 µl U	320 µl yeast + 5 µl U	320 µl yeast + 5 µl U
F	120 µl yeast	120 µl yeast	120 µl yeast	320 µl yeast + 5 µl F	320 µl yeast + 5 µl F	320 µl yeast + 5 µl F	320 µl yeast + 5 µl N	320 µl yeast + 5 µl N	320 µl yeast + 5 µl N	320 µl yeast + 5 µl V	320 µl yeast + 5 µl V	320 µl yeast + 5 µl V
G	120 µl yeast	120 µl yeast	120 µl yeast	320 µl yeast + 5 µl G	320 µl yeast + 5 µl G	320 µl yeast + 5 µl G	320 µl yeast + 5 µl O	320 µl yeast + 5 µl O	320 µl yeast + 5 µl O	320 µl yeast + 5 µl W	320 µl yeast + 5 µl W	320 µl yeast + 5 µl W
H	320 µl yeast + 5 µl ETOH	320 µl yeast + 5 µl ETOH	320 µl yeast + 5 µl ETOH	320 µl yeast + 5 µl H	320 µl yeast + 5 µl H	320 µl yeast + 5 µl H	320 µl yeast + 5 µl P	320 µl yeast + 5 µl P	320 µl yeast + 5 µl P	120 µl gal	120 µl gal	120 µl gal

Figure 1. Microwell Plate Layout and Standard Dilution Curve Preparation for the YES Assay. E2 standards (light gray wells), samples and negative extraction controls (white wells), and vehicle (H1 - 3) and galactose media (H10 - 12) controls (dark gray wells) were all tested in triplicate. An E2 standard curve (light gray wells) was constructed by plating 320 µL of yeast into wells A1 through A3 and 120 µL of yeast into wells B1 through G3. Then, 5 µL of E2 (227.5 nM for yeast that express ERα; 9.75 nM for yeast that express ERβ) were added to the yeast in wells A1 through A3. The yeast + E2 suspension was serially diluted by transferring 205 µL from each well to the well below it, yielding the final E2 concentrations listed in **Table 1**. Note that, at the end of the serial dilution process, 205 µL must be discarded from wells G1 through G3 to achieve a final volume of 120 µL in each well. Sample and negative extraction control wells were prepared by adding 5 µL of each extract (dissolved in 50% ethanol) to 320 µL of yeast. Vehicle control wells (H1 - 3) were prepared by adding 5 µL of 50% ethanol to 320 µL of yeast. All sample and control wells were mixed by pipetting, and 205 µL were removed and discarded to adjust well volumes to 120 µL each. Lastly, media controls were prepared by plating 120 µL of galactose media into wells H10 - 12. To use the LacZ calculator in **Appendix 1** and the R-based application described in **Appendix 2**, the E2 standard curve and vehicle and galactose media controls must be plated as shown. Samples and negative extraction controls can be plated in any of the white wells as long as the order of plating is noted. Please click here to view a larger version of this figure.

