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TITLE:

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

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Generation, assembly, respiratory syncytial virus (RSV), virus-specific, RNA, nucleocapsid (NC), chaperone, ribonucleoprotein

SUMMARY:

For in-depth mechanistic analysis of the respiratory syncytial virus (RSV) RNA synthesis, we report a protocol of utilizing the chaperone phosphoprotein (P) for coexpression of the RNA-free nucleoprotein (N^0) for subsequent *in vitro* assembly of the virus-specific nucleocapsids (NCs).

ABSTRACT:

The use of an authentic RNA template is critical to advance the fundamental knowledge of viral RNA synthesis that can guide both mechanistic discovery and assay development in virology. The RNA template of nonsegmented negative-sense (NNS) RNA viruses, such as the respiratory syncytial virus (RSV), is not an RNA molecule alone but rather a nucleoprotein (N) encapsidated ribonucleoprotein complex. Despite the importance of the authentic RNA template, the generation and assembly of such a ribonucleoprotein complex remain sophisticated and require in-depth elucidation. The main challenge is that the overexpressed RSV N binds non-specifically to cellular RNAs to form random nucleocapsid-like particles (NCLPs). Here, we established a protocol to obtain RNA-free N (N^0) first by co-expressing N with a chaperone phosphoprotein (P), then assembling N^0 with RNA oligos with the RSV-specific RNA sequence to obtain virus-specific nucleocapsids (NCs). This protocol shows how to overcome the difficulty in the preparation of this traditionally challenging viral ribonucleoprotein complex.

INTRODUCTION:

Nonsegmented negative-sense (NNS) RNA viruses include many significant human pathogens, such as rabies, Ebola, and respiratory syncytial virus (RSV)[1,2]. RSV is the leading cause of

respiratory illness such as bronchiolitis and pneumonia in young children and older adults worldwide[3]. Currently, no effective vaccines or antiviral therapies are available to prevent or treat RSV[4]. As part of the life cycle, the RSV genome serves as the template for replication by the RSV RNA dependent RNA polymerase to produce an antigenome, which in turn acts as the template to generate a progeny genome. Both genome and antigenome RNAs are entirely encapsidated by the nucleoprotein (N) to form the nucleocapsids (NCs)[3]. Because the NCs serve as the templates for both replication and transcription by the RSV polymerase, proper NC assembly is crucial for the polymerase to gain access to the templates for RNA synthesis[5]. Interestingly, based on the structural analyses of the NNS viral polymerases, it is hypothesized that several N proteins transiently dissociate from the NCs to allow the access of the polymerase and rebind to RNA after the RNA synthesis[6-12].

Currently, the RSV RNA polymerization assay has been established using purified RSV polymerase on short naked RNA templates[13,14]. However, the activities of the RSV polymerase do not reach optimal, as observed in the non-processive and abortive products generated by the RSV polymerase when using naked RNA templates. The lack of NC with virus-specific RNA is a primary barrier for the further mechanistic understanding of the RSV RNA synthesis. Therefore, using an authentic RNA template becomes a critical need to advance the fundamental knowledge of RSV RNA synthesis. The known structures of the nucleocapsid-like particles (NCLPs) from RSV and other NNS RNA viruses reveal that the RNAs in the NCLPs are either random cellular RNAs or average viral genomic RNAs[15-19]. Together, the main hurdle is that N binds non-specifically to cellular RNAs to form NCLPs when N is overexpressed in the host cells.

To overcome this hurdle, we established a protocol to obtain RNA-free (N^0) first and assemble N^0 with authentic viral genomic RNA into NCLPs[20]. The principle of this protocol is to obtain a large quantity of recombinant RNA-free N (N^0) by co-expressing N with a chaperone, the N-terminal domain of RSV phosphoprotein (P_{NTD}). The purified N^0P could be stimulated and assembled into NCLPs by adding RSV-specific RNA oligos, and during the assembly process, the chaperone P_{NTD} is displaced upon the addition of RNA oligos.

Here, we detail a protocol for the generation and assembly of RSV RNA-specific NCs. In this protocol, we describe the molecular cloning, protein preparation, *in vitro* assembly, and validation of the complex assembly. We highlight the cloning strategy to generate bi-cistronic constructs for protein coexpression for molecular cloning. For protein preparation, we describe the procedures of cell culture, protein extraction, and the purification of the protein complex. Then we discuss the method for *in vitro* assembly of the RSV RNA-specific NCs. Finally, we use size exclusion chromatography (SEC) and negative stain electron microscopy (EM) to characterize and visualize the assembled NCLPs.

PROTOCOL:

1. Molecular cloning

NOTE: Ligase Independent Cloning (LIC) was used to make an RSV bi-cistronic coexpression

construct plasmid. LIC is a method developed in the early 1990s, which uses the 3'-5' Exo activity of the T4 DNA polymerase to create overhangs with complementarity between the vector and the DNA insert[21,22]. The constructs were made using the 2BT-10 vector DNA, which consists of a 10x His tag at the N-terminal of the Open Reading Frame (ORF) (**Figure 1**).

1.1. Perform linearization of LIC vectors using SSPI digestion.

1.1.1. Combine 10 µL of SSPI 10X buffer, 4 µL of SSPI enzyme at a concentration of 5 U/µL, the equivalent volume of 5 µg of vector miniprep DNA, and sterile ddH_2O to 100 µL.

1.1.2. Incubate the digest for 3 hours at 37 °C.

1.1.3. Run the digest on a 1.0% agarose gel for the extraction of vector DNA.

1.1.4. Use a gel extraction kit to perform extraction and purification. Suspend the final volume of vector DNA in 30 µL of ddH_2O and store it at -20 °C.

1.2. Prepare the DNA inserts for N_{1-391} and P_{1-126} using the N_{1-391} Forward Primer, N_{1-391} Reverse Primer, P_{1-126} Forward Primer, and P_{1-126} Reverse Primer (**Table 1**).

NOTE: There is sufficient overlap with the linearized vector to ensure a melting temperature of between 55 °C and 60 °C. For the reverse primer, there is sufficient overlap with the reverse complementary strand of the linearized vector for the same reason.

1.2.1. Perform PCR amplification of the DNA insert using the conditions in **Table 2** and **Table 3**.

1.2.2. Extract the amplified DNA insert. Run the PCR products from the previous step on a 1.0% agarose gel, then extract and purify the bands by gel extraction. Suspend the final volume of extracted DNA in 15 µL of ddH_2O.

1.3. Perfrom T4 DNA polymerase treatment of vector and insert DNA (**Table 4**).

NOTE: Treatment must be performed separately for the vector DNA and the insert DNA.

1.3.1. Incubate the mixture for 40 minutes at room temperature. Then, heat-inactivate the polymerase at 75 °C for 20 minutes. Store the reaction mixture at -20 °C.

1.4. Anneal the LIC vector and the insert vector.

1.4.1. Set up a negative control with 2 µL of LIC vector DNA and 2 µL of sterile ddH_2O.

1.4.2. Combine 2 µL of insert DNA and 2 µL of LIC vector DNA from the previous T4 DNA polymerase reactions in a 0.2 mL tube.

1.4.3. Perform the annealing reaction at room temperature for 10 minutes.

1.4.4. Quench the reaction with 1.3 µL of EDTA at a concentration of 25 mM.

1.4.5. Transform the reaction into 100 µL of *E. coli* Top10 competent cells and plate them on an ampicillin selection plate[23,24].

1.5. Identify the positive constructs.

1.5.1. Prepare the plasmid miniprep solution. This can be done through colony picking and inoculation in LB media. Usually, 3 colonies are sufficient.

