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Screening for Phytoestrogens Using a Cell-based Estrogen Receptor β Reporter Assay

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TITLE:

Screening for Phytoestrogens Using a Cell-based Estrogen Receptor β Reporter Assay

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KEYWORDS:

Plant secondary compounds, Phytosteroid, Estrogenic activity, Herbivory, Environmental endocrinology, Estradiol

SUMMARY:

We have optimized a commercially available estrogen receptor β reporter assay for screening human and nonhuman primate foods for estrogenic activity. We validated this assay by showing that the known estrogenic human food soy registers high, while other foods show no activity.

ABSTRACT:

Plants are a source of food for many animals, but they can produce thousands of chemicals. Some of these compounds affect physiological processes in the vertebrates that consume them, such as endocrine function. Phytoestrogens, the most well studied endocrine-active phytochemicals, directly interact with the hypothalamo-pituitary gonadal axis of the vertebrate endocrine system. Here we present the novel use of a cell-based assay to screen plant extracts for the presence of compounds that have estrogenic biological activity. This assay uses mammalian cells engineered to highly express estrogen receptor beta (ERβ) and have been transfected with a luciferase gene. Exposure to compounds with estrogenic activity results in the cells producing light. This assay is a reliable and simple way to test for biological estrogenic activity. It has several improvements over transient transfection assays, most notably ease of use, the stability of the cells, and the sensitivity of the assay.

INTRODUCTION:

Plants are a necessary source of food for many animals, providing calories and nutrients critical to survival, reproduction, growth, development, and behavior[1]. Plants produce thousands of chemicals, many as adaptations for their own growth, stomatic maintenance, and reproduction. Other compounds, deemed plant secondary metabolites (PSMs), have functions that are less clear, though some are toxic and likely used as a defense against herbivory and parasitism (e.g., alkaloids, tannins)[2,3]. Some of these chemicals have the ability to affect long term physiological

processes in animals, such as endocrine functioning, although why these endocrine active phytochemicals interact with the vertebrate endocrine system is still unclear[2,4].

Phytoestrogens, the most well studied endocrine-active phytochemicals, are polyphenolic PSMs that structurally and functionally mimic estrogens, directly interacting with the hypothalomo-pituitary gonadal axis of the vertebrate endocrine system[5]. Ingestion of phytoestrogens in the human diet is associated with protection against some cancers, heart disease, and menopausal symptoms, though other effects include fertility problems. In fact, the physiological effects of these compounds were discovered in the 1940s when infertility in sheep was attributed to their grazing on phytoestrogen-rich clover (*Trifolium subterrareum*)[6]. When ingested, phytoestrogens can pass into cells and mimic the effects of estrogen. While phytoestrogens had negative effects on sheep fertility, the relationship between phytoestrogens and physiology is not simple. Like sheep, Southern white rhinoceros display sensitivity to estrogenic compounds in feed derived from high quantities of soy and alfalfa. Daughters of females fed this diet during pregnancy are less likely to reproduce[7]. However, other studies have shown that phytoestrogens may have positive effects as well, including maturation of ovarian follicles in older mice[8], prevention of certain cancers, antioxidant activity, and antiproliferative effects[9].

The breadth of effects of phytoestrogens are not surprising given that estrogens affect a wide array of biological functions, including growth and development and regulation of the reproductive system and central nervous system[10]. Although there are many mechanisms of action, phytoestrogens often have the ability to modify, enhance, or disrupt estrogen signaling through their ability to act as ligands for the intranuclear estrogen receptors alpha and beta (ERα and ERβ). Many phytoestrogens have a phenolic ring structure similar to estrogens that allows them to bind estrogen receptors. Those with agonistic estrogenic activity function like estrogen, forming an activated ER-ligand complex that can dimerize and bind to an estrogen response element (ERE) and trigger gene transcription[11]. Thus, estrogens and phytoestrogens regulate cell activity and system functions through their actions as transcription factors.

Here we present the novel use of a cell-based assay to screen plant extracts for the presence of compounds that have estrogenic biological activity. This assay uses Chinese hamster ovary CHO cells engineered to highly express ERβ, which have been transfected with the firefly luciferase gene linked to an ERE promoter[12]. When estrogenic compounds are present, they bind to the ER, dimerize, and bind to the ERE, leading to transcription of the luciferase gene. Upon addition of a substrate solution, the luciferase catalyzes a reaction leading to photon emission. Therefore, positive samples produce light and negative samples do not.

