

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

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## 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)<sup>1,2</sup>. RSV is the leading cause of respiratory illness such as bronchiolitis and pneumonia in young children and older adults worldwide<sup>3</sup>. Currently, no effective vaccines or antiviral therapies are available to prevent or treat RSV<sup>4</sup>. 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)<sup>3</sup>. 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<sup>5</sup>. 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<sup>6,7,8,9,10,11,12</sup>.

Currently, the RSV RNA polymerization assay has been established using purified RSV polymerase on short naked RNA templates<sup>13,14</sup>. 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<sup>15,16,17,18,19</sup>. 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<sup>20</sup>. 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<sup>21,22</sup>. 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. Perform linearization of LIC vectors using SSPI digestion.
  1. Combine 10  $\mu$ L of SSPI 10X buffer, 4  $\mu$ L of SSPI enzyme at a concentration of 5 U/ $\mu$ L, the equivalent volume of 5  $\mu$ g of vector miniprep DNA, and sterile ddH<sub>2</sub>O to 100  $\mu$ L.
  2. Incubate the digest for 3 hours at 37 °C.
  3. Run the digest on a 1.0% agarose gel for the extraction of vector DNA.
  4. Use a gel extraction kit to perform extraction and purification. Suspend the final volume of vector DNA in 30  $\mu$ L of ddH<sub>2</sub>O and store it at -20 °C.

2. Prepare the DNA inserts for N<sub>1-391</sub> and P<sub>1-126</sub> using the N<sub>1-391</sub> Forward Primer, N<sub>1-391</sub> Reverse Primer, P<sub>1-126</sub> Forward Primer, and P<sub>1-126</sub> 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. Perform PCR amplification of the DNA insert using the conditions in **Table 2** and **Table 3**.
  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<sub>2</sub>O.
3. 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. 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.
  4. Anneal the LIC vector and the insert vector.
    1. Set up a negative control with 2 µL of LIC vector DNA and 2 µL of sterile ddH<sub>2</sub>O.
    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.
    3. Perform the annealing reaction at room temperature for 10 minutes.

4. Quench the reaction with 1.3 µL of EDTA at a concentration of 25 mM.
  5. Transform the reaction into 100 µL of *E. coli* Top10 competent cells and plate them on an ampicillin selection plate<sup>23,24</sup>.
5. Identify the positive constructs.
    1. Prepare the plasmid miniprep solution. This can be done through colony picking and inoculation in LB media. Usually, 3 colonies are sufficient.
    2. Incubate the mixture overnight at 37 °C.
    3. Centrifuge the mixture at 4,560 x g for 10 minutes and discard the supernatant.
    4. Resuspend the pellets in 250 µL of P1 buffer and prepare plasmid minipreps using a spin miniprep kit.
    5. Conduct a digestion analysis of the miniprep product using AseI or other restriction enzymes.
    6. Load samples on 0.8% agarose gel and run the digested plasmid. Analyze the gel under a UV lamp.
    7. Use a sequencing service to validate the sequence of the positive product.
  6. Obtain the coexpression DNA insert.
    1. Perform PCR to obtain N<sub>1-391</sub> and P<sub>1-126</sub> using the previously constructed 2BT-10 N<sub>1-391</sub> and 2BT-10 P<sub>1-126</sub> as templates.
    2. Perform the 1<sup>st</sup> PCR to obtain N<sub>1-391</sub> from the 2BT-10 N<sub>1-391</sub> construct using the PCR conditions in **Table 2** and **Table 3** with the N<sub>1-391</sub> Forward primer and Reverse Primer 5'-GTGAAGATCCTGGCTGATGCAATGCGGCGGCGCGCCGCGATCGCGGATCC-3'.