1.5.2. Incubate the mixture overnight at 37 °C.

1.5.3. Centrifuge the mixture at 4,560 x g for 10 minutes and discard the supernatant.

1.5.4. Resuspend the pellets in 250 µL of P1 buffer and prepare plasmid minipreps using a spin miniprep kit.

1.5.5. Conduct a digestion analysis of the miniprep product using AseI or other restriction enzymes.

1.5.6. Load samples on 0.8% agarose gel and run the digested plasmid. Analyze the gel under a UV lamp.

1.5.7. Use a sequencing service to validate the sequence of the positive product.

1.6. Obtain the coexpression DNA insert.

1.6.1. Perform PCR to obtain $N_{1\text{-}391}$ and $P_{1\text{-}126}$ using the previously constructed 2BT-10 $N_{1\text{-}391}$ and 2BT-10 $P_{1\text{-}126}$ as templates.

1.6.2. Perform the 1st PCR to obtain $N_{1\text{-}391}$ from the 2BT-10 $N_{1\text{-}391}$ construct using the PCR conditions in **Table 2** and **Table 3** with the $N_{1\text{-}391}$ Forward primer and Reverse Primer 5′-GTGAAGATCCTGGCTGATGCAATGCGGCGGCGCGCCGCGATCGCGGATCC-3′.

1.6.3. Perform the 2nd PCR to obtain $P_{1\text{-}126}$ from the 2BT-10 $P_{1\text{-}126}$ construct using the Forward primer: 5'-CCGCCGCATTGCATCAGCCAGGATCTTCACTGCAGGACTCGAGTTCTAGA-3′ and the $P_{1\text{-}126}$ Reverse primer (use the PCR conditions in **Table 2** and **Table 3**).

1.6.4. Finally, perfrom overlap PCR on the mixed products of the previous 2 PCR reactions to merge $N_{1\text{-}391}$ and $P_{1\text{-}126}$. Use the $N_{1\text{-}391}$ Forward primer and $P_{1\text{-}126}$ Reverse primer. Use the PCR conditions in **Table 5** and **Table 6**.

1.7. Join the vector and the DNA insert.

1.7.1. Treat the overlap PCR product with T4 DNA polymerase following the protocol in step 1.3.

1.7.2. Anneal the LIC vector and PCR product dollowing the protocol in step 1.4.

1.7.3. Identify the positive constructs following the protocol in step 1.5.

2. Protein expression and purification

NOTE: Use *E. coli* for the bi-cistronic construct of the coexpression of both N and P. Culture the cells at 37 °C, but carry out the expression at a reduced temperature (16 °C) overnight. Purify the protein complexes through a combination of cobalt column, ion exchange, and size exclusion chromatography (**Figure 2**).

2.1. Use the *E. coli* BL21(DE3) strain for protein production. Grow 4 L cell cultures at 37 °C in LB (Luria Broth) medium until OD_{600} reaches 0.6.

2.2. Lower the temperature to 16 °C. An hour later, induce the expression with 0.5 mM isopropyl 1-thio--D-galactopyranoside (IPTG) overnight.

2.3. Centrifuge the cells at 4,104 x g for 25 min and then discard the supernatant.

2.4. Resuspend the cell pellets in 200 mL of lysis buffer A (50 mM sodium phosphate, pH 7.4, 500 mM NaCl, 5 mM imidazole, 10% glycerol, and 0.2% NP40). Use 50 mL of lysis buffer to resuspend the cell pellets form 1 L of cell culture.

2.5. Lyse the cells by sonication for 15 min, 3 seconds on, and 3 seconds off. Then centrifuge cells at 37,888 x g for 40 min.

NOTE: The protocol can be paused by freezing the cells before sonication in a -80 °C freezer.

2.6. Load the supernatant into a cobalt gravity column (diameter x length: 2.5 cm x 10 cm) with ~10mL of beads pre-equilibrated with 5-10 column volumes (CV) of lysis buffer.

2.7. Wash the column with 5 CV of buffer B (50 mM Tris-HCl pH 7.4, 1 M NaCl, 10% glycerol, and 5 mM imidazole) and 5 CV of buffer C (50 mM Tris-HCl pH 7.4, 500 mM NaCl, 10% glycerol, and 5 mM imidazole).

2.8. Elute the protein from the beads using 2 CV of buffer D (50 mM Tris-HCl pH 7.4, 500 mM NaCl, and 250 mM imidazole).

2.9. Dilute the eluted protein 5x with QA buffer (50 mM Tris-HCl pH 8.0, 5% Glycerol) for the Q column.

2.10. Wash the 5 mL of Q column with QA buffer to equilibrate the column, then load the diluted sample into the Q column using the peristaltic pump (e.g., Rabbit).

2.11. Load the Q column into the HPLC machine along with QA buffer and QB buffer(50 mM Tris-HCl pH7.4, 1.5 M NaCl, 5% glycerol). Set up the flow rate as 1 mL/min.

2.12. Run the program (pump wash), first washing the machine with QB buffer then with QA using 1-2 CV of each. Set the system flow to 3 mL/min.

2.13. Set the UV1 to 280 nm and UV2 to 260 nm. Use a 96 deep-well plate to collect the fractions.

2.14. Elute proteins using a stepwise gradient of elution agent (QB Buffer) applying 3-4 CV of each concentration, increasing the percentage by 5% each time starting at 0% QB. N^0P protein complex will come out at 15% QB Buffer.

2.15. Once all of the protein is eluted, wash the column with 100% QB Buffer (2 CV).

2.16. Isolate the protein by gel filtration Superdex 200 Increase 10/300 GL column (diameter x length: 1.0 cm x 30 cm) and equilibrate with buffer E (20 mM HEPES pH 7.4 and 200 mM NaCl).

2.17. Analyze protein-containing fractions by SDS-PAGE.

3. *In vitro* assembly of the virus-specific NC

NOTE: The *in vitro* assembly of the RSV-specific NC (N:RNA) was performed by incubating the prepared N^0P complex with RNA oligos. Then, SEC chromatography was used to separate the assembly complex from the N^0P and excess RNA (**Figure 2**).

3.1. Mix and incubate the purified N^0P complex with RNA oligo with the molecular ratio of 1:1.5 at room temperature for 1 hour, usually 1 mL of the protein N^0P with the concentration of 1 mg/mL is enough for the next step. Set up the control sample, which only contains the same amount of N^0P protein.

3.2. Pre-equilibrate the gel filtration Superdex 200 Increase 10/300 GL column with the buffer E (20 mM HEPES, pH 7.4, 200 mM NaCl).

3.3. Centrifuge the sample with 21,130 *x g* for 15 min, remove any precipitation and load the supernatant to the SEC column.

3.4. Compare the SEC chromatography images of N:RNA assembly sample and N^0P control sample, combine the A_{260}/A_{280} ratio to identify which peaks are the assembled N-RNA, N^0P, and free RNA.

3.5. Collect the peak fractions, run the SDS-PAGE gel, or make grids.

3.6. For the assembly N-RNA complex, collect all the fractions of N-RNA peak, do the RNA extraction and run the Urea-PAGE gel to double-check the length of specific RNA, which is the same as mixed and incubated at the first step.

4. Making negative stain grids

NOTE: Negative stain electron microscopy (EM) is a method in which the molecules are adsorbed to a carbon film and then embedded in a layer of heavy metal atoms. Negative stain EM produces a high image contrast, making it easy to see and computationally align the particles. Another advantage is that adsorption of the particles to a carbon film usually induces the molecules to adhere to the grid with few preferred orientations. When the molecules are in a similar orientation, it is easy to separate them into structurally distinct classes. Negative stain EM is thus the appropriate technique to guide sample preparation[25,26] (**Figure 3**).

4.1. Microwave or heat 5 mL of ddH_2O in a glass tube using a Tungsten heater until it is boiling.

4.2. Weigh 37.5 mg of uranyl formate and add to 5 mL of heated ddH_2O to make a 0.75% uranyl formate staining solution. Stir under an aluminum foil covered beaker to protect from light.