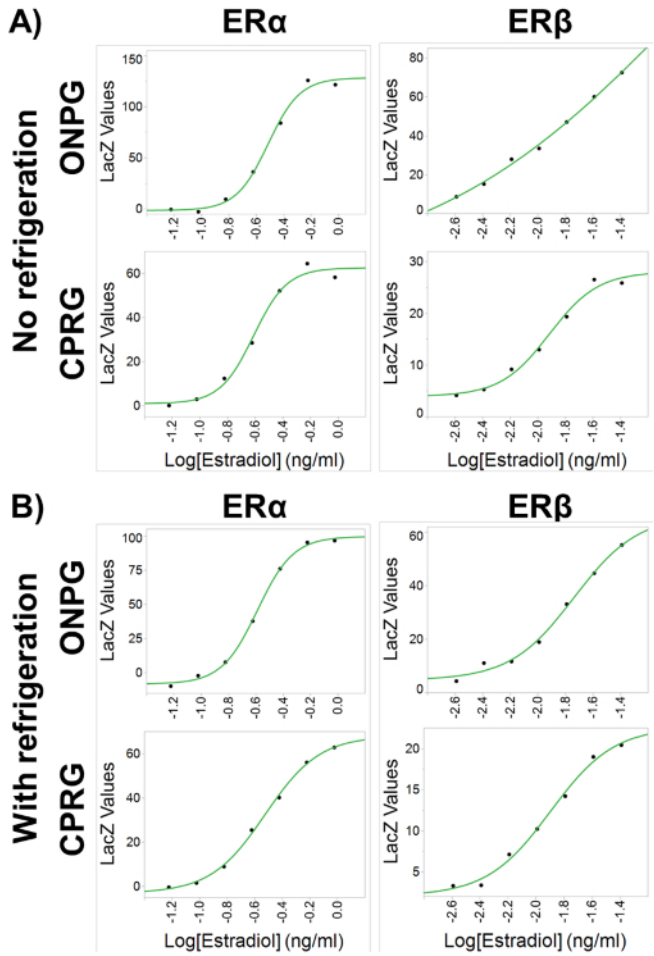

Figure 2. Standard Curves for the YES Plates Generated via 4-parameter Logistic Regression. Two substrates (ONPG & CPRG) were tested using yeast expressing one of two human estrogen receptors (ERα or ERβ) both without (**A**) and with (**B**) a 6 d refrigeration period after the 17 h incubation with 17β-estradiol and sample extracts. All E2 standards and media and vehicle controls were tested in triplicate. Points represent means of triplicate LacZ values, where LacZ values reflect the degree of color change induced by β-galactosidase. Logistic regression model parameters are listed in **Table 3**. ONPG = *ortho*-nitrophenyl-β-D-galactopyranoside; CPRG = chlorophenol red-β-D-galactopyranoside. Please click here to view a larger version of this figure.

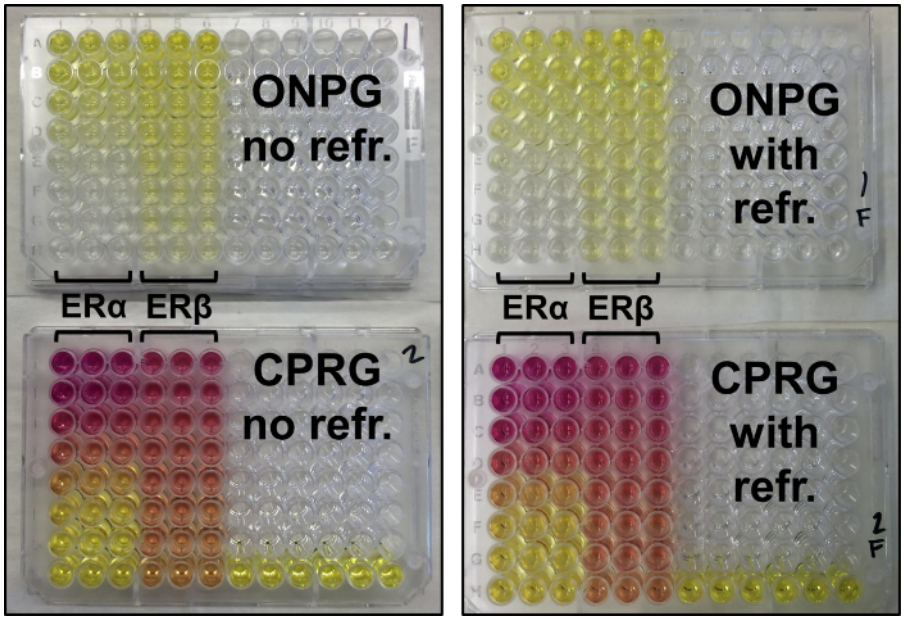

Figure 3. Examples of Developed YES Plates. Two substrates (ONPG & CPRG) were tested using yeast expressing human estrogen receptors (ERα or ERβ), either without (left) or with (right) a six-day refrigeration period after the 17 h incubation with 17β-estradiol or sample extracts. All E2 standards, media and vehicle controls were tested in triplicates. Plates were arranged with the highest E2 concentration in the top row of each plate and controls (50% ethanol + yeast on the left and galactose media on the right) in the bottom row of each plate according to **Figure 1**. ONPG = *ortho*-nitrophenyl-β-D-galactopyranoside; CPRG = chlorophenol red-β-D-galactopyranoside. Please click here to view a larger version of this figure.

