FINAL SCRIPT: APPROVED FOR FILMING

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Title: Extraction and Quantification of Soluble, Radiolabeled Inositol Polyphosphates from Different Plant Species using SAX-HPLC

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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**

Current Protocol Length

Number of Steps: 19
Number of Shots: 49

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Philipp Gaugler:** The protocol demonstrated here can help to elucidate the biosynthesis of inositol polyphosphates in plants and to answer questions regarding their role in certain aspects of plant metabolism and development.

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

1.2. **Philipp Gaugler:** This method is highly sensitive because the use of the radioactively labeled precursor of inositol phosphates allows for reliable detection of all inositol phosphate species from plants.

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

OPTIONAL:

1.3. **Philipp Gaugler:** Visual demonstration of this method is important because most plant biologists are unfamiliar with HPLC analysis and the needed setup. In addition, critical steps of this protocol like the extraction of inositol phosphates are difficult to follow without proper demonstration.

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

Protocol

2. HPLC System Set Up

2.1. Begin by setting up a system consisting of two independent HPLC pumps, one for buffer A and one for buffer B **[1]**. Both pumps need to be controlled together via a computer or a master pump **[2]**. Implement a piston seal wash for both pumps, either via gravitational force or through a third low pressure pump **[3]**.

2.1.1. WIDE: Establishing shot of talent next to the setup.

2.1.2. Computer or master pump that controls the pumps.

2.1.3. Talent implementing a piston seal wash.

2.2. Connect both pumps to a dynamic mixer **[1]**, then connect the mixer to an injection valve with a sample loop that has at least 1-milliliter capacity **[2]**. Use capillaries to connect the injection valve to the column **[3]** and the column to the fraction collector **[4]**.

2.2.1. Pumps connected to the dynamic mixer.

2.2.2. Mixer connected to an injection valve.

2.2.3. Injection valve connected to the column.

2.2.4. Column connected to the fraction collector.

3. Plant Cultivation and Labeling with [^{3}H]-*myo*-inositol

3.1. Sow out *Lotus* seeds in 1 row on square Petri dishes filled with solid growth media consisting of 0.8% bacteriological agar in deionized. **[1]**. Allow the seeds to stratify for at least 3 days at 4 degrees Celsius in the dark **[2]**. Place the seeds in a growth incubator **[3]**.

3.1.1. Talent putting the seeds in the Petri dish.

3.1.2. Talent putting the dish in the fridge.

3.1.3. Talent putting the dish into the growth incubator

3.2. Transfer 10 to 20 seedlings into one well of a 12-well clear flat-bottomed cell culture plate filled with 2 milliliters of half-strength MS salt solution that is supplemented with 1% sucrose and adjusted to pH 5.7 **[1]**. *Videographer: This step is important!*

3.2.1. Talent transferring seedlings into a cell culture plate.

3.3. Add 45 microcurie of [^{3}H]-*myo*-inositol *(pronounce 3-H-my-oh-inositol')* to the plate and swirl it gently **[1-TXT]**. Cover the plate with a lid **[2]** and seal it with microporous surgical tape **[3]**, then place it back into the growth incubator **[4]**.

3.3.1. Talent adding the [^{3}H]-*myo*-inositol to the plate and swirling it. **TEXT: CAUTION! ^{3}H can be a harmful radiation hazard. Always wear gloves.**

3.3.2. Talent covering the plate with a lid.

3.3.3. Talent sealing the plate with surgical tape.

3.3.4. Talent putting the plate in the incubator

3.4. After 5 days of labeling, remove seedlings from the media and wash them briefly with deionized water **[1]**. Dry them with paper towels **[2]** and transfer them into a 1.5-milliliter microcentrifuge tube, making sure to not overfill the tube **[3-TXT]**. Snap-freeze the tube in liquid nitrogen and store it at -80 degrees Celsius until extraction **[4]**. *Videographer: This step is important!*

3.4.1. Talent washing the seedlings with water.

3.4.2. Talent drying the seedlings.

3.4.3. Talent transferring the seedlings into the microcentrifuge tube. **TEXT: 10 – 20 seedlings per tube**

3.4.4. Talent snap-freezing the tube.

4. Extraction of Soluble InsPs

4.1. Take the samples from the freezer and keep them in liquid nitrogen until ready to use **[1]**. Grind them with a microcentrifuge tube pestle until they start thawing **[2]**, then add 500 microliters of ice-cold extraction buffer **[3]**. Continue grinding until the sample is homogenized and the solution is colored**[4]**. *Videographer: This step is difficult and important!*

4.1.1. Talent transferring the samples from the freezer to liquid nitrogen.

4.1.2. Talent grinding a sample.

4.1.3. Talent adding extraction buffer to the sample, with the buffer container in the shot.

4.1.4. Talent continuing to grind until the sample is completely homogenized.

4.2. Centrifuge the samples for 10 minutes at 18000 x *g* and 4 degrees Celsius **[1]**, then transfer the supernatant into a fresh 1.5-milliliter tube **[2]**. The tubes used for extraction are considered solid radioactive waste and need to be disposed of accordingly **[3]**.