4.3. Add 4 μL of 10 M NaOH to the staining solution and continue to stir for 15 min, protected from the light.

4.4. Filter the solution through a 0.22 μm filter into a test tube.

4.5. Use glow discharge to make the continuous carbon-coated EM grids hydrophilic[27].

NOTE: The grids are placed inside a chamber connected to a power supply. When high voltage is applied, the gas within the chamber ionizes, and the negatively charged ions deposit on the carbon grids to make them hydrophilic.

4.6. Cut and fold a parafilm strip. Pipet 2 drops of 40 μL of the buffer on one side of the parafilm, and pipet another 2 drops of 40 μL of staining solution to the other side.

4.7. To make the EM carbon grids, apply 3 μL of protein sample for 1 minute.

4.8. Blot the grids against a blotting paper.

NOTE: The grids are washed 2x with buffer, blotted, and washed 1x with the 0.75% uranyl formate staining solution.

4.9. Hold the grid surface in the 0.75% uranyl formate staining solution for 30 seconds.

4.10. Blot the grid against a blotting paper to remove excess stain solution and allow the grid to air-dry.

4.11. Store the grids in the grid box before imaging.

REPRESENTATIVE RESULTS:

Purification of RNA-free N^0P protein

With this protocol, a large-scale soluble heterodimeric RSV N^0P complex can be obtained. The full length of N and N terminal part of P proteins were co-expressed with 10X His-Tag on the N protein in *E. coli*. N^0P was purified using a cobalt column, ion exchange, and size exclusion chromatography. N^0P contains both the full-length N and N terminal P but did not contain cellular RNA based on the UV absorbance A_{260}/A_{280} ratio[20] (**Figure 4**).

Assembly of N-RNA and checking with negative stain

We then demonstrated that the purified N^0P could be stimulated and assembled into Nucelocapsid-like particles (NCLPs) by incubating with specific RNA oligos. The NCLPs were assembled by incubating the N^0P with RNA oligos with the ratio of 1:1.5 at room temperature for 1 hour and then run through the gel filtration column. When the N:RNA complex forms, it shows three peaks: the 1st peak is N:RNA, the 2nd peak is N^0P, and the 3rd peak is excess free RNA. The highest fraction of the N:RNA peak creates the negative stain grids for checking with EM[20] (**Figure 5**).

FIGURE AND TABLE LEGENDS:

Figure 1. The illustration of the plasmid constructions. A. The construct of the RSV $N_{1\text{-}391}$; B. The construct of the RSV $P_{1\text{-}126}$; C. The bi-cistronic construct for the coexpression of $N_{1\text{-}391}$ and $P_{1\text{-}126}$. The first gene RSV $N_{1\text{-}391}$, the second gene RSV $P_{1\text{-}126}$, the antibiotic-resistant gene (AmpR), and the promoters are highlighted in yellow, cyan, pink, and orange boxes, respectively. In summary, the gene inserts of the RSV $N_{1\text{-}391}$ and the RSV $P_{1\text{-}126}$ are constructed separately and assembled.

Figure 2. The flowchart of the purification of the protein complex $N_{1\text{-}391}P_{1\text{-}126}$. It outlines the inoculation and large scale grow-ups of the *E. coli* cell culture and harvesting the cell by centrifugation. Followed by cell lysis, the protein samples are purified by the affinity chromatography (i.e., Co2+ column), ion-exchange chromatography (i.e., Q column), and gel filtration size exclusion (SEC) chromatography. The protein samples are further analyzed by the SDS-PAGE gel.

Figure 3. Preparation of negative stain EM grids for imaging. A. Glow discharges the grids. B. The

procedure for negative staining grids is shown. The tweezers are used to pick up a grid, followed by applying the protein sample for 1 minute. The grids are blotted with blotting paper. The grid is washed twice with buffer and twice with the 0.75% uranyl formate staining solution. The grids are held in the second staining solution drop for 30 seconds. The grids are blotted after each wash and air-dried after the final blotting. C. The grids are stored in the grid box for imaging.

Figure 4. Representative results of the copurification of the $N_{1\text{-}391}P_{1\text{-}126}$ complex. A. The SEC profile of $N_{1\text{-}391}P_{1\text{-}126}$. B. The SEC profile of the assembly $N_{1\text{-}391}P_{1\text{-}126}$ with RNA. C. The SDS-PAGE gel shows the N protein only for the N-RNA complex and the bands for both N and P proteins of the $N_{1\text{-}391}P_{1\text{-}126}$ complex.

Figure 5. Representative images of N-RNA. A and B are representative negative stain EM images of N-RNA from the $N_{1\text{-}391}$-RNA peak in Figure 4. The N-RNA complexes are stained using the procedure described in Figure 3.

Table 1. Primer sequences.

Table 2. PCR amplification of DNA insert reagents.

Table 3. PCR amplification of DNA insert thermocycling program.

Table 4. T4 DNA polymerase treatment.

Table 5. Overlap PCR reagents.

Table 6. Overlap PCR thermocycling program.

DISCUSSION:

The known nucleocapsid-like particle (NCLP) structures of the nonsegmented negative-sense (NNS) RNA viruses show that the assembled NCLPs are the complex N with host cellular RNAs when overexpressed in bacterial or eukaryotic expression systems[15-19]. Previous studies have attempted to get the RNA free N with a variety of methods, such as the RNase A digestion, high salt washing, or adjusting different pH buffers to remove the nonspecific cellular RNAs[28,29]. However, none of the above methods can be successfully used for the assembly of the virus-specific NCs. To obtain the RNA-free RSV N, we also tried a combination of methods, including RNase A digestion, high salt (1.5M NaCl) wash, adjusting the buffer pH from pH 5.0 to pH 9.0, protein denaturation and renaturation. After many failed trials, we still could not get RNA-free N with the above methods. We will briefly discuss the attempts and potential reasons.

One method to obtain RNA-free N is to digest host cellular RNA in assembled NCLPs with RNase A. In VSV, the incubation of the purified NCLP with RNase A at a final concentration of 1mg/ml at 37 °C for 1 h completely removed RNA from the NC[28]. Purified empty oligomeric N was then incubated with poly-A (250-nt or longer) in a molar ratio of 1:5 in the presence of RNase inhibitors. Analysis of the RNA isolated from reconstituted N:poly-A showed that the RNA was

approximately 90 nt in length. This suggested that the RNA outside the nucleoprotein is susceptible to nonspecific digestion by the contaminated RNase A from the previous step. The strategy of RNase A digestion to remove RNA was not successful when applied to RSV. This may be due to two reasons. First, contaminated RNase A will digest the RSV-specific RNA, which will subsequently be incubated and assembled with N. Second, the efficiency of the RNase A digestion is much lower in RSV. This is because the RNAs assemble differently in different NNS viruses. The known crystal structures of N:RNA show that RNA binds outside of the NC in VSV, but inside of the NC in RSV[30,31]. The configuration of RNA binding inwards of the NC may lead to low efficiency for RNase A to access and digest.

Another method to get RNA-free N is to make the truncations that cut both the N-terminal motif (N-arm) and the C-terminal motif (C-arm) of N. However, this truncated RNA-free N cannot be used to assemble with RNA into a stable NC because the N-arm of N is folded into its neighboring subunits by interacting with the C-terminal domain (CTD) of the precedent N subunit. The extended C-arm is positioned to the CTD of the next N subunit[31].

An additional method to get RNA-free N is to prepare mutant N. For example, the result obtained by Galloux et al. showed that RSV RNA free N^0P complex could be obtained by coexpression of a K170A/R185A double N mutant with the N-terminus of P in bacteria[32]. However, it has two potential issues for further structural characterizations. One issue is the low stability of this mutant complex at high concentrations. The other issue is that the mutant complex lost the RNA binding ability, which cannot be used in the next step of assembly.

Despite the tremendous challenges, we have established and optimized the protocol to obtain virus-specific NCs using the coexpression of N with a chaperone P. Recently, another successful method is to make a chimeric fusion construction encoding for $P_{1\text{-}50}$-TEV-$N_{1\text{-}405}$-8xHis for MeV[33,34]. N^0P can be obtained after the TEV protease cleavage. The purity of N^0P depends on the efficiency of TEV cleavages, which cut the chimeric fusion between N and P protein.