This commercially available assay eliminates the need for laboratories to transfect the mammalian cells with the reporter gene and estrogen receptor[13,14], which was unstable and variable in efficacy. The assay provides a stable transfection platform that allows for quickly and simply determining whether a plant has estrogenic activity via receptor binding.

We test the hypothesis that soybeans have higher estrogenic activity than all other foods given their known concentrations of estrogenic isoflavones[15] using human foods from local grocers.

PROTOCOL:

1. Preparation of plant materials

1.1 Freeze dry plant items that were collected fresh using a lyophilizer.

1.1.1 To protect samples from light, cover chambers with aluminum foil during drying process.

1.1.2 To ensure that samples are completely dry, lyophilize until chambers no longer feel cold to touch and plant materials no longer lose mass when weighed.

1.1.3 Store dried plants in sterile low residue bags in absence of light until grinding.

1.2 Finely grind samples using a grinding mill with 0.85 mm mesh screen.

1.2.1 Store the ground samples in the bags in the absence of light until extraction.

2. Extraction of plant secondary metabolites

2.1 To extract the secondary plant metabolites, use a ratio of 1 g of dried sample to 10 mL of HPLC grade methanol.

2.1.1 Weigh sample on an analytical balance and add it to an appropriately sized Erlenmeyer flask (125 – 250 mL). Then add appropriate volume of methanol. Record the mass of sample extracted.

2.1.2 Cover the plant-methanol solution with aluminum foil, and then set to rotate at 100 rpm speed at room temperature (RT) for 3 days on an orbital shaker, allowing the potentially estrogenic compounds to dissolve into the methanol.

2.1.3 Decant the supernatant into a drip filtration system using filter paper (125 mm).

2.1.4 Using a rotary evaporator, dry the plant extract until the sample is thickened, but pourable, in a 300 mL round-bottom flask. Pour sample into a 50 mL round-bottom flask, rinsing the large flask with a small amount of methanol. Continue to dry the sample in the small flask until the methanol is completely evaporated.

2.1.5 Weigh the sample residue using an analytical balance. Record residue mass.

2.1.6 Dissolve the plant extract in dimethyl sulfoxide (DMSO) at a concentration of 0.1 g of extract to 2 mL of DMSO. Vortex until homogenized.

2.1.7 Store the plant extract-DMSO solution at 4 °C in amber glass vials until the assay.

CAUTION: Plants can produce unknown biologically active chemicals, and DMSO is a vehicle that can transport them across cell membranes. Use appropriate personal protective equipment and care when handling these samples.

3. Human estrogen receptor β transfection assay[12]

NOTE: Aseptic technique and a laminar flow hood is required for Day 1 of the assay protocol.

3.1 Prepare dilutions of 17β-Estradiol for the standard curve.

3.1.1 Transfer the Cell Recovery Medium and Compound Screening Medium (CSM) from the freezer storage and thaw in a 37 °C water bath.

3.1.2 Label microcentrifuge tubes Intermediate 1 and 2 (INT1, INT2) and 1-8.

3.1.3 Fill INT1 with 995 µL of CSM, INT2 with 615 µL of CSM, tube 1 with 900 µL of CSM, and tubes 2-8 with 600 µL of CSM. Set tube 8 aside.

3.1.4 Transfer 5 µL of 100 µM 17β-Estradiol Stock into INT1. Discard the tip. Vortex.

3.1.5 Before each transfer, rinse pipette 3 times, and then transfer 10 µL from INT1 into INT2. Discard the tip.

3.1.6 Rinse pipette 3 times, and then transfer 100 µL from INT2 into tube 1. Discard tip. Transfer 300 µL from tube 1 into tube 2. Repeat for tubes 3 through 7. Discard 300 µL from tube 7 into waste container. Tube 8 is a Zero and does not receive estradiol. Final concentrations of plated standards are: 400, 133.3, 44.44, 14.815, 4.938, 1.646, 0.5487, and 0 pM estradiol.

3.2 Prepare sample compounds.

3.2.1 Vortex samples.

3.2.2 Take 4 µL of each plant sample in DMSO and add to 496 µL of CSM to yield a 0.8% DMSO solution.

3.3 Rapidly thaw Reporter Cells.

3.3.1 Retrieve the tube of Cell Recovery Medium from the 37 °C water bath. Disinfect the outside surface using 70% ethanol.

3.3.2 Retrieve Reporter Cells from -80 °C storage and thaw by transferring 10 mL of the pre-warmed CRM into the tube of frozen cells.

