FINAL SCRIPT: APPROVED FOR FILMING

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

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Emily Chester:** We use this assay to assess whether plant foods contain phytosteroids, which can affect physiological processes in animals, as well as behavior and reproductive success.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Emily Chester:** This assay provides a simple and reliable method for determining whether plants produce estrogenic compounds with biological activity.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Emily Fender:** This method was developed initially to screen drugs with xeno-estrogen activity for applications in medicine, for example inhibiting cell proliferation. We are using it to provide insight into primate ecology and evolution.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Emily Fender:** It is important to have practice working with aseptic technique in a clean environment prior to attempting this assay as it is very sensitive to contamination.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Commented [AG1]: Authors: Journal guidelines dictate that, when possible, an author delivers no more than 2 statements per section. Therefore, it would be helpful if Emily Fender delivered statements 1.3 and 1.4. Furthermore, since an author is introduced with a text overlay as they deliver an interview statement, this will eliminate the need for the introduction of demonstrator statement.

Protocol

2. Extraction of Potential Plant Phytosteroids

2.1. Begin by freeze drying the fresh plant items with a lyophilizer **[1]**, then finely grind them using a grinding mill with a 0.85-millimeter mesh screen **[2]**. Store the ground samples in bags in the dark until extraction **[3]**. *Videographer: This step is important!*

2.1.1. Talent using the lyophilizer.

2.1.2. Talent grinding the samples.

2.1.3. Talent putting a bag of ground sample in the appropriate storage location.

2.2. To extract any potential phytosteroids in the plants, add dried sample to an appropriately sized flask **[1]**, then add HPLC grade methanol **[2-TXT]**. Cover the plant-methanol solution with aluminum foil **[3]** and set it to rotate at 100 rpm for 3 days on an orbital shaker **[4]**.

2.2.1. Talent adding dried sample to a flask.

2.2.2. Talent adding methanol to the flask, with the methanol container in the shot. **TEXT: Use 10 mL methanol per 1 g dried sample**

2.2.3. Talent covering solution with foil.

2.2.4. Talent setting solution to rotate.

2.3. After 3 days, use filter paper to decant the supernatant into a drip filtration system **[1]**. Dry the plant extract with a rotary evaporator until it is thickened, but pourable, in a 300-milliliter round-bottom flask **[2]**.

2.3.1. Talent decanting the supernatant.

2.3.2. Flask in the rotary evaporator.

2.4. Pour the sample into a 50-milliliter flask **[1]** and rinse the large flask with a small amount of methanol **[2]**, then continue to dry the sample in the small flask until the methanol is completely evaporated **[3]**. *Videographer: This step is important!*

2.4.1. Talent pouring the sample into a 50mL flask.

2.4.2. Talent rinsing the large flask with methanol.

2.4.3. Talent putting the flask in the rotary evaporator.

2.5. When finished, weigh the sample residue with an analytical balance and record the mass **[1]**. Dissolve the plant extract in DMSO at a concentration of 0.1 gram of extract to 2 milliliters of DMSO **[2]** and vortex it until homogenized **[3]**.

2.5.1. Talent weighing the residue and writing down the mass.

2.5.2. Talent adding the extract to DMSO.

2.5.3. Talent vortexing the mixture.

2.6. **Emily Fender**: DMSO is not toxic on its own, but it is a vehicle that allows other substances to cross membranes. Since we do not know if our plants contain toxic chemicals, all are treated as if they are hazardous. Do not allow the solution to touch skin, replace gloves if contaminated, and wear thick close-toed shoes and lab coats.

2.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot. *Videographer: Have talent look more directly at the camera because this is a warning statement.*

2.7. Store the sample at 4 degrees Celsius in amber glass vials until ready to use **[1]**.

2.7.1. Talent putting the sample into the refrigerator and closing the door.

3. Human Estrogen Receptor β Transfection Assay

3.1. Vortex the samples, then add 4 microliters of each sample to 496 microliters of compound screening medium to yield a 0.8% DMSO solution **[1]**. Disinfect the outside surface of a pre-warmed Cell Recovery Medium tube with 70% ethanol **[2]** and transfer 10 milliliters of the medium into a tube of frozen Reporter Cells to thaw them **[3]**.

3.1.1. Talent vortexing the sample and then adding 4 microliters to a tube with CSM.

3.1.2. Talent wiping the tube of CRM with ethanol.

3.1.3. Talent adding CRM to frozen cells.

3.2. Close the tube of Reporter Cells and place it in a 37-degree Celsius water bath for 5 to 10 minutes **[1]**. After retrieving the cells from the water bath, gently invert the tube several times to break up aggregates of cells and produce a homogenous suspension **[2]**, then clean the surface of the tube with 70% ethanol **[3]**.