Instead of using the chimeric fusion method, we designed a bi-cistronic coexpression construct. Specifically, the coexpression constructs of the N^0P complex have been designed and engineered in two open reading frames, comprising the full length of N (1-391) with a 10x His-tag at N-terminal in the first ORF, and the N-terminal peptides (1-126) from P in the second ORF[20]. Briefly, the overall procedure of the N^0P purification is to purify His-tagged N^0P and N-RNA from cellular lysis samples with cobalt beads, remove the nonspecific RNA and N:cellular RNA complex with Q column, and get a pure N^0P complex with the SEC column. In the SEC step, the ratio of A_{260}/A_{280} can be monitored and double-checked with RNA extraction from the N^0P peak fractions.

Collectively, in this protocol, the most critical steps are the strategy to design the construction of the coexpression of N^0P complex and using a series affinity and ion-exchange column to separate the RNA free N^0P complex from the other N-cellular RNA complexes. The efficiency of the coexpression strategy to get N^0P complex is relatively low; around 50% N protein is still N-cellular RNA complex. The protocol may also be applied for getting RNA free N^0P and assembly with specific RNA with N to get N:RNA complex of other NNS viruses.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1.tif

Background

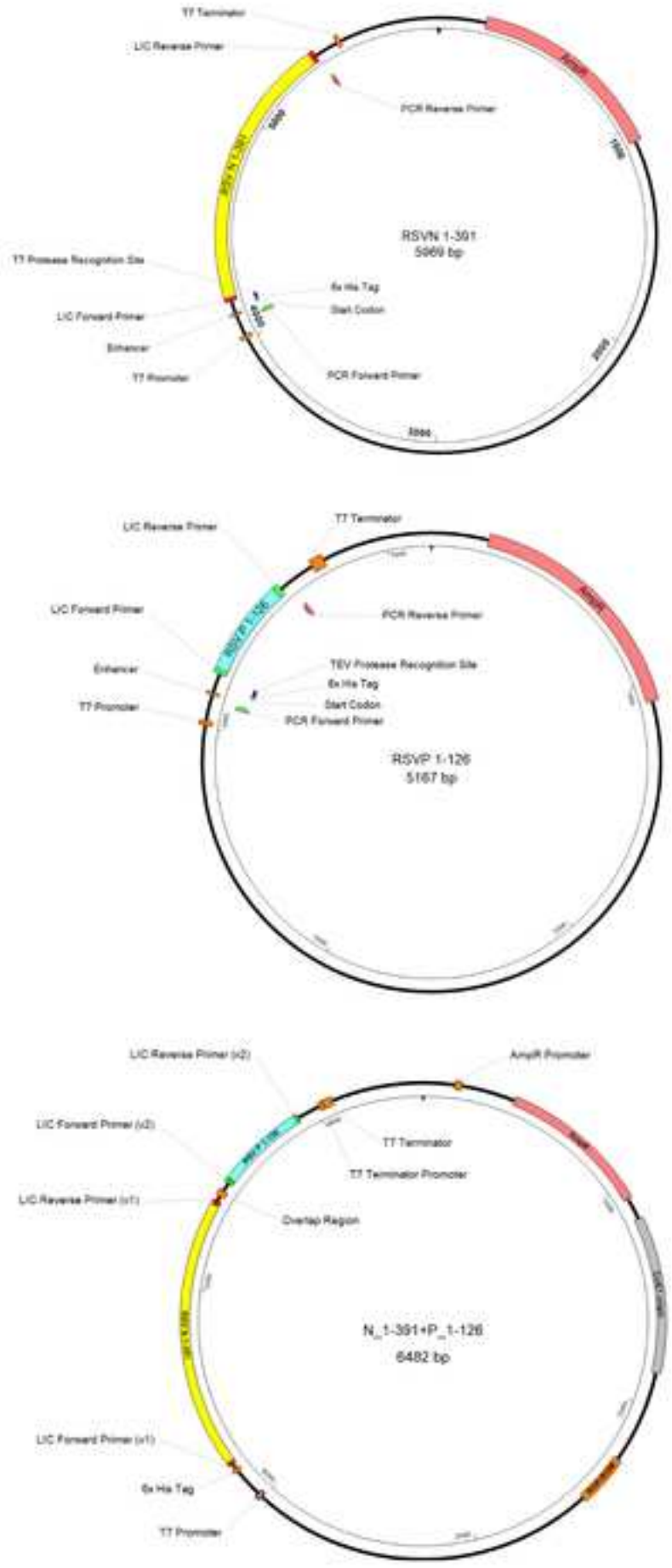
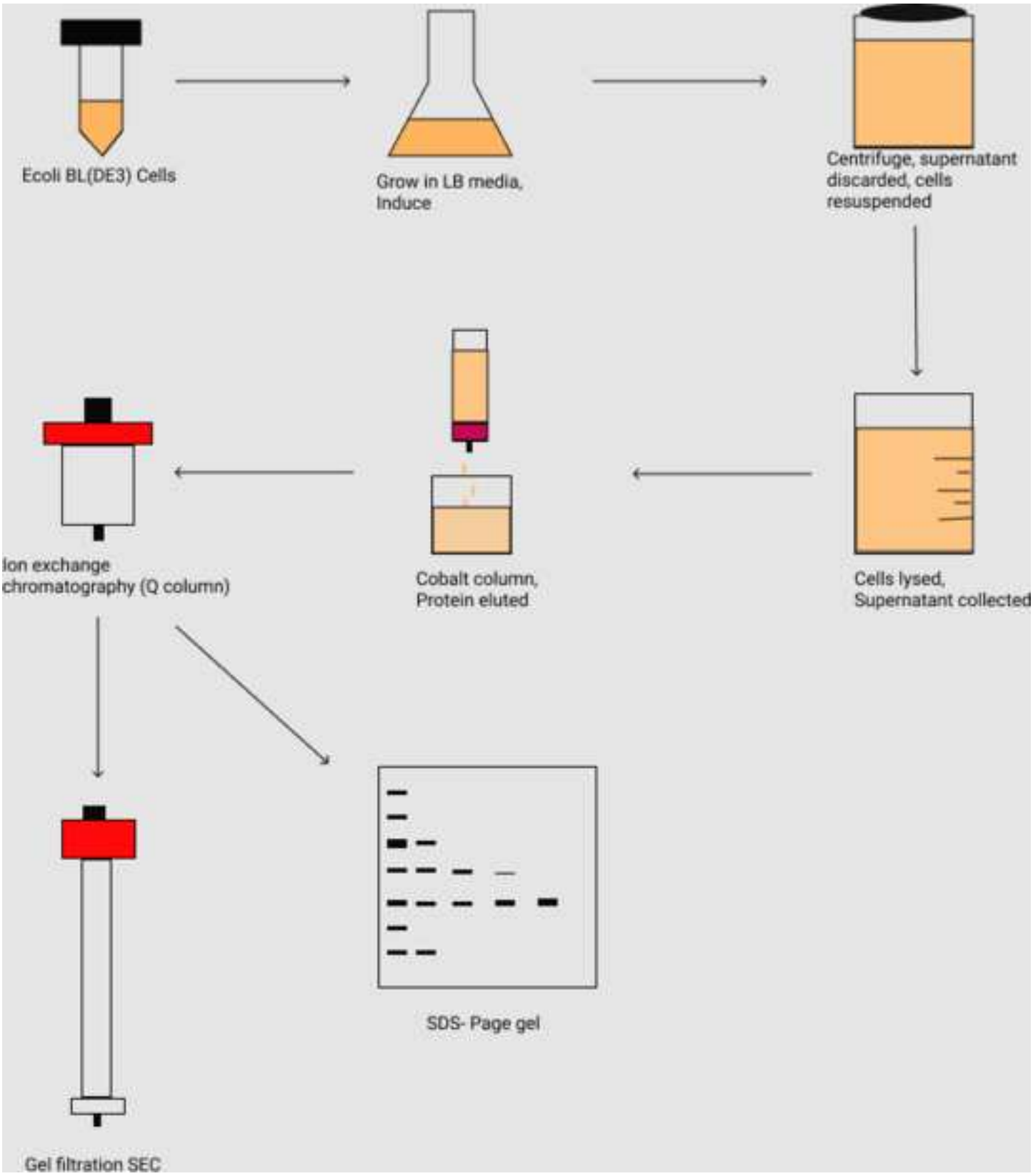