Standard Number	[E2] for ERα yeast (pM)	[E2] for ERβ yeast (pM)
1	3500	150
2	2208	94.6
3	1393	59.7
4	878	37.6
5	554	23.7
6	349	15
7	220	9.45

Table 1. Final Concentrations of E2 Standards used in the YES.
Powdered E2 was dissolved in anhydrous ethanol and then diluted to working stock concentrations of 227.5 nM (for yeast that express ERα) and 9.75 nM (for yeast that express ERβ) in 50% ethanol. Working stocks were then added to microplate wells containing yeast in galactose media and serially diluted to the concentrations shown in the table.

Samples Tested		Assay Conditions		Estrogenic Equivalents (EEQs) of PCPs			
PCP #	Sample Type	Receptor	Substrate	EEQ 1 (ng/g)	EEQ 2 (ng/g)	EEQ 3 (ng/g)	Mean EEQ (ng/g)
1	Hair cream	ERα	ONPG	ND	0.379	ND	<0.379
1	Hair cream	ERα	CPRG	ND	ND	ND	ND
1	Hair cream	ERβ	ONPG	0.528	0.338	0.363	0.410
1	Hair cream	ERβ	CPRG	ND	0.215	ND	<0.215
4	Sunscreen	ERα	ONPG	6.803	1.390	ND	<4.097
4	Sunscreen	ERα	CPRG	ND	ND	ND	ND
4	Sunscreen	ERβ	ONPG	1.321	0.838	0.818	0.992
4	Sunscreen	ERβ	CPRG	0.651	0.591	0.725	0.656
7	Foundation	ERα	ONPG	ND	ND	ND	ND
7	Foundation	ERα	CPRG	ND	ND	ND	ND
7	Foundation	ERβ	ONPG	ND	0.158	ND	<0.158
7	Foundation	ERβ	CPRG	ND	ND	ND	ND
9	Shave cream	ERα	ONPG	ND	ND	ND	ND
9	Shave cream	ERα	CPRG	ND	ND	ND	ND
9	Shave cream	ERβ	ONPG	ND	0.256	0.295	<0.276
9	Shave cream	ERβ	CPRG	0.507	0.392	0.560	0.486
10	Nail Polish	ERα	ONPG	ND	ND	ND	ND
10	Nail Polish	ERα	CPRG	ND	ND	ND	ND
10	Nail Polish	ERβ	ONPG	0.916	0.503	0.554	0.658
10	Nail Polish	ERβ	CPRG	0.532	0.628	0.594	0.585
11	Lotion	ERα	ONPG	ND	ND	2.327	<2.327
11	Lotion	ERα	CPRG	ND	ND	ND	ND
11	Lotion	ERβ	ONPG	Exceeds range	2.599	1.845	>2.222
11	Lotion	ERβ	CPRG	--	1.986	1.236	1.611
13	Sunscreen	ERα	ONPG	14.069	11.494	10.189	11.917
13	Sunscreen	ERα	CPRG	4.773	5.790	5.850	5.471
13	Sunscreen	ERβ	ONPG	Exceeds range	2.580	Exceeds Range	>2.580
13	Sunscreen	ERβ	CPRG	0.292	0.240	ND	<0.266
15	Lip balm	ERα	ONPG	ND	ND	0.431	<0.431
15	Lip balm	ERα	CPRG	ND	ND	ND	ND
15	Lip balm	ERβ	ONPG	1.202	1.060	0.887	1.050
15	Lip balm	ERβ	CPRG	0.820	0.871	0.851	0.847

Table 2. EEQs of PCPs with non-zero EEQs. Three aliquots of 15 PCPs and three extraction controls were homogenized in anhydrous ethanol, evaporated, and reconstituted in 50% ethanol for a total of 48 samples, each of which was analyzed in triplicate using the YES assay. Eight PCPs had at least 1 non-zero EEQ, while 7 PCPs were not found to be estrogenic at the tested concentrations. EEQ 1, EEQ 2, and EEQ 3 refer to the three aliquots of each PCP, each tested in triplicate on a separate plate. Values for EEQ 1, EEQ 2, and EEQ 3 are the means of the triplicates for each aliquot. Yeast used in the assay expressed 1 of 2 isoforms of human estrogen receptors (ERα or ERβ). Two colorimetric substrates, ONPG and CPRG, were tested using the non-refrigerated protocol. EEQs were determined using four-parameter logistic regressions (with R^2 values ≥ 0.98) of LacZ values at each of 7 concentrations of E2. ND = below detection limit of the assay. -- = lost replicate.

