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Title: Generation and Assembly of Virus-Specific Nucleocapsids of the Respiratory Syncytial Virus

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **49**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Dr. Bo Liang**: This method utilizes the virus' way to tackle the recombinant viral protein expression challenges: using one viral protein (P) as a chaperone for another viral protein (N) to obtain RNA-free N proteins **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera Note: Authors keep adding back "Dr"

REQUIRED:

1.2. **Dr. Bo Liang**: The RNA-free RSV N protein is obtained before assembling the virus-specific RNA into nucleocapsid in vitro. The method can also be used with other, non-segmented, negative-sense RNA viruses **[1]**.

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

OPTIONAL:

1.3. **Yunrong Gao**: The ligation independent cloning method is a quick technique for acquiring coexpression constructs, while negative stain electron microscopy is a quick method for checking large assemblies in vitro **[1]**.

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

1.4. **Yunrong Gao**: We want to give you two tips:
Chloe Von Hoffmann: First: Get high yield and solid results for each step before moving to the next step.
Claire Ogilvie: Second: Set up proper programs for chromatography and practice picking up grids with forceps. No notes from authors or videographer - I assume this was all included in one shot.

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

Protocol

2. Protein Expression and Purification

2.1. For bi-cistronic construction of the N and P coexpression, grow four, 1-liter cultures of *E. coli* BL21(DE3) (B-L-twenty-one-D-E-three) strain cells in LB (L-B) medium at 37 degrees Celsius **[1-TXT]**.

2.1.0 Added shot: Antibiotics being added to culture flasks Note: No narrative text was provided for this shot

2.1.1. WIDE: Talent adding cells to culture flask **TEXT: LB: Luria broth**

2.2. When the optical density at 600 nanometers reaches 0.6 **[1]**, lower the temperature to 16 degrees Celsius **[2]**. After 1 hour, induce protein expression by treatment with 0.5-millimolar IPTG (eye-P-T-G) overnight **[3-TXT]**.

2.2.0. Added shot: Sample being gathered Note: No narrative text was provided for this shot

2.2.1. Talent adding sample to the spectrophotometer

2.2.2. Talent lowering temperature

2.2.3. Talent adding IPTG to the flask, with IPTG container visible in frame as possible **TEXT: IPTG: isopropyl 1-thio-D-galactopyranoside**

2.3. The next morning, collect the cells by centrifugation **[1-TXT]** and resuspend the pellets in 200 milliliters of lysis buffer **[2-TXT]**.

2.3.1. Talent placing tube(s) into centrifuge **TEXT: 25 min 4140 x g, RT**

2.3.2. ~~Shot of pellet(s), then buffer being added to the tube, with buffer container visible in frame~~ B buffer being added **TEXT: See text for all buffer and solution preparation details**

2.4. Lyse the cells by sonication for 15 minutes with 3-seconds-on, 3-seconds-off pulses **[1]** and collect the lysates by centrifugation **[2-TXT]**.

2.4.1. Talent sonicating cells

2.4.2. Talent adding tube(s) to centrifuge **TEXT: 40 min, 37,888 x g, RT**

2.5. Load the supernatant into a 2.5- x 10-centimeter cobalt gravity column with approximately 10 milliliters of beads pre-equilibrated with 5-10 column volumes of lysis buffer **[1]** and wash the column with 5 column volumes of buffer B and 5 volumes of buffer C **[2]**.

2.5.1. Talent transferring supernatant to the column, with centrifuge tube(s) visible in frame
2.5.2. Talent adding buffer B to the column, with buffer B and C containers visible in frame

2.6. Use 2 volumes of buffer D to elute the protein from the beads **[1]** and equilibrate the Q column with five, 1-milliliter volumes of ~~dilute the eluted protein 5 times with~~ QA (Q-A) buffer ~~for the Q column~~ **[2]**. Note: 2.6.2. and 2.7.1. merged

2.6.1. Talent adding buffer to column, with buffer D container visible in frame
~~2.6.2. Talent adding buffer to column, with buffer QA container visible in frame~~

2.7. ~~Equilibrate the Q column with 5 milliliters of QA buffer **[1]** and~~ use a peristaltic pump to load the diluted sample onto the column **[2]**. Note: 2.6.2. and 2.7.1. merged

2.7.1. Talent adding buffer to the column, with buffer container visible in frame
2.7.2. Talent pumping sample onto the column

2.8. Mount the loaded Q column onto the HPLC (H-P-L-C) machine **[1-TXT]** along with fresh QA and QB buffers **[2]**. ~~After the wash, set the flow rate to 1 milliliter/minute **[3]**~~.