Figure 2.png



JoVE Article Image-6.png

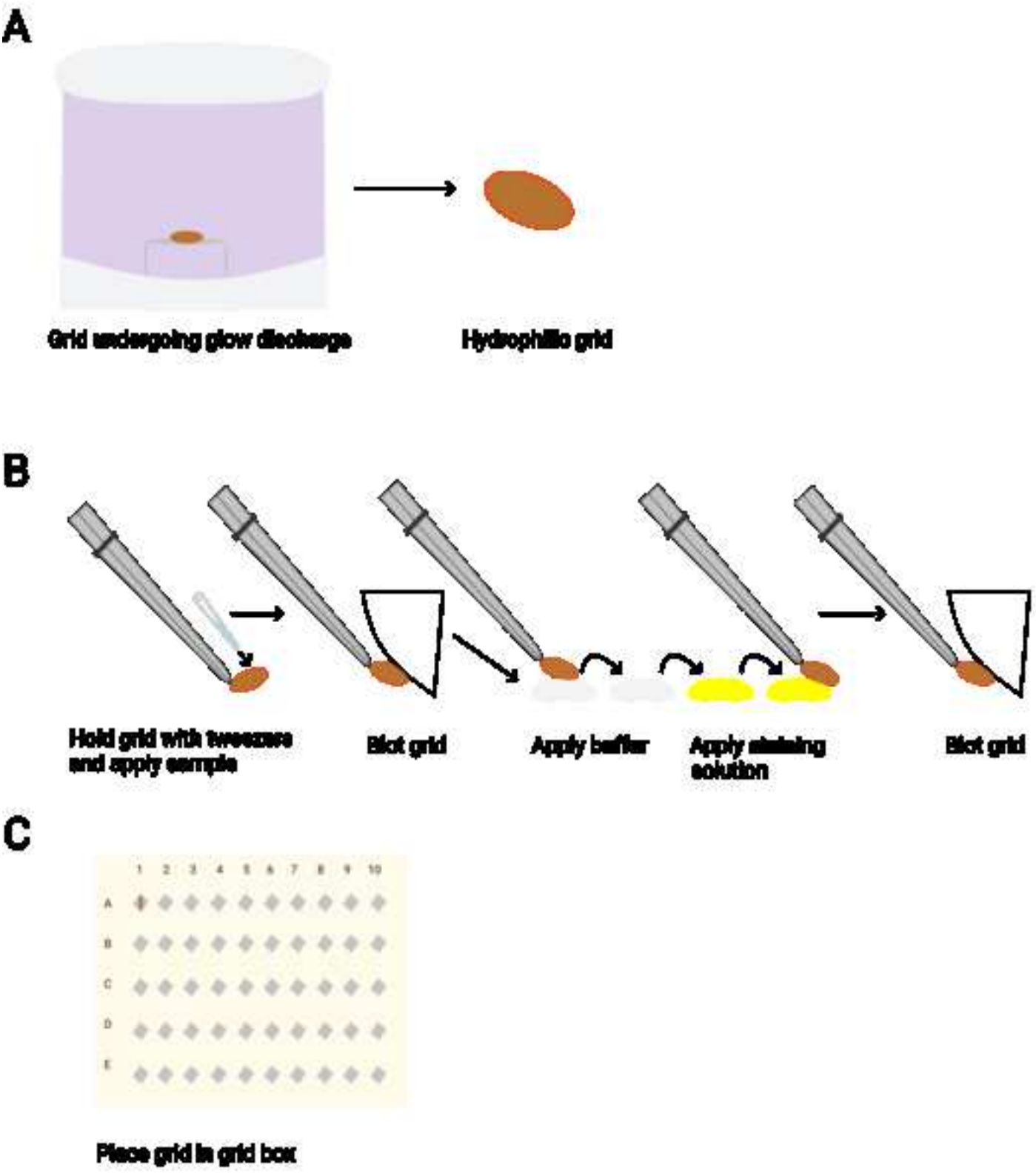


Figure 4

Click here to access/download;Figure;Figure 4.tif

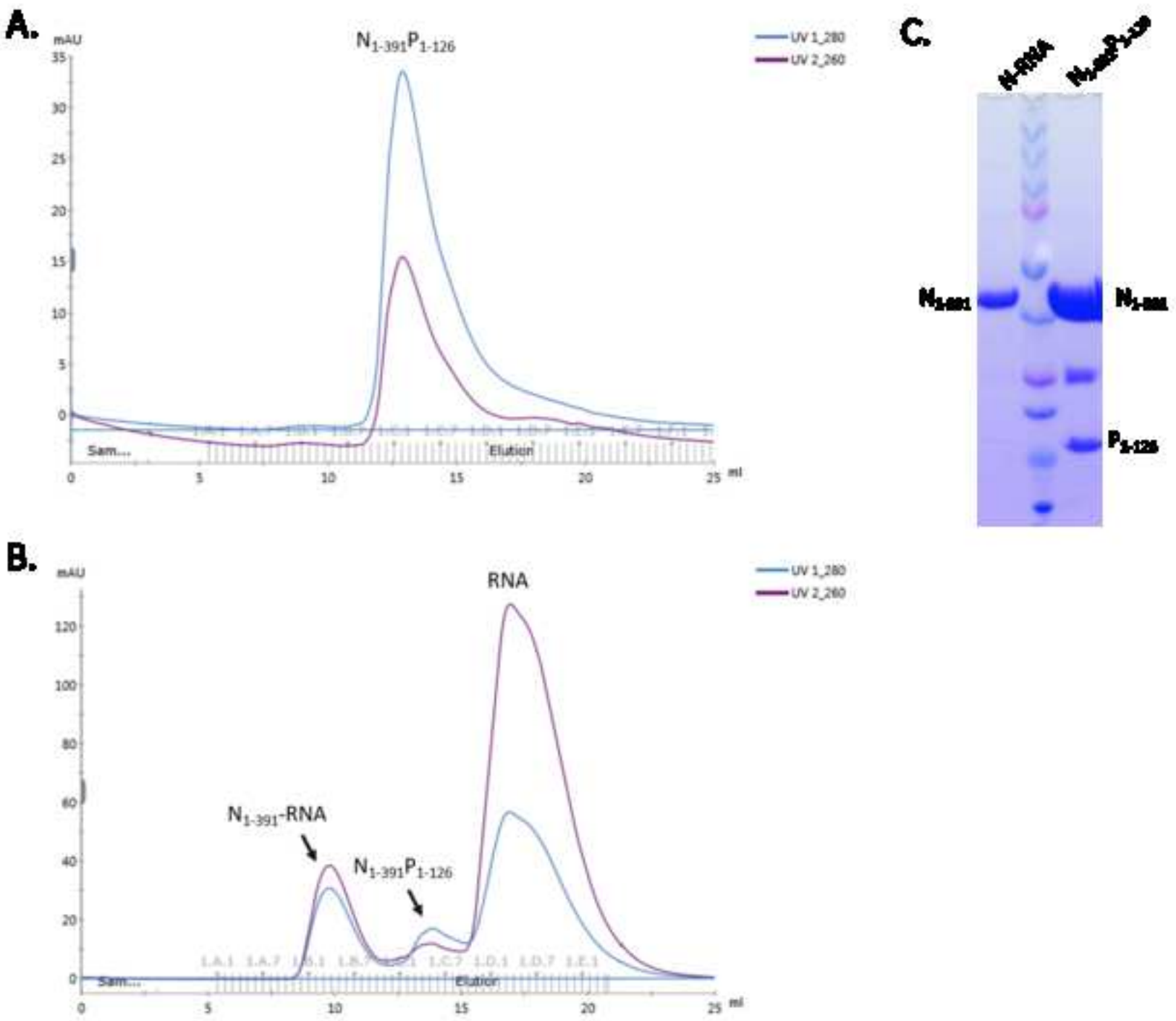


Figure 5

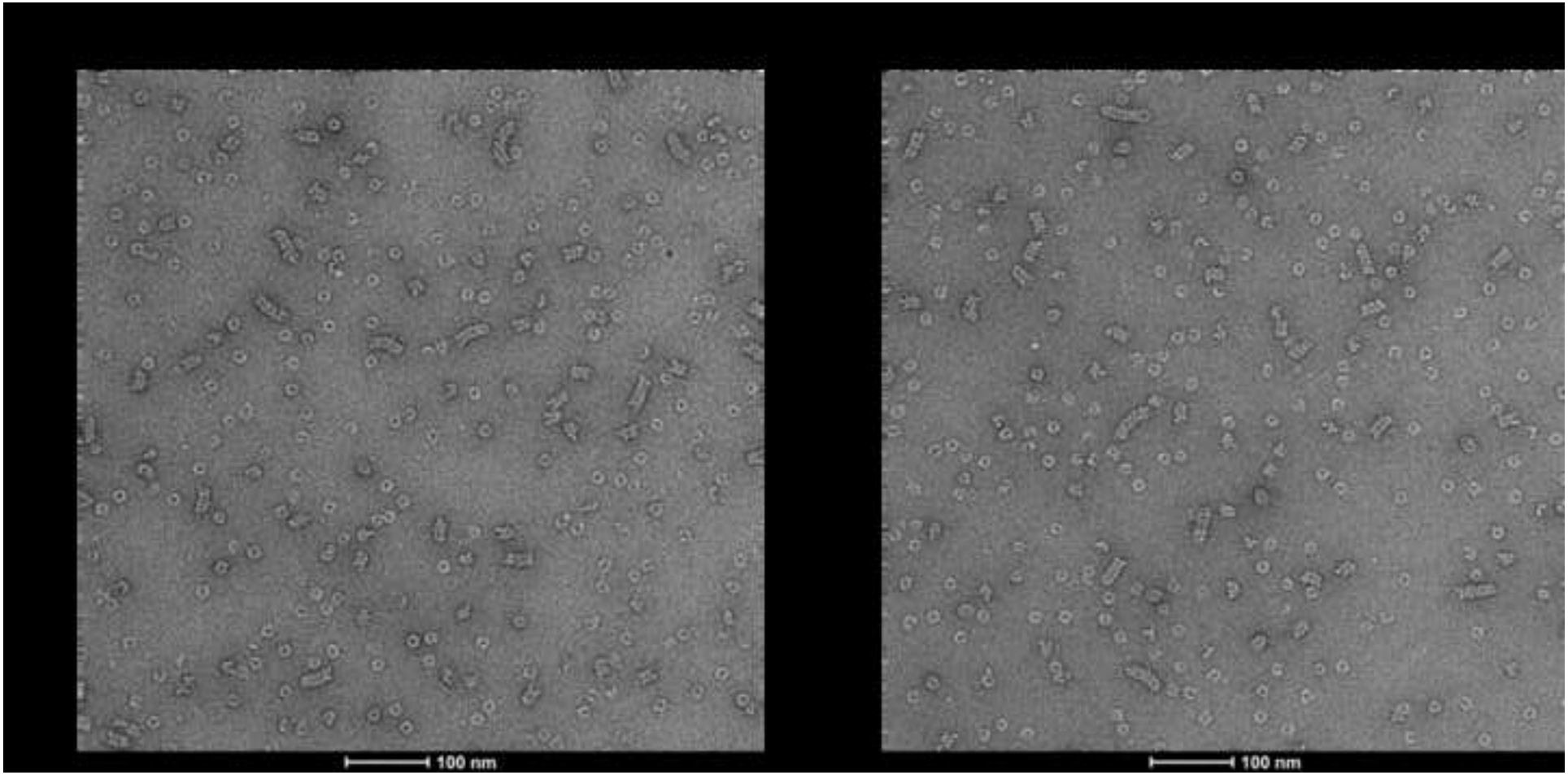


Table 1

Primers	
N_{1-391} Forward	5’-TACTTCCAATCCAATGCAATGGCCCTGAGCAAAGTGAAG-3’
N_{1-391} Reverse	5’-TTATCCACTTCCAATGTTATTACAGTTCCACGTCGTTGTCCTTGG-3'
P_{1-126} Forward	5’-TACTTCCAATCCAATGCAATGGAAAAGTTCGCCCCCGAG-3'
P_{1-126} Reverse	5’-TTATCCACTTCCAATGTTATTACTGGTCGTTGATTTCCTCGTAGC-3’

PCR amplification of DNA insert

15 µL	10x *Pfu* polymerase reaction buffer
3 µL	Forward primer (100 µM concentration)
3 µL	Reverse primer (100 µM concentration)
15 µL	dNTP mix at 2.5 mM concentration
6 µL	Plasmid DNA contains the gene of N or P (100ng/ µL)
7 µL	DMSO
3 µL	*Pfu* polymerase at 2.5U/µL
Volume to fill to 150 µL	Sterile ddH_2O

PCR amplification of DNA insert

Step	Time	Temperature	Cycles
Denaturation	4 min.	95 °C	1
Denaturation	45 sec.	95 °C	30
Annealing*	1 min.	62 °C	