Assay Conditions			Model Parameters				
Receptor	**Substrate**	**Arrest at 4 °C for 6 d**	**Model R^2**	**Growth Rate (LacZ units/ng/ml)**	**Inflection Point (ng/mL)**	**Lower Asymptote (LacZ units)**	**Upper Asymptote (LacZ units)**
ERα	ONPG	NO	>0.99	8.548 ±1.633	-0.512 ±0.025	-1.623 ±4.408	128.11 ±6.31
ERα	ONPG	YES	>0.99	7.618 ±0.758	-0.587 ±0.014	-8.378 ±2.223	99.53 ±2.528
ERα	CPRG	NO	>0.99	8.256 ±2.182	-0.610 ±0.033	0.853 ±3.333	62.52 ±3.473
ERα	CPRG	YES	>0.99	5.068 ±0.616	-0.523 ±0.022	-3.147 ±1.946	68.06 ±3.069
ERβ	ONPG	NO	>0.99	1.041 ±2.004	-0.898 ±4.410	-32.98 ±86.62	248.6 ±778.9
ERβ	ONPG	YES	>0.99	4.327 ±1.289	-1.736 ±0.087	4.654 ±3.087	66.37 ±10.75
ERβ	CPRG	NO	= 0.99	5.673 ±1.764	-1.914 ±0.052	3.925 ±1.679	28.05 ±2.334
ERβ	CPRG	YES	>0.99	4.412 ±1.142	-1.894 ±0.051	2.052 ±1.303	22.64 ±2.047

Table 3. Model Parameters of 4-parameter Logistic Regressions for the Standard Curves in Figure 2. Two substrates, ONPG and CPRG, were tested using yeast expressing 1 of 2 human estrogen receptors (ERα or ERβ) both without and with a six-day refrigeration period after the 17 h incubation. Parameters are reported as estimates ±standard errors.

Appendix 1. Application for Calculating LacZ Values. To use the application, first download free Java software (as noted in **Table of Materials**). Then open the LacZ application. The plate layout used with the application must be identical to the plate layout presented in **Figure 1**. If some sample wells were not used, retain absorbance readings from the empty wells as place holders in the absorbance dataset being pasted into the LacZ application. This preserves the spatial layout of the plate in the application and ensures that media control wells are in their required location. Additionally, the application will not execute if there are empty cells. Paste in OD_{405} readings (for ONPG) or OD_{574} readings (for CPRG) of all wells using keyboard command “control (ctrl) + v” or “command + v,” depending on the computer platform being used. Enter the amount of incubation time in **hours** for the assay to produce color (from step 5.4; for example, 0.66667 h for 40 min). Click next. Paste in OD_{610} readings from step 5.3. Click submit. LacZ results will be displayed and can be copied by pressing “control (ctrl) + c” or “command + c,” depending on the computer platform being used. Please click here to download this file.

Appendix 2. Statistical Software Options for Calculating EEQs. EEQs can be calculated using one of three presented options. Directions for using 1. JMP software or 2. R code are provided in Appendix 2. The R code has also been converted to 3. an application format available at https://furmanbiology.shinyapps.io/YESapp/. Please click here to download this file.

Appendix 3. Sample Data Collected using Yeast that Express ERα and CPRG as the Substrate. Measurements of OD_{610} and OD_{574} for each of seven E2 standards, vehicle and media controls, and a single representative PCP sample are included, along with calculated LacZ values. LacZ values of standards were used to construct a four-parameter logistic regression model of the standard curve as described in steps 7A & B above. The model was used to interpolate triplicate estimates of EEQs of the representative sample in ng/mL in the microplate wells. These values were then converted to EEQs expressed as ng/g of sample (step 8.1). Please click here to download this file.