3. Perform the 2<sup>nd</sup> PCR to obtain P<sub>1-126</sub> from the 2BT-10 P<sub>1-126</sub> construct using the Forward primer: 5'-CCGCCGCATTGCATCAGCCAGGATCTTCACTGCAGGACTCGAGTTCTAGA-3' and the P<sub>1-126</sub> Reverse primer (use the PCR conditions in **Table 2** and **Table 3**).
  4. Finally, perform overlap PCR on the mixed products of the previous 2 PCR reactions to merge N<sub>1-391</sub> and P<sub>1-126</sub>. Use the N<sub>1-391</sub> Forward primer and P<sub>1-126</sub> Reverse primer. Use the PCR conditions in **Table 5** and **Table 6**.
  7. Join the vector and the DNA insert.
    1. Treat the overlap PCR product with T4 DNA polymerase following the protocol in step 1.3.
    2. Anneal the LIC vector and PCR product following the protocol in step 1.4.
    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**).
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<sub>600</sub> reaches 0.6.
  2. Lower the temperature to 16 °C. An hour later, induce the expression with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) overnight.
  3. Centrifuge the cells at 4,104 x g for 25 min and then discard the supernatant.
  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 from 1 L of cell culture.
  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.
  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.
  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).
  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).
  9. Dilute the eluted protein 5x with QA buffer (50 mM Tris-HCl pH 8.0, 5% Glycerol) for the Q column.
  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).

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.
12. Run the “pump wash” program to wash the machine with QB buffer followed by QA buffer (1-2 CVs/each). Set the system flow to 3 mL/min.
13. Set the UV1 to 280 nm and UV2 to 260 nm. Use a 96 deep-well plate to collect the fractions.
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<sup>0</sup>P protein complex will come out at 15% QB Buffer.
15. Once all of the protein is eluted, wash the column with 100% QB Buffer (2 CV).
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).
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<sup>0</sup>P complex with RNA oligos. Then, SEC chromatography was used to separate the assembly complex from the N<sup>0</sup>P and excess RNA (**Figure 2**).

1. Mix and incubate the purified N<sup>0</sup>P 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<sup>0</sup>P with a concentration of 1 mg/mL is enough for the next

step. Set up the control sample, which only contains the same amount of N<sup>0</sup>P protein.

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. Centrifuge the sample with 21,130 x g for 15 min, remove any precipitation and load the supernatant to the SEC column.
4. Compare the SEC chromatography images of N:RNA assembly sample and N<sup>0</sup>P control sample, combine the A<sub>260</sub>/A<sub>280</sub> ratio to identify which peaks are the assembled N-RNA, N<sup>0</sup>P, and free RNA.
5. Collect the peak fractions, run the SDS-PAGE gel, or make grids.
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 the 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<sup>25,26</sup> (**Figure 3**).

1. Microwave or heat 5 mL of ddH<sub>2</sub>O in a small glass beaker using a Tungsten heater until it is boiling.
2. Weigh 37.5 mg of uranyl formate and add to 5 mL of heated ddH<sub>2</sub>O to make a 0.75% uranyl formate staining solution. Stir under an aluminum foil covered beaker to protect from light.
3. Add 4 μL of 10 M NaOH to the staining solution and continue to stir for 15 min, protected from the light.
4. Filter the solution through a 0.22 mm filter into a test tube.
5. Use glow discharge to make the continuous carbon-coated EM grids hydrophilic<sup>27</sup>.

**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.

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.
7. To make the EM carbon grids, apply 3 μL of protein sample for 1 minute.
8. Blot the grids against a blotting paper.

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

9. Hold the grid surface in the second drop of 0.75% uranyl formate staining solution for 30 seconds.
10. Blot the grid against a blotting paper to remove excess stain solution and allow the grid to air-dry.
11. Store the grids in the grid box before imaging.