Extension**	90 sec.	72 °C	
Extension	10 min.	72 °C	1
Hold		4 °C	1

The 150 µL mixture can be run in three separate PCR reactions (3 50 µL).

**For the *Pfu* DNA polymerase, 1 kb /min is the recommended speed for the ext

T4 DNA polymerase treatment

Vector/Insert DNA (0.1pmol vector or 0.2pmol insert)	5 µL
dNTP* at 25 mM	2 µL
DTT at 100 mM	1 µL
T4 DNA polymerase (LIC qualified)	0.4 µL (1.25U)
Sterile ddH_2O	9.6 µL

*dGTP was used for the vector, and dCTP was used for the insert DNA.

Table 5

Overlap PCR

15 µL	10x *Pfu* polymerase reaction buffer
3 µL	Forward primer (100 µM concentration)
3 µL	Reverse primer (100 µM concentration)
15 µL	dNTP mix at 2.5 mM concentration
6 µL	Plasmid DNA contains the gene of N or P (100ng/ µL)
7 µL	DMSO
3 µL	*Pfu* polymerase at 2.5U/µL
Volume to fill to 150 µL	Sterile ddH_2O

Overlap PCR

Step	Time	Temperature	Cycles
Denaturation	4 min.	95 °C	1
Denaturation	45 sec.	95 °C	30
Annealing*	1 min.	62 °C	
Extension**	2 min.	72 °C	
Extension	10 min.	72 °C	1
Hold		4 °C	1