3.3.3 Close the tube of Reporter Cells and transfer to a 37°C water bath for 5-10 min.

3.3.4 Retrieve the tube of Reporter Cell Suspension from the water bath. Invert the tube of cells several times gently to break up aggregates of cells and produce a homogenous suspension. Clean the surface of the tube with 70% ethanol.

3.4 Assay plating

3.4.1 Dispense 100 µL of the Reporter Cell Suspension into each well using a multichannel pipette.

3.4.2 Dispense 100 µL of samples in triplicate into appropriate assay wells.

3.4.3 Transfer the plate into a 37 °C, humidified 5% CO_2 incubator for 22-24 h.

4.1 Thaw Detection Substrate and Detection Buffer in a dark refrigerator overnight to prepare for Day 2.

5.1 Just prior to the end of the plate incubation, remove Detection Substrate and Detection Buffer from refrigerator and place in low light area until equilibrated to RT. Once at RT, invert each tube gently several times to thoroughly mix solutions.

5.1.1 Immediately before the incubation is complete, pour the entire contents of the Detection Buffer into the tube of Detection Substrate to create Luciferase Detection Reagent. Mix gently so as not to produce foam.

5.1.2 Once the incubation is complete, invert the plate to discard content into an appropriate waste container. Gently tap the plate on a clean absorbent paper towel to remove the last droplets from the wells.

5.1.3 Add 100 µL of the Luciferase Detection Reagent to each well. Allow the assay plate to rest at RT for 15 min. Do not shake the plate.

5.2 Quantify luminescence using a 96-well plate-reading luminometer.

REPRESENTATIVE RESULTS:

Twenty-two extracts of fruits and vegetables commonly found in human diets were screened for the presence of estrogenic compounds. A variety of foods were assayed, including legumes, such as soybeans, snow peas, and snap peas, as the pea family is a known source of phytoestrogens[16], as well as figs, dates, corn, carrots, apples, bananas, strawberries, tomato, kale, and cabbage. Endocrine disrupting compounds are found in common substances (e.g., plastics and pesticides) and some are biologically active through ERs[17]. When possible, both organic and nonorganically grown items were assayed to account for the possibility that pesticides with estrogenic activity could have affected the results.

Each plant food item was plated in triplicate and the luminometer reported each well's activity

in Relative Light Units (RLUs). Background levels of RLUs are determined in the standard curve with Standard 8, the zero concentration, and used for reference. The fold activation values, which is the multiplier above the RLU for the Zero point on the curve.

$$\text{Fold Activation} = \text{Unknown (RLU)} \div \text{Standard 8 (RLU)}$$

For interpretive purposes, estrogenic activity is presented in an ordinal, qualitative manner of High, Med, Low, or No Activity. High levels of activity register above the Standard 4 fold activation value. Medium falls between Standard 5 and Standard 4, and Low values are between Standard 6 and Standard 5. Any samples with fold activation values below Standard 7 are considered No Activity. Referring to **Table 1**, soybeans, both organic and non-organic, screened at high levels of activity, while all other fruit and vegetable items registered no activity. Comparing soybean results to the standard curve (**Figure 1**), shows that, whether grown organically or not, they score high off the curve estradiol activity levels at this concentration. Soybean extract, a known potent source of the isoflavones daidzein and genistein[9], was further used to determine the dilution yielding a 50% signal to the maximum (**Figure 2**). This extract requires 422 times more dilution to produce half the signal of our standard dilution protocol.

FIGURE AND TABLE LEGENDS:

Table 1. Representative results of the ERβ Reporter Assay System for screening of fruit and vegetable items for phytoestrogen activity. Positive activity is indicated by High, Med, Low, or No Activity.

Figure 1. Serial dilution of 17β-Estradiol standard (Standard 1 through 8 concentrations = 400, 133.3, 44.44, 14.815, 4.938, 1.646, 0.5487, and 0 pM, respectively) using the ERβ Reporter Assay System.

Figure 2. The ERβ Reporter Assay using a serial dilution of soybean extract to determine the dilution that yielded a signal-to-background ratio that is 50% of the maximum signal. From the standard extraction method dissolving the plant extract in dimethyl sulfoxide (DMSO) at a concentration of 0.1 g of extract to 2 mL of DMSO, soybean has to be diluted 422 times to elicit a signal 50% of the maximum response.