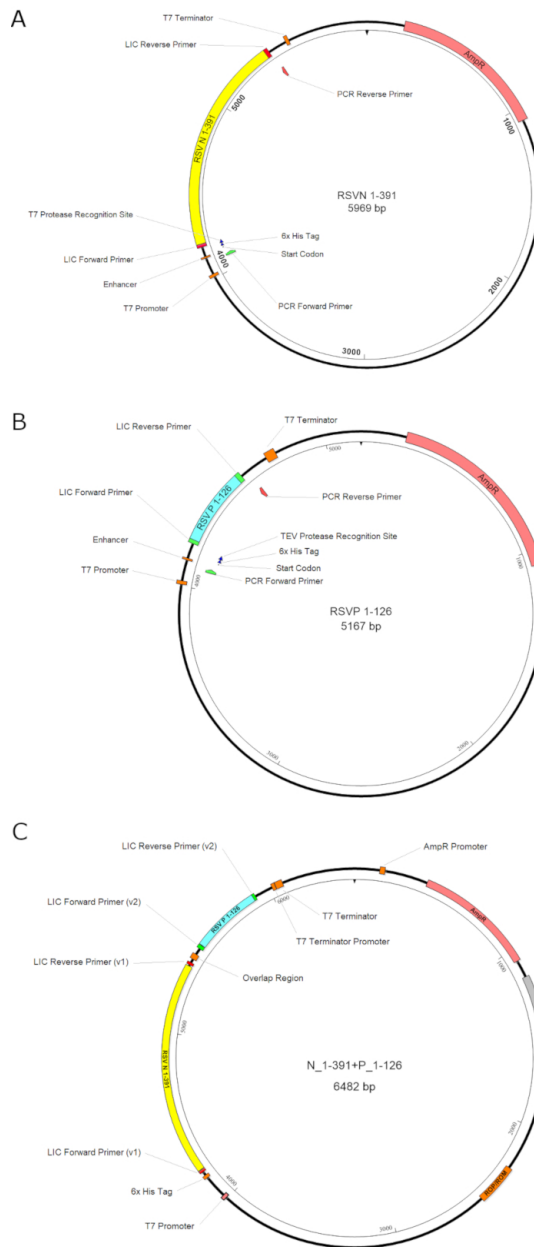
## Representative Results

### Purification of RNA-free N<sup>0</sup>P protein

With this protocol, a large-scale soluble heterodimeric RSV N<sup>0</sup>P 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<sup>0</sup>P was purified using a cobalt column, ion exchange, and