*The 150 µL mixture can be run in three separate PCR

**For the *Pfu* DNA polymerase, 1 kb /min is the recom

Name of Material/Equipment	Company
Agarose	SIgma
Amicon Ultra-15 Centrifugal Filter Unit	Millipore
Ampicillin sodium	GOLD BIOTECHNOLOGY
AseI	NEB
Cobalt (High Density) Agarose Beads	Gold Bio
Corning LSE Digital Dry Bath Heater	CORNING
dCTP	Invitrogen
dGTP	Invitrogen
Glycerol	Sigma
HEPES	Sigma
HiTrap Q HP	Sigma
Imidazole	Sigma
IPTG (Isopropyl-beta-D-thiogalactopyranoside)	GOLD BIOTECHNOLOGY
Microcentrifuge Tubes	VWR
Misonix Sonicator XL2020 Ultrasonic Liquid Processor	SpectraLab
Negative stain grids	Electron Microscopy Sciences
New Brunswick Innova 44/44R	eppendorf
Nonidet P 40 Substitute	Sigma
OneTaq DNA Polymerase	NEB
QIAquick Gel Extraction Kit	QIAGEN
SSPI-HF	NEB
Superose 6 Increase 10/300 GL	Sigma
T4 DNA polymerase	Sigma
Thermo Scientific Sorvall RC 6 Plus Centrifuge	Fisher Scientific
Trizma hydrochloride	Sigma
Uranyl Formate	Electron Microscopy Sciences

Catalog Number	Comments/Description
A9539-500G	making construct using LIC method
UFC901024	concentrate the protein sample
5118.111317A	antibiotic for cell culture
R0526S	making construct using LIC method
H-310-500	For purification of His-tag protein
6885-DB	Heate the sample
10217016	making construct using LIC method
10218014	making construct using LIC method
G5516-4L	making solution
H3375-100G	making solution
GE29-0513-25	Protein purification
I5513-100G	making solution
1116.071717A	induce the expression of protein
47730-598	for PCR
MSX-XL-2020	sonicator for lysing cell
CF400-Cu-TH	For making negative stain grids
M1282-0000	Shaker for culturing the cell
74385-1L	making solution
M0480L	PCR
28706	Purify DNA
R3132S	making construct using LIC method
GE29-0915-96	Protein purification
70099-3	making construct using LIC method
36-101-0816	Centrifuge, highest speed 20,000 rpm
T3253-250G	making solution
22451	making negative stain solution

Editorial comments:

Responses to the editor: Thank you very much. We appreciate your effort in this review and your constructive suggestions.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Responses to the editor: We have thoroughly proofread the manuscript and have corrected the spelling and grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side.

Responses to the editor: Yes, we have double-checked the format according to the instructions.

3. Xx

Responses to the editor: N/A.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Responses to the editor: We have replaced the “should be” to the proper phrase in the protocol section. No “could be” or “would be” are used in the protocol section.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: QIAquick® Gel Extraction Kit (Qiagen); Novagen®; Spin Miniprep Kit (Qiagen); NEB; Eurofins Genomics; Thermo Fisher Scientific; GE Healthcare, etc

Responses to the editor: We have changed the names with trademark symbols into generic terms throughout the manuscript. They are referenced in the Table of Materials and Reagents.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your

protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Responses to the editor: There are a total of 4 subsections of the protocol. 1.Molecular cloning; 2. Protein Expression and Purification; 3. *In vitro* assembly of the virus-specific NC; and 4. Making negative stain grids.

In summary, we want to focus on filming the last three parts (2-4). For part 1, we could selectively show the positive results, e.g., sequencing result, AseI digestion gel images.

7. 1.1.5: How much T4 DNA polymerase and dGTP are to be used? How do you determine that bases until the first G residue have been removed?

Responses to the editor: Based on the activity of T4 DNA polymerase, and it has dGTP in the reaction solution, the 5′→ 3′ extension, and 3′→ 5′ exonuclease activity of T4 DNA polymerase will be at a dynamic balance.

8. 1.2.3: What are the PCR conditions?

Responses to the editor:

15 µL	10x *Pfu* polymerase reaction buffer
3 µL	Forward primer (100 µM concentration)
3 µL	Reverse primer (100 µM concentration)
15 µL	dNTP mix at 2.5 mM concentration
6 µL	Plasmid DNA contains the gene of N or P (100ng/ µL)
7 µL	DMSO
3 µL	*Pfu* polymerase at 2.5U/µL
Volume to fill to 150 µL	Sterile ddH_2O

Step	Time	Temperature	Cycles
Denaturation	4 min.	95 ℃	1
Denaturation	45 sec.	95 ℃	30
Annealing*	1 min.	62 ℃	
Extension**	90 sec.	72 ℃	
Extension	10 min.	72 ℃	1
Hold		4 ℃	1

The 150 µL mixture can be run in three separate PCR reactions (3 50 µL).

**For the *Pfu* DNA polymerase, 1 kb /min is the recommended speed for the extension phase. Here both the lengths of the N_{1-391} gene or the P_{1-126} gene are shorter than 1.5 Kb. Thus, we use 90 seconds for the extension step.

9. 1.3.1 (and 1.7.1): How much T4 DNA polymerase and dCTP are to be used?

Responses to the editor:

Vector/Insert DNA (0.1pmol vector or 0.2pmol insert)	5 µL
dNTP* at 25 mM	2 µL
DTT at 100 mM	1 µL
T4 DNA polymerase (LIC qualified)	0.4 µL (1.25U)
Sterile ddH_2O	9.6 µL

* dGTP was used for the vector, and dCTP was used for the insert DNA.

10. 1.4.5: Please mention strain and other details for competent cells. For your video, will you be filming this step? If so, please provide as many details as possible to facilitate filming. Otherwise, please cite a reference.

Responses to the editor: Yes, we will film this step. The reaction is transformed into 100 µL *E. coli* Top10 competent cells and plated on the ampicillin selection plate. We have also updated the references.

References:

Hanahan D (1983) Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* 166(4): 557–580.

Green R, Rogers EJ (2013) Transformation of chemically competent E. coli. *Methods Enzymol* 529: 329–336.

11. 1.5.3; 2.3; 2.5; 3.3: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Responses to the editor: We have converted the speeds of the centrifuges to centrifugal force (x g) in the main text.

12. 1.5.4: How much buffer P1 is to be used?

Responses to the editor: Resuspend the pellets in buffer 250 µL P1 and prepare plasmid minipreps using a Spin Miniprep Kit. We have updated the main text.

13. 1.6: How much of the vector and primers are to be used? What are the PCR conditions?

Responses to the editor:

Step 1.6.2 and 1.6.3 are using the same PCR conditions as step 1.2.3

Step 1.6.4 PCR conditions are as follow:

15 µL	10x *Pfu* polymerase reaction buffer
3 µL	Forward primer (100 µM concentration)
3 µL	Reverse primer (100 µM concentration)
15 µL	dNTP mix at 2.5 mM concentration
6 µL	Plasmid DNA contains the gene of N or P (100ng/ µL)
7 µL	DMSO
3 µL	*Pfu* polymerase at 2.5U/µL
Volume to fill to 150 µL	Sterile ddH_2O

Step	Time	Temperature	Cycles
Denaturation	4 min.	95 ℃	1
Denaturation	45 sec.	95 ℃	30
Annealing*	1 min.	62 ℃	
Extension**	2 min.	72 ℃	
Extension	10 min.	72 ℃	1
Hold		4 ℃	1

The 150 µL mixture can be run in three separate PCR reactions (3 50 µL).

**For the *Pfu* DNA polymerase, 1 kb /min is the recommended speed for the extension phase. Here the total length of the N_{1-391} gene and the P_{1-126} gene is shorter than 2.0 Kb. Thus, we use 2 minutes for the extension step.

14. 2.4: How much lysis buffer A is to be used?

Responses to the editor: Resuspend the cell pellets in 200 mL lysis buffer A (50 mM sodium phosphate, pH 7.4, 500 mM NaCl, 5 mM imidazole, 10 % glycerol, and 0.2 % NP40). Usually, using a 50 mL lysis buffer to resuspend the cell pellets form 1L cell culture. We have updated the main text.

15. 2.6 and 2.16: Please mention dimensions of the cobalt and Superdex 200 columns; the criteria for choosing these columns for these experiments (volumes to be loaded, eluted etc), how much of buffer to use for equilibriation etc. If this will not be filmed, please cite a reference to allow replication.