DISCUSSION:

The ERβ reporter assay developed to individually screen pharmaceutical agents is also suitable for screening plant foods for phytoestrogens biologically active through the ERβ. Important considerations in the protocol include treating the plant samples with care: fresh plant material needs to be dried swiftly to prevent molding or other biological degradation, and it needs to be kept away from light to prevent photolysis of the compounds[18]. The assay protocol[12] provided by the manufacturer is clear and needs very few modifications for screening purposes. The standard curve suggested by the manufacturer has been modified in this protocol to increase the number of points that fall in the exponential range of the curve (**Figure 1**), while preserving the top and bottom plateaus. It is possible to use this assay for quantitative analysis, but our purpose is to associate plants with high activity to biological effects, food choice, and other behaviors in the

animals that consume them.

To further illustrate the effectiveness of the extraction and assay we include a dose response curve with soybean extract (**Figure 2**) and determined that given the potency of the normal extraction protocol, soy must be diluted extensively before the signal drops to 50% maximum. This highlights the fact that at high concentrations of phytoestrogens the signal plateaus at a stable maximum signal. At very low concentrations the signal may not be strong enough to be distinguished from background. It is important to work with high concentrations of extracts, in order to detect phytoestrogens present in low amounts in a sample, minimizing false negatives. Initially the laboratory used a greater volume of DMSO relative to the plant residue from the methanol extraction (i.e., 10 mL of DMSO to 0.1 g of plant residue). The samples were too dilute to induce a strong luminescence in positive samples. Due to a maximum DMSO percentage for reporter cell viability and volume constraints within the wells on the plate, sample extract concentration should be optimized when adding DMSO to the plant residues. A positive control such as soy should be included on every plate, to confirm that cells are viable and capable of luminescence, and that the extract concentration is sufficient to elicit a response.

This assay detects compounds that bind to ERβ, but not all phytoestrogens have the same mechanism of action. This assay protocol can be modified by incubating the cells with a combination of estradiol and the plant compounds to detect if there is antiestrogen activity in a sample[9,12]. Estradiol has great affinity to ER, so the presence of phytoestrogens may have antiestrogenic biological activity in the presence of estradiol by blocking the receptors, which reduces the response to estrogens. Antiestrogenic activity would be detected by a reduction in total activation with increasing concentration of plant extract. This assay will not detect other methods of action, such as binding to membrane-bound ERs[19]. Furthermore, some phytoestrogens are not biologically active until they have been metabolized by gut microbes[20]. It is possible that some plants that have no or low estrogenic activity in their unmetabolized state have higher estrogenic activity post-metabolization that this assay would not detect.

The ERβ reporter assay has been chosen to exemplify the screening of phytoestrogens for activity in plants because phytoestrogens compete for binding with estradiol more strongly to ERβ than they do to ERα[21]. Screening for ERα activity is possible through a similar assay, wherein the cells are transfected with the ERα gene rather than ERβ.

Following a positive screening for active phytoestrogens, the active compounds can be identified with chromatography methods. Indeed, at that point the isolated compounds can be tested using this assay and the half maximal effective concentrations (EC_{50}) can be determined using a dilution series as a measure of potency of the compound.

This assay is a reliable and simple way to test for biological estrogenic activity, keeping in mind its limitations in the breadth of mechanisms of estrogenic activity. It has several improvements over transient transfection assays, most notably ease of use, the stability of the cells, and the sensitivity of the assay.

Little is known about the prevalence of phytoestrogens in wild plant foods consumed by humans or wild animals[22], but studies show that exposure to estrogenic PSMs in diet can have long lasting effects[23]. Having a simple robust assay that detects these compounds, in conjunction with studies assessing amounts eaten and when they are eaten, is a powerful step in determining the function of including estrogenic foods in the diet and the effects of these compound on physiological systems.

ACKNOWLEDGMENTS:

Authors are thankful to Dale Leitman for initial training in use of transient transfection assays to determine estrogenic activity of primate plant foods. Thanks to Bradford Westrich and C. Eric Johnson for helping to set up laboratory equipment and training students in extraction methods. Finally, thank you to Indiana University for funding this research.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Wasserman, M. D. et al. Estrogenic plant consumption predicts red colobus monkey (*Procolobus rufomitratus*) hormonal state and behavior. *Hormones and Behavior.* **62** (5), 553-562 (2012).

2 Wasserman, M. D., Milton, K., Chapman, C. A. The roles of phytoestrogens in primate ecology and evolution. *International Journal of Primatology.* **34** (5), 861-878 (2013).