size exclusion chromatography. N<sup>0</sup>P contains both the full-length N and N terminal P but did not contain cellular RNA based on the UV absorbance A<sub>260</sub>/A<sub>280</sub> ratio<sup>20</sup> (**Figure 4**).

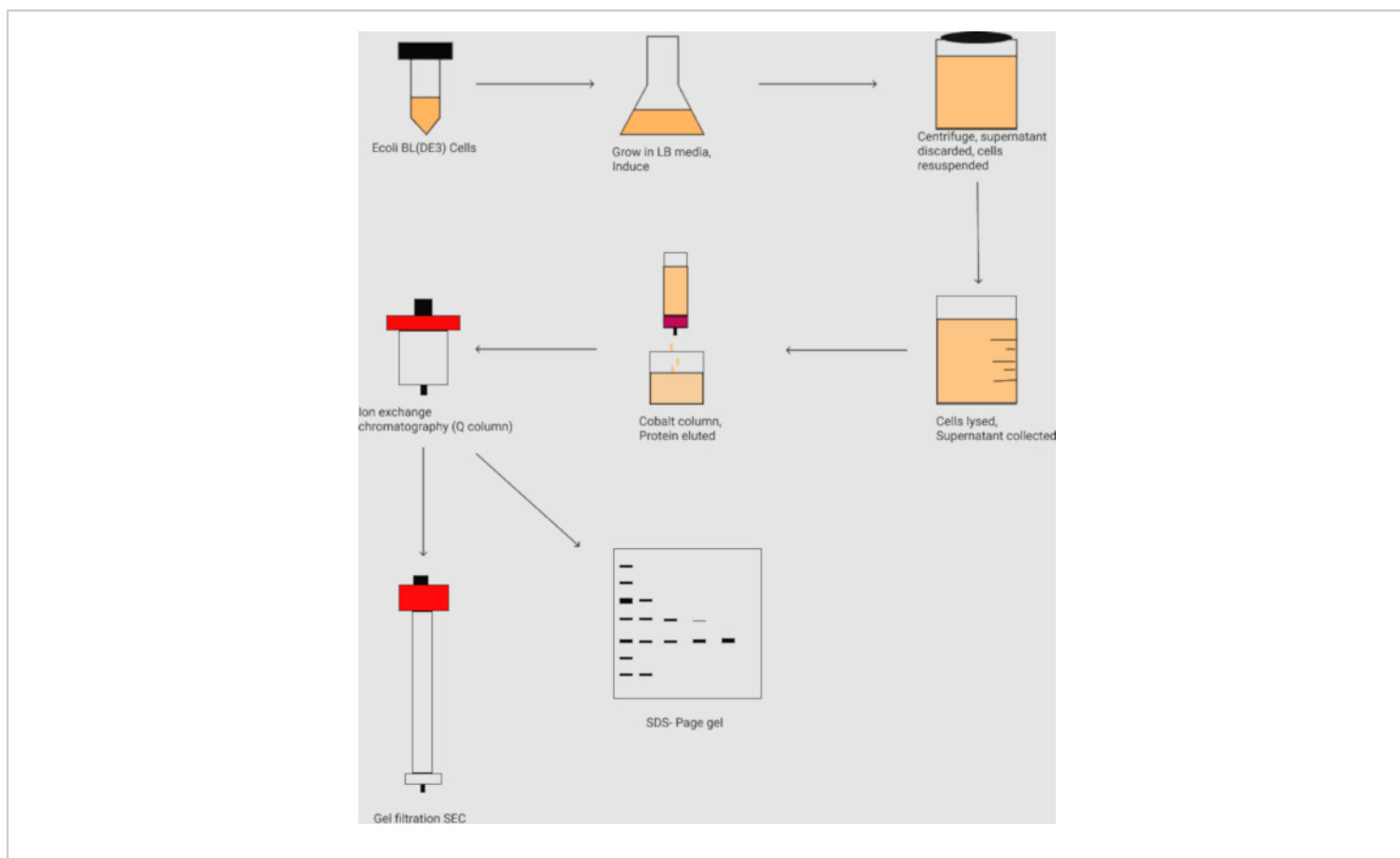
### Assembly of N-RNA and checking with negative stain