2.8.1. Talent loading column onto the machine *Videographer: Important step* **TEXT: HPLC: high pressure liquid chromatography**
2.8.2. Talent loading buffer(s) *Videographer: Important step*
~~2.8.3. Talent setting flow rate~~

2.9. Run the pump wash to successfully wash the machine with 1-2 volumes of QB and QA buffers ~~at a 3 milliliter/minute flow rate~~ **[1]**.

2.9.1. ~~Talent running wash~~ SCREEN: 2.9.1: 00:02-02:30 *Video Editor: please speed up*

2.10. After the last wash, set the flow rate to 1 milliliter/minute [0] and set UV1 to 280 nanometers and UV2 to 260 nanometers **[1]**, ~~and~~ using a 96 deep-well plate to collect the fractions **[2]**.

2.10.0 Added shot: SCREEN: 2.10.1_t2: 00:02-00:10

2.10.1. ~~Talent setting UV parameters~~ SCREEN: 2.10.1_t2: 00:12-00:33 *Video Editor: please speed up*
2.10.2. Talent placing collection plate *Videographer: Important step*

2.11. Then use a stepwise gradient application of 3-4 volumes of each concentration of elution agent to elute the proteins, starting at a 0% QB concentration and increasing the percentage by 5% each time **[1]**. The N^0P (N-zero-P) protein complex will elute at the 15% QB Buffer concentration **[2]**.

2.11.1. ~~Talent setting gradients/concentration~~ SCREEN: 2.11.1: 00:07-00:28 *Video Editor: please speed up*
2.11.2. ~~Protein being eluted~~ SCREEN: 2.11.1: 11:22-11:32

2.12. When all of the protein has been eluted, wash the column with 2 volumes of 100% QB buffer **[1]** and isolate the protein by gel filtration in a 1- x 30-centimeter, small scale purification column equilibrated with buffer E **[2]**.

2.12.1. Talent adding buffer to column, with buffer container visible in frame
2.12.2. Talent adding sample to superdex column Note: Use 2.13.1.

2.13. Then analyze the protein-containing fractions by SDS-PAGE (S-D-S page) **[1-TXT]**.

2.13.1. ~~Talent adding sample~~
2.13.2. Added shot: Talent loading sample onto gel Note: Use for 2.13.1. (multiple takes provided) **TEXT: SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis**

3. In Vitro Virus-Specific Nucleocapsid (NC) Assembly

3.1. For virus-specific nucleocapsid assembly, mix and incubate the purified N^0P complex with RNA oligo at a 1:1.5 molecular ratio at room temperature for 1 hour **[1]**.

3.1.1. WIDE: Talent mixing complex, with protein and oligo containers visible in frame

3.2. Pre-equilibrate a small-scale purification gel filtration column with buffer E **[1]** and remove any precipitation by centrifugation **[2-TXT]**.

3.2.1. Talent adding buffer to column, with buffer container visible in frame Note: Use 2.12.1.
3.2.2. Talent adding sample to centrifuge **TEXT: 15 min, 21,310 x g, RT**

3.3. Load the supernatant to the size exclusion chromatography column **[1]** and compare the size exclusion chromatography images of the protein:RNA assembly and protein

alone control samples, combining the nucleic acid purity ratios to identify which peaks are the assembled N-RNA, N^0P, and free RNA **[2]**.

3.3.1. Talent loading supernatant onto column Note: Use 2.12.2. – not sure if there is a shot labeled 2.12.2. or if the videographer means use the shot FOR 2.12.2. (2.13.1.)
3.3.2. Talent at computer, comparing ratios OR LAB MEDIA: Figure 4B *Video editor: please add/emphasize peak labeling texts*

3.4. To assemble an N-RNA complex, collect all of the N-RNA peak fractions **[1]**, perform an RNA extraction **[2]**, and run a urea-PAGE gel to double-check the length of the specific RNA **[3]**.

3.4.1. Talent collecting fraction
3.4.2. Talent opening RNA extraction kit
3.4.3. Talent adding sample to gel

4. Negative Stain Grid Preparation

4.1. To prepare a negative stain grid for negative stain electron microscopy, use a Tungsten heater to heat 5 milliliters of double distilled water to boiling **[1]** and add 37.5 milligrams of uranyl formate to the water to obtain a 0.75% uranyl formate staining solution **[2]**.

4.1.1. WIDE: Talent heating water
4.1.2. Talent adding uranyl formate to tube, with urany formate container visible in frame

4.2. Transfer the solution to an aluminum foil covered beaker **[1]** and add 4 microliters of 10-molar sodium hydroxide **[2]**.