3 DeGabriel, J. L., Moore, B. D., Foley, W. J., Johnson, C. N. The effects of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. *Ecology.* **90** (3), 711-719 (2009).

4 Wasserman, M. D., Steiniche, T., Després-Einspenner, M.-L. in *Primate Diet & Nutrition* eds Joanna E Lambert & Jessica M Rothman) (University of Chicago Press, *In Press*).

5 Benavidez, K. M., Chapman, C. A., Leitman, D. C., Harris, T. R., Wasserman, M. D. Intergroup variation in oestrogenic plant consumption by black-and-white colobus monkeys. *African Journal of Ecology* (2019).

6 Bennetts, H. W., Underwood, E. J., Shier, F. L. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. *Australian Veterinary Journal.* **22** (1), 2-12 (1946).

7 Tubbs, C. W. et al. Estrogenicity of captive southern white rhinoceros diets and their association with fertility. *General and Comparative Endocrinology.* **238**, 32-38 (2016).

8 Shen, M. et al. Observation of the influences of diosgenin on aging ovarian reserve and function in a mouse model. *European Journal of Medical Research.* **22** (1), 42 (2017).

9 Boué, S. M. et al. Evaluation of the estrogenic effects of legume extracts containing phytoestrogens. *Journal of Agricultural and Food Chemistry.* **51** (8), 2193-2199 (2003).

10 Klinge, C. M. Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Research.* **29** (14), 2905-2919 (2001).

11 Nishikawa, J.-i. et al. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. *Toxicology and Applied Pharmacology.* **154** (1), 76-83 (1999).

12 Human Estrogen Receptor Beta (ERb; ESR2; NR3A2) Reporter Assay System. (Indigo Biosciences, State College, PA).
13 Wasserman, M. D. et al. Estrogenic plant foods of red colobus monkeys and mountain gorillas in uganda. *American Journal of Physical Anthropology.* **148** (1), 88-97 (2012).
14 Vivar, O. I., Saunier, E. F., Leitman, D. C., Firestone, G. L., Bjeldanes, L. F. Selective activation of estrogen receptor-β target genes by 3, 3'-diindolylmethane. *Endocrinology.* **151** (4), 1662-1667 (2010).
15 Whitten, P. L., Patisaul, H. B. Cross-species and interassay comparisons of phytoestrogen action. *Environmental Health Perspectives.* **109** (suppl 1), 5-20 (2001).
16 Di Gioia, F., Petropoulos, S. A. in *Advances in Food and Nutrition Research* (Academic Press Inc., 2019).
17 Lutz, I., Kloas, W. Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. *Science of The Total Environment.* **225** (1), 49-57 (1999).
18 Felcyn, J. R., Davis, J. C. C., Tran, L. H., Berude, J. C., Latch, D. E. Aquatic Photochemistry of Isoflavone Phytoestrogens: Degradation Kinetics and Pathways. *Environmental Science & Technology.* **46** (12), 6698-6704 (2012).
19 Jeng, Y.-J., Kochukov, M. Y., Watson, C. S. Membrane estrogen receptor-alpha-mediated nongenomic actions of phytoestrogens in GH3/B6/F10 pituitary tumor cells. *Journal of Molecular Signaling.* **4**, 2-2 (2009).
20 Dixon, R. A. Phytoestrogens. *Annual Review of Plant Biology.* **55** (2004).
21 Kuiper, G. G. J. M. et al. Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor β. *Endocrinology.* **139** (10), 4252-4263 (1998).
22 Wasserman, M. D. *Feeding on Phytoestrogens: Implications of Estrogenic Plants for Primate Ecology*, UC Berkeley (2011).
23 Jefferson, W. N., Patisaul, H. B., Williams, C. J. Reproductive consequences of developmental phytoestrogen exposure. *Reproduction (Cambridge, England).* **143** (3), 247-260 (2012).