Discussion

The YES is a low cost method used to detect estrogenic ligands in environmental samples, such as water, food, plant tissues, or personal care products. Data presented here compare 2 estrogen receptors (ERα and ERβ), 2 substrates (ONPG and CPRG), and 2 timelines (2 d protocol without refrigeration and seven-day protocol with refrigeration) for measuring estrogenicity in personal care products via the YES assay. The 7 d, refrigerated protocol using CPRG and yeast expressing ERα and/or ERβ best quantifies EEQs while also being compatible with the time constraints imposed by undergraduate laboratory courses that meet only once/week for several h. In fact, compared to the 2 d assay without refrigeration, the seven-day refrigerated assay was associated with reduced variance across plate replicates. In addition, the linear part of the standard curve was slightly expanded for data collected using the refrigerated assay. The linear portion of the standard curve defines the assay detection range and is the only portion of the standard curve that can be used to interpolate sample EEQs.

In all but one of the tested samples, EEQs measured using CPRG were lower than those quantified with ONPG. With a higher extinction coefficient and lower K_m and V_{max}, CPRG is ten times more sensitive than ONPG[17]. Thus, CPRG can be used at lower concentrations and can be used to detect lower amounts of β-galactosidase. For these reasons, CPRG is generally preferred over ONPG[18,19]. However, greater substrate sensitivity does not explain the lower EEQ values detected with CPRG. The higher EEQ values detected with ONPG could be due to matrix interference with the assay, as noted by other authors[2,18]. Yellow pigments from personal care product extracts could inflate EEQ values detected with ONPG. Others have circumvented this problem by including pigment controls in their experimental design[2], an approach that doubles the number of plates in an experiment. When matrix interference may be problematic, CPRG may be a preferable substrate for the YES assay, although it is more expensive and requires longer incubation times than ONPG. Furthermore, the color change induced by the cleavage of substrate by β-galactosidase is more dramatic with CPRG, making it easier for students to visualize results.

Miller *et al.* (2010), who engineered the yeast used in this protocol, noted that yeast expressing ERβ were 30x more sensitive to 17β-estradiol than yeast expressing ERα, a finding substantiated by our data[14]. Apart from potential nuances in plasmid construction, Miller *et al.* (2010) could not explain this difference in sensitivity[14]. One difference between the two plasmid constructs is that ERα expression is regulated by galactose, whereas ERβ expression is regulated by either glucose or galactose. The yeast used in the YES assay are cultured in glucose media and only given galactose when they are diluted at the start of the assay. Therefore, yeast expressing ERβ might accumulate higher copy numbers of receptor proteins prior to the start of the assay, thereby conferring higher sensitivity to estrogenic ligands.

The lower sensitivity of ERα-expressing yeast may explain the higher rates of non-detection of estrogenicity in samples measured with ERα compared with ERβ. To increase the likelihood that sample EEQs will be detected, users could employ different extraction solvents and methods or add higher volumes of sample to the yeast. If higher sample volumes are used, the concentrations of E2 standards should be adjusted such that the same volume of standards and samples can be used in the assay. One limitation of adding more sample is that yeast can tolerate only 6 - 10% ethanol, with better tolerance at incubation temperatures of 30 *Vs.* 35 °C[20]. To control for effects of ethanol on yeast, investigators should add triplicate "yeast only" wells to the plate layout and confirm that the OD_{610} of these "yeast only" wells is comparable to the OD_{610} of vehicle control wells immediately after the addition of LacZ buffer. Alternatively, samples dissolved in ethanol can be added to dry microwell plates and the ethanol evaporated off before yeast are added. Dimethylsulfoxide (DMSO) is also used as a sample solvent in YES assays, but the final working concentration of DMSO with yeast should be limited to 1%[12].