Responses to the editor: Load the supernatant into a cobalt gravity column (diameter x length: 2.5 cm x 10 cm) with ~10mL of beads pre-equilibrated with 5-10 column volumes (CV) lysis buffer. The Superdex 200 Increase 10/300 GL column (diameter x length: 1.0 cm x 30 cm) is also used.

16. 2.11: As you are using HPLC, please specify pumping rate, volumes of QB and QA buffers to elute, gradient used (starting % and time—ending% and time)

Responses to the editor: We have updated the text as follows.

2.11. Load the Q column into the HPLC machine along with QA buffer and QB buffer(50 mM Tris-HCl pH7.4, 1.5 M NaCl, 5% glycerol). Set up the flow rate as 1 mL/min.

2.12. Run the program (pump wash) and first wash the machine with QB buffer then QA using 1-2 column volumes (CV) of each. Set the system flow to 3 mL/min.

2.13. Set the UV1 to 280 and UV2 to 260. Use a 96 deep-well plate to collect the fractions.

2.14. Elute proteins using a stepwise gradient of elution agent (QB Buffer) applying 3-4 CV of each concentration, increasing the percentage by 5% each time starting at 0%QB. N^0P protein complex will come out at 15% QB Buffer.

17. 4.5: How are the grids made hydrophilic by glow discharge? If this is not critical for filming, please cite a reference.

Responses to the editor: Use glow discharge to make the continuous carbon-coated EM grids hydrophilic. We have also updated the reference below.

Reference: **Abei U and Pollard TD.** A glow discharge unit to render electron microscopic grids and other surfaces hydrophilic. *Journal of Electron Microscopy Techniques*. 1987;7:29-33.

18. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Responses to the editor: As question #7.

There are a total of 4 subsections of the protocol. 1.Molecular cloning; 2. Protein Expression and Purification; 3. *In vitro* assembly of the virus-specific NC; and 4. Making negative stain grids.

In summary, we want to focus on filming the last three parts (2-4). For part 1, we could selectively show the positive results, e.g., sequencing result, AseI digestion gel images.

19. Please add scale bars to figure legends, e.g., Figure 5.

Responses to the editor: We have the scale bars in the negative stain images.

20. As we are a methods journal, please add limitations of your technique to the discussion section.

Responses to the editor: The details of this protocol are for RSV and may be applied to the other viruses in the same family. The limitations have been discussed in the main text.

21. Please do not abbreviate journal names in the reference list.
Responses to the editor:
We have download the JoVE format using ENDNote.
https://endnote.com/style_download/journal-of-visualized-experiments-jove/. However, it seems the journal names are still being abbreviated.

22. Please sort the Materials Table alphabetically by the name of the material.
Responses to the editor: We have resorted the Material Table alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper is a detailed methodology to obtain nucleocapsid-like particles for Respiratory Syncytial Virus, the main cause of bronchiolitis. This work has been already published in JBC recently (DOI10.1074/jbc.RA119.011602). The methodology is very well and precisely described. It is an important work since the obtaining of nucleocapsid-like complexes in vitro will paves the way for in vitro assays with the viral RNA-dependent RNA polymerase and tests with antiviral compounds: the viral polymerase uses a N-RNA complex as a template instead of naked RNA. I have only two main remarks:

Responses to reviewer #1: Thank you very much for the positive opinion on our work. We appreciate your effort in this review and your constructive suggestions.

Major Concerns:

(1) The authors encapsidated RNA in vitro with a N°-like complex; although the proteins are very well described, there is nothing about the RNA template: length? Sequence? Did they compare with a non-relevant RNA (non-viral sequence)? This is critical if the authors want to obtain RSV-like nucleocapsids that could be used as template for in vitro polymerase assays.
Responses to the reviewer: When we assemble the RNA with NP complex, we did different RNA templates, which contain non-viral sequences (e.g., polyA) and viral RNA sequence (e.g., Tr70). Both of the RNA oligos worked and formed the N-RNA ring particle in the solution. Our next plan uses the nucleocapsid, which contains trailer RNA, as the template to do the *in vitro* polymerase assays.

(2) On Figure 5, the EM images show ring-like structures and also some sorts of small helices. So far, nucleocapsid-like complexes that were obtained for RSV are always rings structures containing 10 or 11 N protomers and RNAs of 70 or 77 bases, respectively. Did the authors use

an RNA template longer than 77 bases? Did they test the RNA length encapsidated? Can they use any technique to determine if there are some helices present after RNA encapsidation by N°? Can they comment on these results?

Responses to the reviewer: When we do the nucleocapsid assembly *in vitro* using different lengths of RNA sequences, we found that the RNAs with shorter length than 70 or 77bases can also form the ring structures. For the longer RNA, we used the viral RNA sequence (e.g. Trailer70, Trailer77, Trailer84) to get the nucleocapsid-like particles, which can be determined by EM. We will try to get the detail information about the N-longer RNA complex structure by the CryoEM.

Minor Concerns:

Line 71 : specify that P is an RSV protein

Responses to the reviewer: We have updated the main text. "The principle of this protocol is to obtain a large quantity of recombinant RNA-free N (N^0) by co-expressing N with a chaperone, the N-terminal domain of RSV phosphoprotein (P_{NTD})".

Reviewer #2:

Manuscript Summary:

This manuscript describes the preparation of ribonucleocapsids (NC) derived from recombinant respiratory syncytial virus (RSV) proteins and synthetic RNA oligonucleotides. Previous methods of isolating RSV NC relied on purification from infected cells or used recombinant proteins that produced NCs which did not include an authentic viral RNA. The method presented here allows for production of RSV NCs that resemble authentic NCs, albeit truncated.

Responses to reviewer #2: Thank you very much for the positive opinion on our work. We appreciate your effort in this review and your constructive suggestions.

Major Concerns:

1. Description of the essential elements of the 2BT-10 vector beyond highlighting them in Figure 1 would allow researchers to select other suitable vectors.

Responses to the reviewer: 2BT-10 is an empty vector that can be inserted with the gene of interest with a LIC cloning protocol. It has ampicillin resistance and a TEV-cleavable N-terminal His6 fusion tag. To clone in this vector, add LIC v1 tag to the 5' end of your PCR primers, Forward - 5'TACTTCCAATCCAATGCA3', Reverse - 5'TTATCCACTTCCAATGTTATTA3'

2. Section 1.6 should include detail on the essential elements of the spacer region between N and P(NTD) encoded in the PCR primers.

Responses to the reviewer: We have filled the details in the manuscript.

3. Section 2 should include typical volumes used for cultures, resuspension, washes, etc.

Responses to the reviewer: We have added all the details, as suggested.

4. Section 3 should include a brief description of the oligonucleosides used including sizes (or range of sizes) and essential sequence elements, if applicable.
Responses to the reviewer: The efficiency of the N-RNA assemble *in vitro* depends on the length and the type of RNA. For the length of RNA, we test the RNA length between 7nt and 84nt. 7nt RNA oligo has the lowest efficiency of assembly. The type of RNA we test includes the virus genomic RNA(e.g. Trailer RNA) and non-viral RNA(e.g. PolyA). with the same length, like 14nt, PolyA14 has a higher efficiency than Tr14. For the PolyU14, which even can not be assembled with N^0. In this protocol, we used Tr14 as an example. The RNA sequence is 5'-ACGAGAAAAAAAGU

Minor Concerns:
1. The last sentence in Section 1.3.1 mentions PCR, but probably should read "T4 DNA pol treatmeny".
Responses to the reviewer: We have updated the main text, as suggested.

2. Section 1.7.2 should probably read anneal LIC vector and PCR product (insert).
Responses to the reviewer: We have updated the main text, as suggested.

3. The ratio of N0P to oligo should be specified (molar ratio? w/w?).
Responses to the reviewer: Molecular ratio. We have updated the main text.

4. The manuscript needs to be proof-read for grammar, verb tense agreement.
Responses to the reviewer: We have proofread the manuscript to correct the tense and grammar issues.