Figure 1

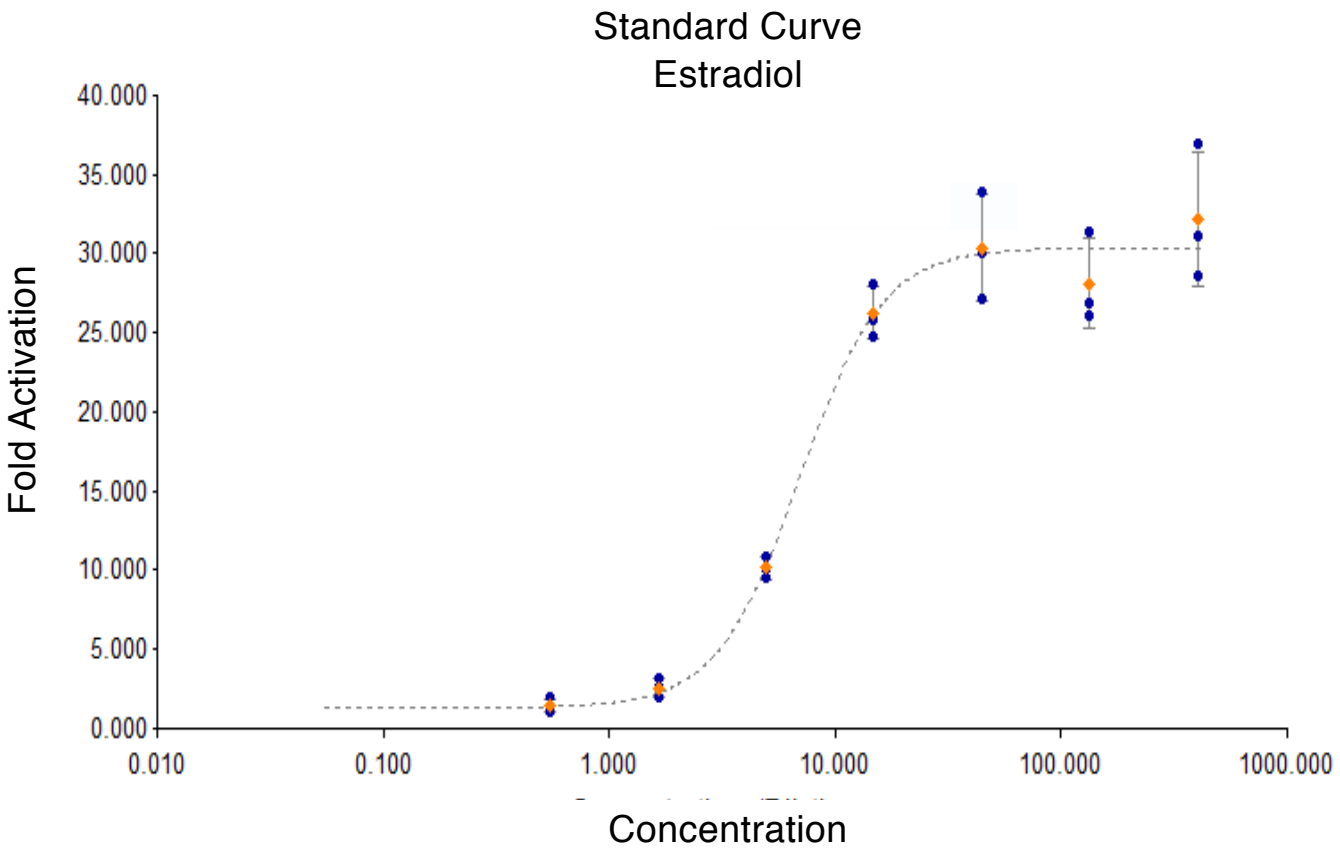

Figure 2

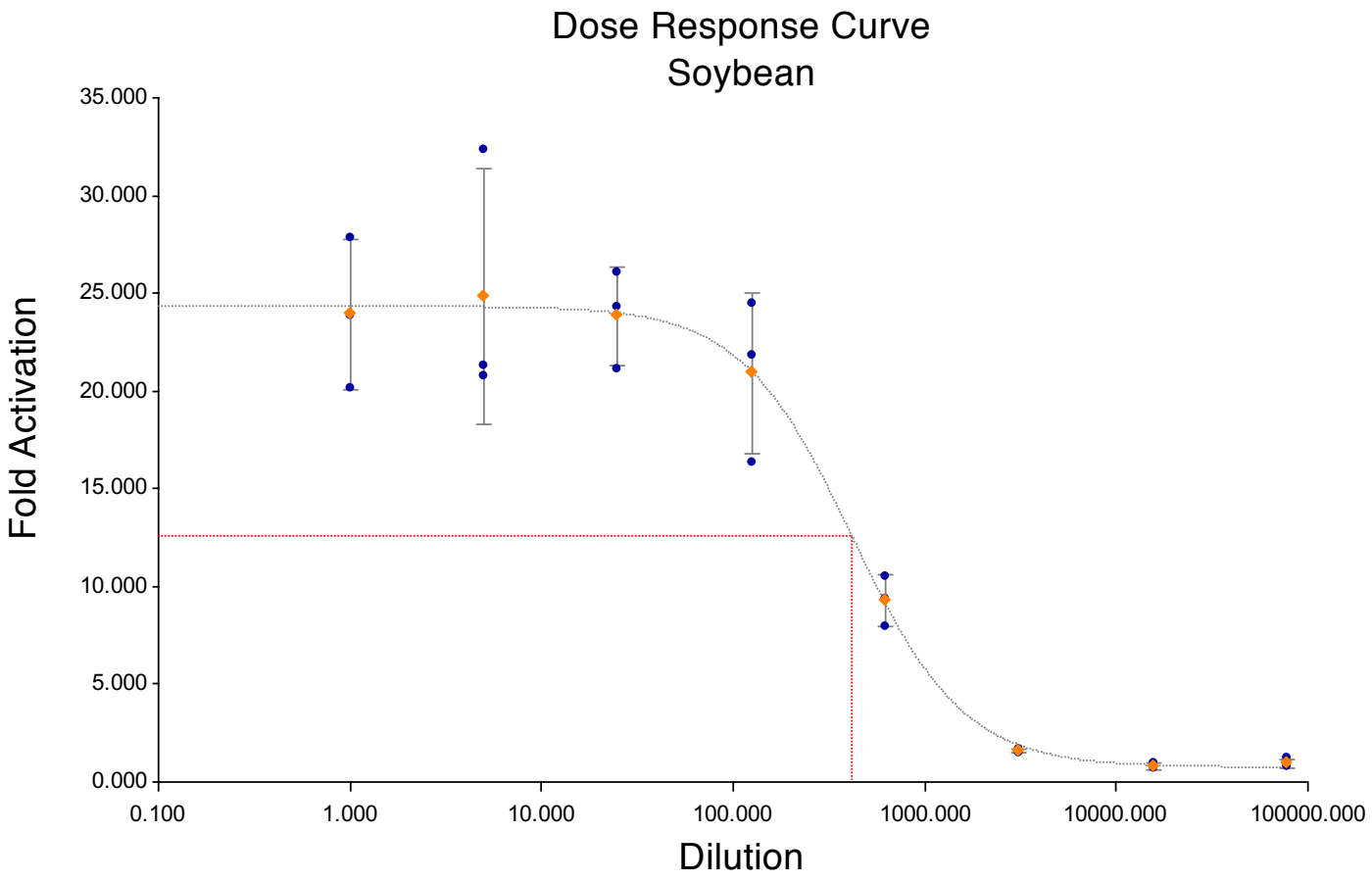

Table 1

Produce Item	Organic/ Non-organic	Relative Light Units (Lum)	Fold Activation	Fold Activation (Mean)	Phytoestrogen Activity
Soybeans	Organic	1687	29.016	31.06	High
		2023	34.796		
		1706	29.353		
Soybeans	Non-organic	2041	35.106	32.05	High
		1956	33.647		
		1593	27.399		
Snow Peas	Non-organic	53	0.919	0.92	No Activity
		59	1.015		
		49	0.836		
Snap Peas	Non-organic	66	1.142	1.21	No Activity
		60	1.032		
		85	1.462		
Corn	Non-organic	29	0.502	0.53	No Activity
		30	0.513		
		33	0.575		
Strawberry	Non-organic	35	0.609	0.77	No Activity
		47	0.808		
		51	0.884		
Strawberry	Organic	56	0.956	0.88	No Activity
		59	1.015		
		39	0.678		
Banana	Organic	32	0.544	0.52	No Activity
		28	0.489		
		31	0.533		
Banana	Non-organic	33	0.564	0.60	No Activity
		41	0.712		
		31	0.533		
Plantain	Non-organic	37	0.64	0.70	No Activity
		39	0.667		
		47	0.805		
Kale	Organic	26	0.447	0.47	No Activity
		26	0.444		
		30	0.519		
Kale	Non-organic	40	0.685	0.63	No Activity
		28	0.485		
		42	0.719		
Cabbage	Organic	33	0.568	0.54	No Activity
		27	0.468		
		34	0.588		
Cabbage	Non-organic	44	0.757	0.66	No Activity
		34	0.585		
		36	0.626		
Apple	Organic	30	0.523	0.49	No Activity
		25	0.437		
		30	0.509		
Apple	Non-organic	41	0.705	0.62	No Activity
		31	0.53		
		37	0.63		
Tomato	Organic	51	0.874	0.87	No Activity
		57	0.974		