The YES assay is a powerful screening tool for detecting estrogenicity in environmental samples. Specifically, the YES detects ligands that bind nuclear estrogen receptors that interact with estrogen response elements to direct gene expression[14]. However, the YES also has important limitations. The YES does not detect EEs that work through non-nuclear mechanisms such as membrane estrogen receptors or mechanisms that involve additional elements such as Activator Protein 1 (AP-1) transcription factors. Moreover, because yeast do not have the same metabolic capacity as mammalian cells, the YES cannot detect ligands that require metabolic activation to be estrogenic.

In addition, the YES assay cannot readily differentiate between estrogenic and anti-estrogenic ligands in complex samples. Instead, it measures *net* estrogenicity, which is the sum effect of estrogenic and anti-estrogenic ligands. To quantify the concentration of ER antagonists or evaluate the inhibitory activity of mixtures, the assay can be modified by incubating yeast with both the standard agonist (17β-estradiol) and a range of test sample concentrations[12]. This process determines if antagonists in the samples can diminish agonist-induced reporter activity and provides a useful screen for identifying the presence of ER antagonists in samples.

The protocol presented here can accommodate a variety of sample types, although some samples may require modifications to the extraction and sample preparation steps. For example, EEQs varied widely among replicates of some personal care products. The more variable samples tended to contain oil droplets or were otherwise not entirely homogeneous, indicating that a more lipophilic solvent such as diethyl ether would be helpful. Alternatively, oils and wax in personal care products could be excluded by extracting samples with 50% ethanol instead of 100% ethanol. A 50% ethanol extraction will also capture more water soluble estrogenic ligands (*e.g.*, some pigments). However, 50% ethanol evaporates more slowly than 100% ethanol and thus may extend extraction time. Additionally, some samples (such as soaps) were cytotoxic to yeast, resulting in reduced cell density measurements (OD_{610}). Fox *et al.* (2008) suggest that such samples should be diluted and retested if cell density differences exceed 30% compared to vehicle control wells[12].

If the YES assay is used for analytical research purposes, dilution curves of sample pools can be tested to preemptively determine appropriate volumes of extract to be added to yeast in step 4.5. Alternatively, extracts can be simultaneously tested at multiple volumes (*e.g.*, 5 µL and 20 µL extract added to yeast in step 4.5) or dilutions that span orders of magnitude (*e.g.*, 0.2 µL, 2 µL, and 20 µL). "Appropriate" volumes of extract are those that identify estrogenicity by matching LacZ values along the linear part of the standard curve. Optimization prevents problems caused by adding too much or too little sample to the yeast, such as cytotoxicity, false negatives, or estrogenicity that exceeds the standard curve. As mentioned above, the volumes of samples and E2 standards should be adjusted when different amounts of ligand are used such that yeast are exposed to a consistent volume and concentration of ethanol or other vehicle across the plate.

Despite the potential for false negatives, the YES assay has been identified as a Tier 3 testing tool for endocrine disruptors by Schug *et al.* (2013), who developed a comprehensive Tiered Protocol for Endocrine Disruption (TiPED)[21]. For undergraduate education, the assay is valuable for teaching concepts related to endocrine disruption, cell culture, receptor binding, enzyme activity, genetic engineering, statistics, and experimental design. Students who use the assay also practice fundamental and broadly applicable laboratory skills such as serially diluting standards; extracting samples; making solutions; constructing and interpolating standard curves; calculating concentrations; making solutions; demonstrating sterile technique; culturing cells; identifying variables and controls; collecting, organizing, and analyzing data; constructing and interpreting graphs; and using common laboratory equipment such as micropipettors and spectrophotometers.

Disclosures

The authors have nothing to disclose.

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References

1. Agradi, E., Vegeto, E., Sozzi, A., Fico, G., Regondi, S., Tome, F. Traditional healthy Mediterranean diet: estrogenic activity of plants used as food and flavoring agents. *Phytother Res.* **20**(8), 670 - 675 (2006).
2. Morgan, H.E., Dillaway, D., Edwards, T.M. Estrogenicity of soybeans (*Glycine max*) varies by plant organ and developmental stage. *Endocr Disruptors.* **2**(1), e28490-1-8 (2014).
3. Myers, S.L., Yang, C.Z., Bittner, G.D., Witt, K.L., Tice, R.R., Baird, D.D. Estrogenic and anti-estrogenic activity of off-the-shelf hair and skin care products. *J Exp. Sci Environ Epidemiol.* **25**(3), 271-7 (2015).