We then demonstrated that the purified N<sup>0</sup>P could be stimulated and assembled into Nucleoplasmid-like particles (NCLPs) by incubating with specific RNA oligos. The NCLPs were assembled by incubating the N<sup>0</sup>P 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 1<sup>st</sup> peak is N:RNA, the 2<sup>nd</sup> peak is N<sup>0</sup>P, and the 3<sup>rd</sup> peak is excess free RNA. The highest fraction of the N:RNA peak creates the negative stain grids for checking with EM<sup>20</sup> (**Figure 5**).



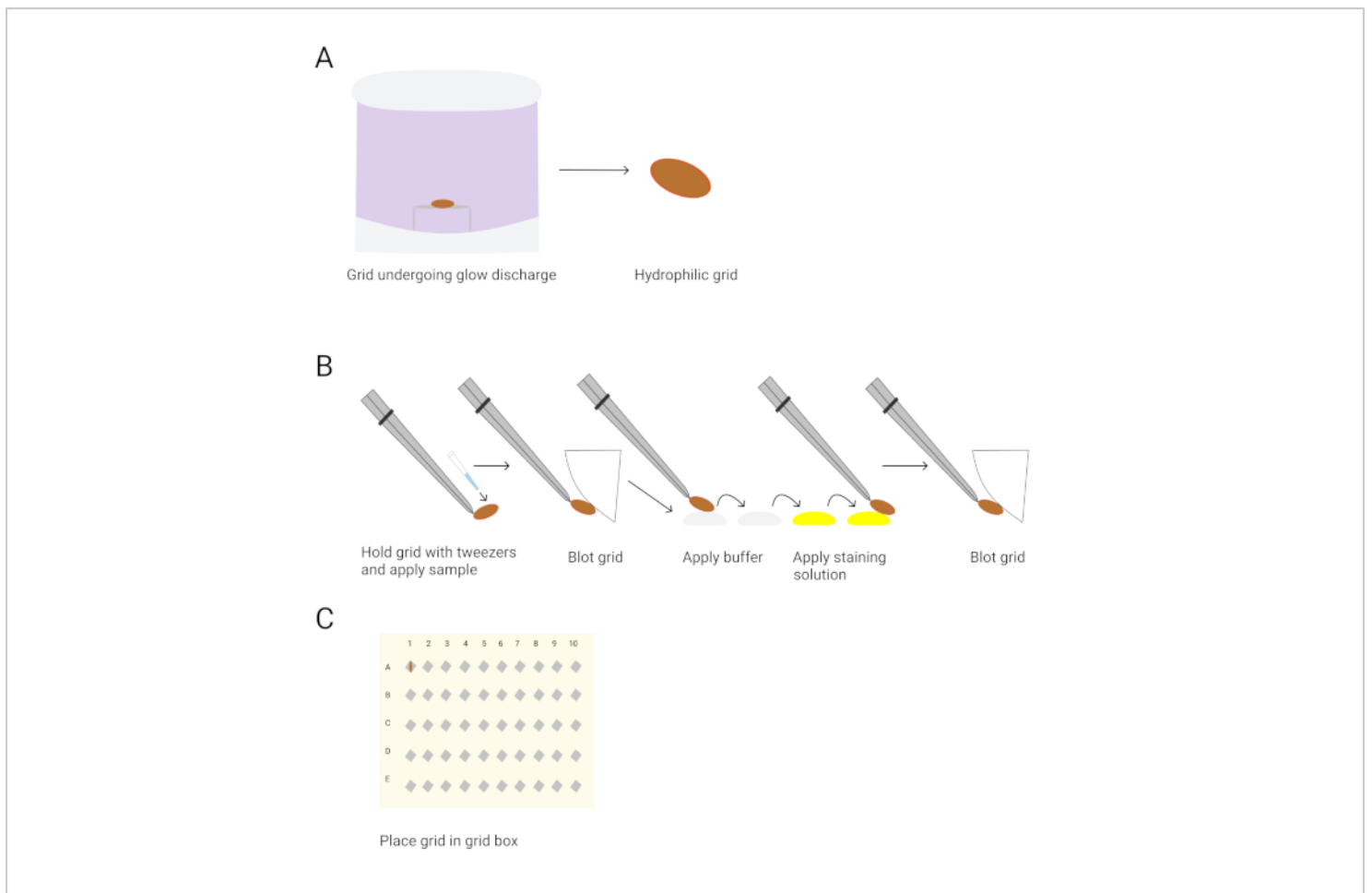
**Figure 1. The illustration of the plasmid constructions.** A. The construct of the RSV N<sub>1-391</sub>; B. The construct of the RSV P<sub>1-126</sub>; C. The bi-cistronic construct for the coexpression of N<sub>1-391</sub> and P<sub>1-126</sub>. The first gene RSV N<sub>1-391</sub>, the second gene RSV P<sub>1-126</sub>, 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<sub>1-391</sub> and the RSV P<sub>1-126</sub> are constructed separately and assembled. [Please click here to view a larger version of this figure.](#)