		44	0.76		
Tomato	Non-organic	61	1.056	1.19	No Activity
		81	1.386		
		66	1.128		
Carrot	Organic	33	0.575	0.51	No Activity
		33	0.561		
		22	0.382		
Carrot	Non-organic	31	0.53	0.52	No Activity
		21	0.365		
		38	0.657		
Fig	Non-organic	29	0.506	0.61	No Activity
		42	0.716		
		36	0.619		
Dates	Non-organic	29	0.495	0.59	No Activity
		39	0.667		
		35	0.602		

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1000 µL pipette			
20 µL pipette			
200 µL pipette			
37 ℃ water bath			
37 ℃, humidified 5% CO_2 incubator			
70% ethanol			
analytical balance			
cell culture-rated laminar flow hood			
dimethyl sulfoxide			
disposable media basin, sterile			
drip filtration system			
Erlenmeyer flasks			125 mL and 250 mL
HPLC grade methanol			
Human ERβ Reporter Assay System, 1 x 96-well format assays	Indigo Biosciences	IB00411	Assay kit - analyzes 24 samples plus standard curve
lyophilizer			
multi-channel pipette			
orbital shaker			
plate-reading luminometer			ex. Bioteck Synergy HTX
rotory evaporator			
round bottom flasks			50 mL and 300 mL
sterile microcentrifuge tubes or sterile multi-channel media basins			
sterile tips			200 µL and 1000 µL
Whatman grade 1 paper			
whirl-pak bags			sterile polyethylene bags

DEPARTMENT OF ANTHROPOLOGY
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February 23, 2020

Dear Dr. Nguyen,

We thank you and the five reviewers for the very helpful comments that we think have helped improve the quality of our manuscript. We have closely examined the comments provided by the reviewers and have made the following changes to the manuscript.

Editorial Comments:

1. The text has been proofread for spelling and grammar.
2. Textual overlap: The selected lines in the methods section have been rewritten.
3. Protocol highlight: Approximately 2 pages of the text has been highlighted that would visualize the protocol well.
4. Discussion:
5. Commercial language: The commercial language has been removed from the text of the document and replaced with generic words.

Reviewer #1

1. Unfortunately, the color-coded comments as alluded to in the revision statement did not show up, but differences between their version of the manuscript and the original were assessed and changes for clarity were included in the methods.
2. The acknowledgements section was modified to include the suggested wording.

Reviewer #2

1. Major concerns: none
2. Minor concern: The reviewer suggests that the catalog number be included in the manuscript. Respectfully, the number is included in the table of materials and it seems that is the most appropriate place for it.

Reviewer #3

1. The review suggests that a flow chart of the protocol would clarify the process to the readers. However, given the nature of the journal with a video portion detailing the protocol, the authors do not think that a flow chart will add any more clarification.

Reviewer #4

1. The reviewer correctly states that this assay would not detect estrogenic activity if the phytosteroids bind the ERα receptor alone or in addition to the ERβ. We have clarified that point and suggested that the ERα Reporter Assay would be the correct assay to screen for ERα activity. As stated in the manuscript, the decision to only explore ERβ was based on previous research: Wasserman et al. 2012. These cells are transfected with only the ERβ gene, therefore they do not express ERα. The reviewer also asks us to expand on the idea that some phytoestrogens are not active or highly active until they have been metabolized by intestinal bacteria. We have clarified the section on assay limitations, but within this paper we are only interested in ERβ-selective agonists.
2. Though we do not have red clover to extract, soy, a plant rich in isoflavones was included as a positive control. We have added a dose response curve of soy, that shows the potency of our normal extraction protocol through the need to dilute the extract extensively before signal drops to 50% of maximum.
3. We have extended the discussion section and included clarified and extended explanations of the scope of the assay and interpretation. We have also included more suggestions for successful application of the method.
4. We have included a discussion of antiestrogenic properties and expanded the explanation of the assay modification and interpretation of these results. The antiestrogenic properties are beyond the scope of paper.
5. We have provided more detail in the figure 1 caption.
6. We have clarified the language describing the fold activation calculation and added the equation for reference.

Reviewer #5

1. The assay was not changed from the manufacturer's method with the noted exception of the standard curve modification as noted in the first paragraph of the discussion. However, we did add details regarding the optimization of DMSO:sample ratio in the discussion as well.

Thank you again for the constructive feedback. We look forward to continuing to work with you during the next phase of this manuscript.

Sincerely,
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