4.2.1. Talent putting the sample in the centrifuge and closing the lid.

4.2.2. Talent transferring the supernatant to a fresh tube.

4.2.3. Talent properly disposing of the extraction tube.

4.3. Carefully add 300 microliters of neutralization buffer to the extract, which will immediately cause precipitation of proteins and bubbling **[1]**. After a minute, mix the sample with a pipette tip **[2]** and pipet 5 microliters onto pH paper to make sure that the pH is between 7 and 8 **[3]**. *Videographer: This step is difficult and important!*

4.3.1. Talent adding neutralization buffer to the sample.

4.3.2. Talent mixing the sample with a pipette.

4.3.3. Talent pipetting a small amount of sample on the pH strip.

4.4. If necessary, add small amounts of either neutralization buffer or extraction buffer until the desired pH is reached and let the samples rest on ice for at least 1 hour with an open lid **[1]**. Then, centrifuge them for 10 minutes **[2-TXT]** and transfer the supernatant into a fresh 1.5-milliliter tube **[3]**.

4.4.1. Talent putting the sample on ice and leaving it there.

4.4.2. Talent putting the sample in the centrifuge and closing the lid. **TEXT: 4 °C ; ≥ 18,000 x *g*.**

4.4.3. Talent transferring the supernatant to a fresh tube.

5. HPLC

5.1. Equip the fraction collector with 96 small scintillation vials **[1]** and fill each vial with 2 milliliters of a suitable scintillation cocktail that is compatible with low pH buffers and high ammonium phosphate concentrations **[2]**.

5.1.1. Talent putting the vials in the fraction collector.

5.1.2. Talent filling a few vials.

5.2. Start the HPLC system **[1]**, then activate the piston seal wash and keep it activated during the whole run **[2]**. Manually inject the sample with a suitable syringe **[3]**. Start the pumps and the gradient **[4].**

5.2.1. Talent starting the HPLC system.

5.2.2. Talent activating the piston seal.

5.2.3. Talent manually injecting the sample.

5.2.4. Added shot: Talent starting the pumps and the gradient

5.3. While the HPLC run is ongoing, check the pressure regularly **[1]**. The starting pressure should be around 18 to 24 bar and should slowly rise to 50 to 60 bar once 100% buffer B is reached **[2]**.

5.3.1. Talent checking the pressure.

5.3.2. Pressure rising.

5.4. After the run, close the vials tightly **[1]** and vigorous shake the fractions with the scintillation cocktail to mix **[2]**. If not proceeding directly with the measurement, keep the vials in an upright position in the dark **[3]**.

5.4.1. Talent closing the vials.

5.4.2. Talent shaking the fractions.

5.4.3. Talent putting the vials in a dark place for storage.

5.5. To measure the fractions, insert the vials into the scintillation counter racks, using racks that fit the small vials **[1]**, and measure each vial for 5 minutes in a liquid scintillation counter **[2]**.

5.5.1. Talent putting the vials in the rack.

5.5.2. Talent measuring a vial.

6. Data Analysis

6.1. Prepare a 2-D line chart where the measured counts per minutes, or cpm, are plotted against the retention time **[1]**. *Videographer: This step is important!*

6.1.1. Talent at the computer preparing the line chart and summing the cpm from the fractions.

6.2. To compare samples with each other, normalize the data by summing up the cpm from each eluted fraction from minute 25 to 96 for each individual sample and by dividing the total cpm from that sample by the total cpm of the other samples **[1]**. *Videographer: This step is important!*

6.2.1. Talent performing the normalization.

6.3. To perform relative quantifications of certain inositol polyphosphate peaks and to subsequently create bar graphs that contain data of replications for statistical analyses, continue the analysis with a specialized software that can calculate peak areas of chromatograms **[1]**.

6.3.1. Talent opening the specialized software to analyze the peaks.

Results

7. Results: InsP profiles of *A. thaliana* and *L. japonicus*

7.1. A complete inositol polyphosphate spectrum obtained from *A. thaliana* extracts after scintillation counting is shown here. The peaks are nicely separated and can be assigned to different isomers **[1]**. When an aged column is used, a clear reduction of inositol hexakisphosphate and the absence of inositol pyrophosphates is visible **[2-TXT]**.

7.1.1. LAB MEDIA: Figure 1 A – C.

7.1.2. LAB MEDIA: Figure 1 B and D. *Video Editor: Label B "***New Column***" and D "***Aged Column***".*

7.2. SAX-HPLC *(pronounce 'sax-H-P-L-C')* analysis was also performed on *L. japonicus* seedlings that were grown and labeled under the same conditions as the *Arabidopsis* seedlings **[1]**.

7.2.1. LAB MEDIA: Figure 2 A – C.

7.3. While presumably all inositol polyphosphate species and peaks that are known from *Arabidopsis* can be seen, there are differences in the relative amounts of specific inositol polyphosphate isomers between the 2 species **[1]**.

7.3.1. LAB MEDIA: Figure 2 D.

7.4. To demonstrate that samples do not have to be analyzed immediately after extraction, one sample was split in 2 and half of it was analyzed on the next day after storage at -80 degrees Celsius **[1]**.

7.4.1. LAB MEDIA: Figure 3 A – C.

7.5. The differences between SAX-HPLC profiles of the fresh and frozen samples were not significant, demonstrating that one freeze-thaw cycle does not harm the sample and that the method itself generates reproducible results **[1]**.

7.5.1. LAB MEDIA: Figure 3.

Conclusion

8. Conclusion Interview Statements

8.1. **Philipp Gaugler:** When attempting this protocol, always grind the samples thoroughly, frozen and with extraction buffer, and aim for a close to neutral pH after neutralization to ensure a maximum recovery of radiolabeled inositol phosphates for SAX-HPLC analysis.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.2, 4.1.4.*

8.2. **Philipp Gaugler:** It is possible to purify radiolabeled InsPs from a SAX-HPLC run for later use, for example in *in vitro* reactions, by collecting fractions without scintillation cocktail and measuring only small aliquots.

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