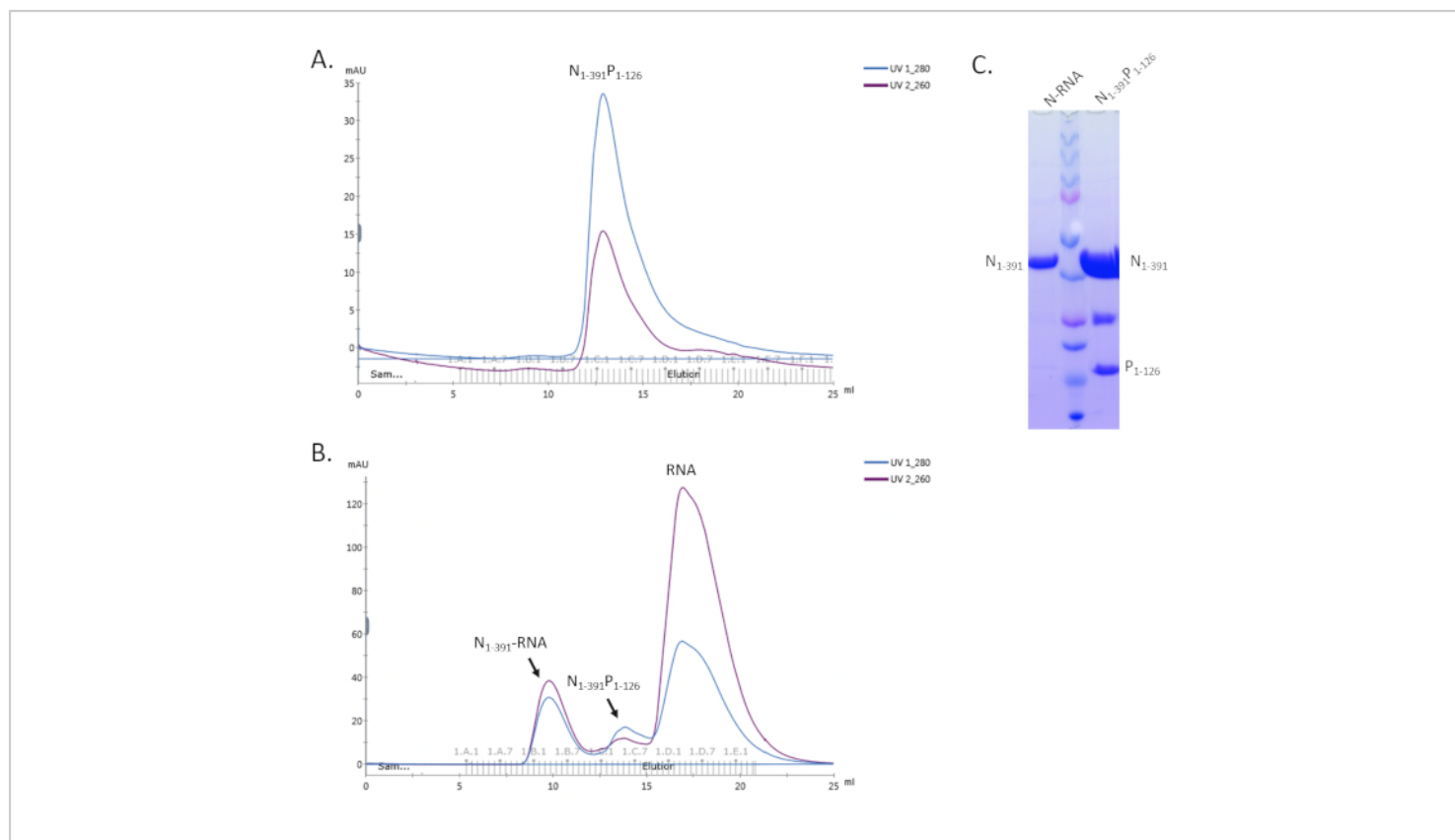


**Figure 2. The flowchart of the purification of the protein complex N<sub>1-391</sub>P<sub>1-126</sub>.** It outlines the inoculation and large scale grow-ups of the *E. coli* cell culture and harvesting the cell by centrifugation. Followed by cell lysis, the protein samples are purified by the affinity chromatography (i.e., Co<sup>2+</sup> 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. [Please click here to view a larger version of this figure.](#)

