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Title: Nonradioactive Assay to Measure Polynucleotide Phosphorylation of Small Nucleotide Substrates

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NOTE: This is an APF

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? **Yes, just for the gel imaging step.**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps.

3. **Filming location:** Will the filming need to take place in multiple locations? **No, same building different floors.**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Robin Stanley:** This method can help answer key questions about the phosphorylation of the 5' end of DNA and RNA molecules by an enzyme known as poly-nucleotide kinase.

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

1.2. **Monica Pillon:** The main advantage to this technique is that it has the resolution to detect a very small change in a short DNA or RNA substrate.

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

Protocol

2. In Vitro RNA Kinase Reaction

2.1. Begin by preparing the RNA-enzyme kinase reactions **[1]**. For each reaction, combine 1 microliter of 500 nanomolar RNA substrate, 8.3 microliters of 130 nanomolar Las1-Grc3 *(pronounce 'LAS-1-G-R-C-3')*, and 0.2 microliters of 5 millimolar EDTA **[2]**.

2.1.1. WIDE: Establishing shot of talent at the lab bench preparing the reaction.

2.1.2. Talent combining reagents in one tube.

2.2. Set the heat block to 37 degrees Celsius **[1]**, then mix 0.5 microliters of an ATP substock from the concentration series with one RNA-enzyme mixture **[2]** and place the reaction on the heat block **[3]**. Continue mixing the reactions and placing them on the heat block at 10 second intervals **[4]**.

2.2.1. Talent setting the heat block to the appropriate temperature.

2.2.2. Talent mixing the reagents.

2.2.3. Talent placing the tube with the reaction on the heat block.

2.2.4. Talent placing another tube on the heat block, with a number of tubes already on the heat block.

2.3. After a 60-minute incubation on the heat block, quench each reaction by spiking it with 10 microliters of urea loading dye **[1]**. Immediately perform downstream analysis or store the reactions at -20 degrees Celsius to be analyzed at a later date **[2]**.

2.3.1. Talent adding loading dye to a reaction.

2.3.2. Talent putting the reaction tubes in the freezer and closing the door.

3. Gel Electrophoresis

3.1. To prepare a 15% denaturing acrylamide gel solution, combine 22.5 milliliters of premixed 40% 29 to 1 acrylamide-bis-acrylamide solution, 6 milliliters of 10 X TBE, 28.8 grams of urea, and RNase-free water to a total volume of 59 milliliters **[1-TXT]**, then gently stir the solution **[2]**.

3.1.1. Talent adding acrylamide, TBE, urea, and water to a 150mL glass beaker, with the acrylamide, TBE, and urea containers in the shot and labeled, if possible. **TEXT: CAUTION: Acrylamide is a neurotoxin!**

3.1.2. Talent stirring the solution.

3.2. Heat the solution in the microwave for 20 seconds **[1]**, stir it, and immediately return it to the microwave for another 20 seconds **[2]**. Gently stir the solution until the urea is completely dissolved **[3]**. *Videographer: This step is important!*

3.2.1. Talent starting the microwave with the beaker inside.

3.2.2. Talent taking the beaker out of the microwave, stirring it, and putting it back in.

3.2.3. Talent stirring the solution with the urea completely dissolved.

3.3. Place the glass beaker into a shallow water bath containing cold water for 5 minutes, making sure that the level of cold water surrounding the glass beaker is above the level of the solution inside the glass beaker **[1]**. *Videographer: This step is important!*

3.3.1. Talent placing the beaker in the water bath.

3.4. When the solution is cool, filter and degas it with a 0.22-micrometer disposable filtration unit to remove particulates and microscopic air bubbles **[1]**.

3.4.1. Talent filtering and degassing the solution.

3.5. Wash a short and long glass plate with soap and water **[1]**, then spray each plate with 95% ethanol and wipe the glass to remove any moisture **[2]**. Elevate the long plate off the benchtop by placing it on top of a box **[3]**, then position 0.4-millimeter spacers along the long edges of the plate **[4]**.

3.5.1. Talent washing the plates.

3.5.2. Talent spraying the plates with ethanol and wiping them.

3.5.3. Talent placing the long plate on top of a box.

3.5.4. Talent placing spacers on top of the plate.

3.6. Lay the short plate on top of the long plate, making sure that the edges of the short plate, long plate, and spacers are aligned **[1]**. Then, clamp each side with 3 evenly spaced metal clamps **[2]**. *Videographer: This step is important!*

3.6.1. Talent putting the short plate on top of the long one and making sure they are aligned.

3.6.2. Talent clamping the plates together.

3.7. Add 24 microliters of TEMED *(pronounce 'tee-med')* to the acrylamide solution and mix it **[1]**, then add 600 microliters of 10% APS **[2]** and immediately pour the solution between the glass plates **[3]**. *Videographer: This step is difficult and important!*

3.7.1. Talent adding TEMED to the acrylamide and mixing it, with the TEMED container in the shot.

3.7.2. Talent adding APS to the acrylamide and mixing it, with the APS container in the shot.

3.7.3. Talent pouring the solution between the plates while tapping the glass sandwich.

3.8. **Monica Pillon**: Pouring the acrylamide solution between the glass plates can be challenging. To avoid air bubbles, tap the glass plate sandwich as you pour the solution.