4.2.1A Added shot: Talent measuring solution Note: No narrative text provided for this shot
4.2.1B Talent adding solution to beaker/covering beaker with foil
4.2.1. Talent adding NaOH to beaker, with NAOH container visible in frame

4.3. After 15 minutes of stirring protected from light, filter the solution through a 0.22-micron filter into a test tube **[1]**.

4.3.1. Talent filtering solution

4.4. To make the continuous carbon-coated electron microscopy grids hydrophilic, place the grids in a chamber connected to a power supply **[1]** and apply negatively charged ions to the grids **[2]**.

4.4.1. Talent placing grids into chamber *Videographer: Important step*
4.4.2. Talent applying ions *Videographer: Important step* Note: You want to see purple (I assume use part of shot when grid shows purple)

4.5. Cut and fold a 2- x 2-inch parafilm strip **[1]**. Add two, 40-microliter drops of distilled water to one end of the strip **[1]** and two, 40-microliter drops of 0.75% uranyl formate staining solution to the other end **[3]**. 4.5.1-.4.5.3 are merged

4.5.1. Talent cutting strip
4.5.2. Talent adding buffer droplets to strip *Videographer: Important step*
4.5.3. Talent adding stain to strip *Videographer: Important step*

4.6. Add 3 microliters of protein sample to each grid **[1]**. After 1 minute, blot the grids against blotting paper **[2]** and dip the grids two times in the distilled water droplets **[3-TXT]**. 4.6.3.-4.7.2. are merged

4.6.1. Talent adding sample to grid *Videographer: Important step*
4.6.2. Talent blotting grid *Videographer: Important step*
4.6.3. Talent adding buffer or solution to grid, with buffer and solution container visible in frame *Videographer: Important step* **TEXT: Blot between each immersion**

4.7. Then immerse the grid in each staining solution droplet for 30 seconds **[1]** before blotting the grids against blotting paper to remove any excess stain solution **[2]** and allowing the grids to air dry before imaging **[3-TXT]**. 4.6.3.-4.7.2. are merged

4.7.1. Grid being immersed in stain
4.7.2. Grid being blotted
4.7.3. Talent placing grid to dry **TEXT: Optional: Store grid in grid box before imaging**
4.7.4. Added shot: Grid being placed into box Note: No narrative text provided for this shot

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
2.8., ~~2.10., 2.11.,~~ 4.4.-4.6.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
Making the construct with high yield; expression of N^0P very low post-purification

Results

5. Results: Representative $N_{1-391}P_{1-126}$ Complex Copurification

5.1. Using this protocol, a large-scale soluble heterodimeric respiratory syncytial virus N^0P complex can be obtained **[1]**.

5.1.1. LAB MEDIA: Figure 4A *Video Editor: please emphasize data lines*

5.2. In *E. coli*, the full length of N and N terminal portions of the P proteins **[1]** are co-expressed with a 10X His-Tag (hiss-tag) on the N protein **[2]**.

5.2.1. LAB MEDIA: Figure 4C
5.2.2. LAB MEDIA: Figure 4C *Video Editor: please emphasize wide N1-391 and P1-126 bands in N1-391P1-126 lane*

5.3. Based on the UV absorbance nucleic acid purity ratio **[1]**, N^0P contained both the full-length N and N terminal P **[2]** but did not contain cellular RNA **[3]**.

5.3.1. LAB MEDIA: Figure 4B
5.3.2. LAB MEDIA: Figure 4B *Video Editor: please add/emphasize N1-391P1-126 text and arrow*
5.3.3. LAB MEDIA: Figure 4B *Video Editor: please add/emphasize N1-391-RNA text and arrow*

5.4. The purified N^0P could then be stimulated and assembled into nucleocapsid-like particles on electron microscopy grids via incubation with specific RNA oligos as demonstrated **[1]**.

5.4.1. LAB MEDIA: Figure 5

Conclusion

6. Conclusion Interview Statements

6.1. **Claire Ogilvie**: When purifying the protein, avoid air bubbles during the column purification and dilute the sample with QA buffer to adjust the pH and salt concentration before loading the sample onto the column **[1]**.

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

6.2. **Chloe Von Hoffmann**: Take care to hold the electron microscopy grids at the outer edge to avoid contaminating the grids **[1]**.

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

6.3. **Yunrong Gao**: This will help us determine the long puzzled RSV genome packing question: whether it is a left-handed or right-handed helical structure **[1]**.

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

6.4. **Dr. Bo Liang**: Following those procedures, an authentic N-RNA template for the RSV polymerase activity assay can be performed, and the RSV viral-specific nucleocapsid can be used for the high-resolution cryo-EM analysis **[1]**.

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