3.2.1. Talent closing the tube of cells and placing it in the water bath.

3.2.2. Talent taking the tube from the water bath and inverting it.

3.2.3. Talent cleaning the outside of the tube with ethanol.

3.3. Use a multichannel pipette to dispense 100 microliters of the Reporter Cell Suspension into each well of a 96-well plate **[1]**, then dispense 100 microliters of samples in triplicate into the appropriate wells **[2]**. Incubate the plate at 37 degrees Celsius and 5% carbon dioxide for 22 to 24 hours **[3]**. *Videographer: This step is important!*

3.3.1. Talent dispensing Reporter Cells into the plate.

3.3.2. Talent adding samples to the plate.

3.3.3. Talent putting the plate in the incubator and closing the door.

3.4. Just prior to the end of the plate incubation, remove Detection Substrate and Detection Buffer from the refrigerator **[1-TXT]** and place them in a low light area until equilibrated to room temperature **[2]**. Then, gently invert each tube to mix the solutions **[3]**.

3.4.1. Talent taking the detection substrate and buffer out of the refrigerator, **TEXT: Thaw Detection Substrate and Detection Buffer in a dark refrigerator overnight**

3.4.2. Talent placing the substrate and buffer in an appropriate spot to equilibrate to room temperature.

3.4.3. Talent gently inverting the tubes.

3.5. Immediately before the plate incubation is complete, pour the entire contents of the Detection Buffer into the tube of Detection Substrate to create Luciferase Detection Reagent **[1]**. Mix the tube gently so as not to produce foam **[2]**.

3.5.1. Talent pouring the detection buffer into the substrate.

3.5.2. Talent mixing the detection reagent tube.

3.6. Discard the contents of the sample plate into an appropriate waste container **[1]** and gently tap it on a clean absorbent paper towel to remove the last droplets from the wells **[2]**.

3.6.1. Talent discarding the contents of the plate into a waste container.

3.6.2. Talent tapping the plate on a clean paper towel.

3.7. Add 100 microliters of the Luciferase Detection Reagent to each well **[1]** and allow the assay plate to rest at room temperature for 15 minutes **[2]**. Then, quantify the luminescence using a 96-well plate-reading luminometer **[3]**. *Videographer: This step is important!*

3.7.1. Talent adding the detection reagent to a few wells.

3.7.2. Plate resting at room temperature.

3.7.3. Talent using the plate reader.

Results

4. Results: ERβ Reporter Assay System for Phytoestrogen Activity Screening of Fruits and Vegetables

4.1. Twenty-two extracts of fruits and vegetables commonly found in human diets were screened for the presence of estrogenic compounds **[1]**. Estrogenic activity is presented in an ordinal, qualitative manner of High, Medium, Low, or No Activity **[2]**.

4.1.1. LAB MEDIA: Table 1, uploaded as Jove table. *Video Editor: To make the table easier to visualize, get rid of columns C and D (relative light units and fold activation).*

4.1.2. LAB MEDIA: Table 1, uploaded as Jove table. *Video Editor: Emphasize the Phytoestrogen Activity column.*

4.2. Organic and non-organic soybeans screened at high levels of activity, while all other fruit and vegetable items registered no activity **[1]**. Comparing soybean results to the standard curve also shows that they have high estrogenic activity levels at this concentration **[2]**.

4.2.1. LAB MEDIA: Table 1, uploaded as Jove table. *Video Editor: Zoom in on the Soybean data, rows 2 - 7.*

4.2.2. LAB MEDIA: Figure 1.

4.3. Soybean extract, a known potent source of the isoflavones daidzein and genistein, was further used to determine the dilution yielding a 50% signal to the maximum **[1]**. This extract requires 422 times more dilution to produce half the signal of our standard dilution protocol **[2]**.

4.3.1. LAB MEDIA: Figure 2.

4.3.2. LAB MEDIA: Figure 2. *Video Editor: Emphasize the point where the red line meets the black curve.*

Conclusion

5. Conclusion Interview Statements

5.1. **Emily Fender:** Samples identified with activity could be sent for analysis by mass spectrometry to identify the active chemicals. Using an antagonist method, you can also determine if the plant foods contain chemicals that reduce the binding and activity of endogenous estrogens.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

5.2. **Emily Chester:** This technique allows us to further our understanding of how prevalent estrogenic foods are in primate diets, as well as how consumption of phytoestrogens affects physiology and behavior when the assay is combined with fecal hormone analyses and field data.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.