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

3.9. Carefully add a clean, 32-well comb to the top of the glass plate sandwich and allow the acrylamide to polymerize for 30 minutes **[1]**. To run the gel, set the heat block to 75 degrees Celsius **[2]**, remove the metal clamps **[3]**, and thoroughly wash and dry the glass plate sandwich **[4]**.

3.9.1. Talent putting a comb in the gel and leaving it to polymerize.

3.9.2. Talent setting the heat block to the appropriate temperature.

3.9.3. Talent removing the metal clamps.

3.9.4. Talent washing the plate sandwich.

3.10. Position the plate sandwich in the gel apparatus with the short plate facing forward **[1]** and prepare 0.5 X TBE running buffer by combining 100 milliliters of 10 X TBE with 1.9 liters RNase-free water **[2]**. Add 600 milliliters of the running buffer to the upper and lower chambers of the apparatus **[3]**.

3.10.1. Talent putting the gel in the apparatus.

3.10.2. Talent preparing the TBE running buffer, with the 10X TBE and water containers in the shot.

3.10.3. Talent pouring buffer into the gel apparatus.

3.11. Gently remove the comb from the gel and thoroughly rinse the wells with a syringe **[1]**. Pre-run the gel at 50 watts for 30 minutes **[2-TXT]**, then rinse the wells again **[3]**. Pulse spin the quenched reactions and incubate them at 75 degrees Celsius for 3 minutes **[4]**. *Videographer: This step is important!*

3.11.1. Talent removing the comb and rinsing the wells.

3.11.2. Talent programming the gel apparatus and starting the run. **TEXT: Caution: Gel runs at a high wattage!**

3.11.3. Talent rinsing the wells.

3.11.4. Talent pulse-spinning the reaction tubes and putting them in the heat block.

3.12. Repeat the pulse spin and immediately load 10 microliters of each sample onto the gel **[1]**, then run the gel for 3 hours at 50 watts **[2]**. When the run has finished, turn off the power supply **[3]** and drain the upper chamber of the apparatus **[4]**.

3.12.1. Talent loading sample into a few wells.

3.12.2. Talent starting the gel run.

3.12.3. Talent turning off the power supply.

3.12.4. Talent draining the upper chamber of the apparatus.

3.13. Wash and dry the outer side of the glass plate sandwich **[1]**, then cover it with foil and transfer it to a laser scanner for imaging **[2]**. Mount the glass plate sandwich onto the stage of a laser scanner **[3]**, set the excitation and emission wavelengths for the desired fluorophore, and image the gel **[4]**. *Videographer: This step is important!*

3.13.1. Talent washing the plate.

3.13.2. Talent covering the plate with foil.

3.13.3. Talent mounting the plate on the laser scanner.

3.13.4. SCREEN: Imaging settings adjusted and gel imaged. NOTE: Authors provided 2 screen shots for this one instead of a video

Results

4. Results: Quantification of RNA Phosphorylation

4.1. Shown here is a representative successful denaturing gel of a titration of ATP with a fixed amount of Las1-Grc3 *(pronounce 'LAS-1-G-R-C-3')* complex **[1]**. Addition of enzyme resulted in Las1-mediated RNA cleavage of the SC-ITS2 *(pronounce 'Saccharomyces cerevisae-I-T-S-2')* RNA substrate, leading to a defined RNA fragment **[2]**. Upon the addition of ATP, the C2 RNA fragment was phosphorylated by Grc3 PNK **[3]**.

4.1.1. LAB MEDIA: Figure 1.

4.1.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize the 5′-OH C2 RNA bands.*

4.1.3. LAB MEDIA: Figure 1. *Video Editor: Emphasize the 5′-P C2 RNA bands.*

4.2. To visualize the phosphorylation of the C2 RNA fragment, the relative amount of unphosphorylated and phosphorylated C2 RNA was plotted against the ATP concentration **[1]**.

4.2.1. LAB MEDIA: Figure 2. *Video Editor: Emphasize the grey line when VO says "unphosphorylated" and the brown line when VO says "phosphorylated".*

4.3. A representative unsuccessful denaturing gel is shown here **[1]**. The 21-nucleotide RNA substrate contained degradation products **[2]**, which overlapped with the phosphorylated product and made it impossible to accurately quantify phosphorylation **[3]**.

4.3.1. LAB MEDIA: Figure 3.

4.3.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize the first lane of the gel, marked with X.*

4.3.3. LAB MEDIA: Figure 3.

4.4. In contrast, the shortest RNA degradation product could be successfully analyzed because this area of the gel did not contain any additional RNA species that hindered accurate quantification of its phosphorylated counterpart **[1]**.

4.4.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the section of the gel that the grey arrows are pointing to.*

Conclusion

5. Conclusion Interview Statements

5.1. **Monica Pillon:** The most important thing to remember when attempting this procedure is that you must rinse the wells of the gel to ensure even loading of your sample.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.11.1.*

5.2. **Monica Pillon:** Following this procedure, an RNA turnover experiment could be performed to measure rates of RNA decay. Phosphorylation of RNA is often the signal to initiate decay of the RNA substrate.

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

5.3. **Robin Stanley:** This technique paves the way for asking detailed questions about the specificity, activity, and enzyme kinetics of a special class of enzymes called poly-nucleotide kinases.

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