4. Wagner, M., Oehlmann, J. Endocrine disruptors in bottled mineral water: estrogenic activity in the E-Screen. *J Steroid Biochem Mol Biol.* **127**(1-2), 128-35 (2011).
5. Arnold, S.F., Robinson, M.K., Notides, A.C., Guillette, L.J. Jr, McLachlan, J.A. A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. *Environ Health Perspect.* **104**(5), 544-8 (1996).
6. Odum, J., *et al.* The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. *Regul Toxicol Pharmacol.* **25**(2), 176-88 (1997).
7. Mellanen, P., *et al.* Wood-derived estrogens: studies in vitro with breast cancer cell lines and in vivo in trout. *Toxicol Appl Pharmacol.* **136**(2), 381-388 (1996).
8. Bergman, A., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T. *State of the science of endocrine disrupting chemicals - 2012. Inter-Organisation Programme for the Sound Management of Chemicals (IOMC).* http://www.who.int/ceh/publications/endocrine/en/ (2013).
9. Balsiger, H.A., de la Torre, R., Lee, W.Y., Cox, M.B. A four-hour yeast bioassay for the direct measure of estrogenic activity in wastewater without sample extraction, concentration, or sterilization. *Sci Total Environ.* **408**(6), 1422-1429 (2010).
10. Coldham, N.G., Dave, M., Sivapathasundaram, S., McDonnell, D.P., Connor, C., Sauer, M.J. Evaluation of a recombinant yeast cell estrogen screening assay. *Environ Health Perspect.* **105**(7), 734-742 (1997).
11. De Boever, P., Demaré, W., Vanderperren, E., Cooreman, K., Bossier, P., Verstraete, W. Optimization of a yeast estrogen screen and its applicability to study the release of estrogenic isoflavones from a soy germ powder. *Environ Health Perspect.* **109**(7), 691-697 (2001).
12. Fox, J.E., Burow, M.E., McLachlan, J.A., Miller III, C.A. Detecting ligands and dissecting nuclear receptor-signaling pathways using recombinant strains of the yeast Saccharomyces cerevisiae. *Nat Protoc.* **3**(3), 637 - 645 (2008).
13. Routledge, E.J., Sumpter, J.P. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ Toxicol Chem.* **15**(3), 241-248 (1996).
14. Miller III, C.A., Tan, X., Wilson, M., Bhattacharyya, S., Ludwig, S. Single plasmids expressing human steroid hormone receptors and a reporter gene for use in yeast signaling assays. *Plasmid.* **63**(2), 73-8 (2010).
15. Delfosse, V., Grimaldi, M., Cavaillès, V., Balaguer, P., Bourguet, W. Structural and functional profiling of environmental ligands for estrogen receptors. *Environ Health Perspect.* **122**(12), 1306-13 (2014).
16. Environmental Working Group. *Skin Deep Cosmetics Database.* http://www.ewg.org/skindeep/ (2016).
17. Eustice, D.C., Feldman, P.A., Colberg-Poley, A.M., Buckery, R.M., Neubauer, R.H. A sensitive method for the detection of beta-galactosidase in transfected mammalian cells. *Biotechniques.* **11**(6), 739-40, 742-3 (1991).
18. Buller, C., Zang, X.P., Howard, E.W., Pento, J.T. Measurement of beta-galactosidase tissue levels in a tumor cell xenograft model. *Methods Find Exp Clin Pharmacol.* **25**(9), 713-716 (2003).
19. Pelisek, J., Armeanu, S., Nikol, S. Evaluation of beta-galactosidase activity in tissue in the presence of blood. *J Vasc Res.* **37**(6), 585-93 (2000).
20. Gray, W.D. Studies on the alcohol tolerance of yeasts. *J Bacteriol.* **42**(5), 561-74 (1941).
21. Schug, T.T., *et al.* Designing endocrine disruption out of the next generation of chemicals. *Green Chem.* **15**(1), 181-198 (2013).