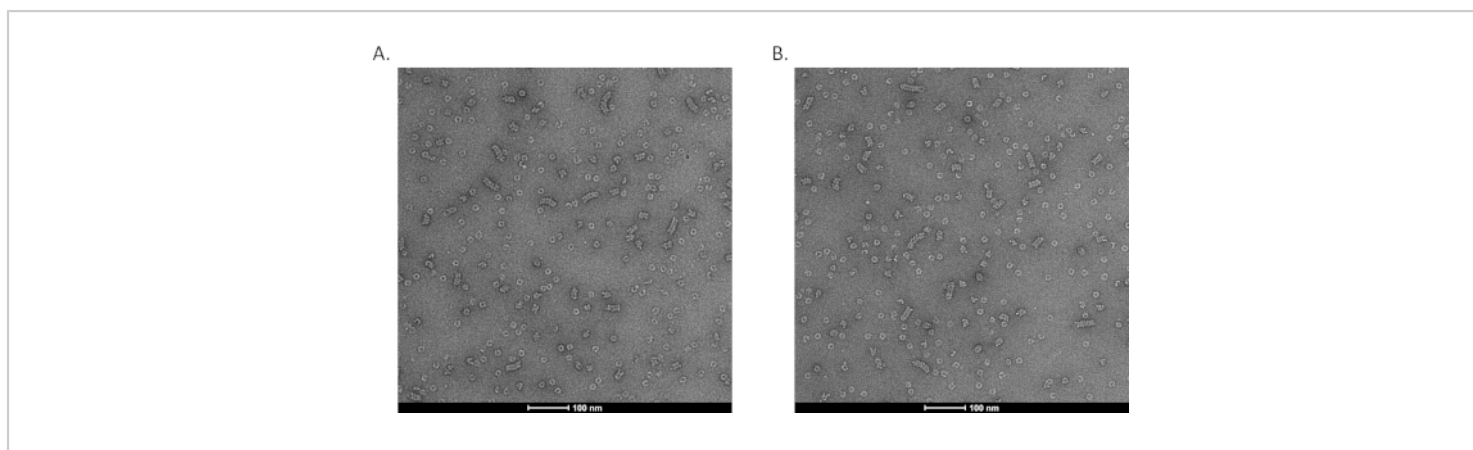




**Figure 3. Preparation of negative stain EM grids for imaging.** A. Glow discharges the grids. B. The procedure for negative staining grids is shown. The tweezers are used to pick up a grid, followed by applying the protein sample for 1 minute. The grids are blotted with blotting paper. The grid is 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. [Please click here to view a larger version of this figure.](#)



**Figure 4. Representative results of the copurification of the N<sub>1-391</sub>P<sub>1-126</sub> complex.** A. The SEC profile of N<sub>1-391</sub>P<sub>1-126</sub>. B. The SEC profile of the assembly N<sub>1-391</sub>P<sub>1-126</sub> 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<sub>1-391</sub>P<sub>1-126</sub> complex. [Please click here to view a larger version of this figure.](#)



**Figure 5. Representative images of N-RNA.** A and B are representative negative stain EM images of N-RNA from the N<sub>1</sub>-391-RNA peak in **Figure 4**. The N-RNA complexes are stained using the procedure described in **Figure 3**. [Please click here to view a larger version of this figure.](#)

Primers	
N <sub>1</sub> -391 Forward	5'-TACTTCCAATCCAATGCAATGGCCCTGAGCAAAGTGAAG-3'
N <sub>1</sub> -391 Reverse	5'-TTATCCACTTCCAATGTTATTACAGTTCCACGTCGTTGTCCTTGG-3'
P1-126 Forward	5'-TACTTCCAATCCAATGCAATGGAAAAGTTCGCCCCCGAG-3'
P1-126 Reverse	5'-TTATCCACTTCCAATGTTATTACTGGTCGTTGATTTCTCGTAGC-3'

**Table 1. Primer sequences.**

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 $\mu$ L	Sterile ddH <sub>2</sub> O
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**Table 2. PCR amplification of DNA insert reagents.**

PCR amplification of DNA insert			
Step	Time	Temperature	Cycles
Denaturation	4 min.	95 °C	1
Denaturation	45 sec.	95 °C	30
Annealing	30 sec.	62 °C	
Extension*	90 sec.	72 °C	
Extension	10 min.	72 °C	1
Hold	$\infty$	4 °C	1
The 150 $\mu$ L mixture can be run in three separate PCR reactions (3 x 50 $\mu$ L).			
*For the Pfu DNA polymerase, 1 kb/min is the recommended speed for the extension phase. Here, both the lengths of the N <sub>1</sub> -391 gene or the P <sub>1</sub> -126 gene are shorter than 1.5 Kb.			
Thus, 90 seconds was used for the extension step.			

**Table 3. PCR amplification of DNA insert thermocycling program.**

T4 DNA polymerase treatment	
10 x Buffer	2 $\mu$ L
Vector/Insert DNA (0.1 pmol vector or 0.2 pmol insert)	5 $\mu$ L
dNTP* at 25 mM	2 $\mu$ L
DTT at 100 mM	1 $\mu$ L
T4 DNA polymerase (LIC qualified)	0.4 $\mu$ L (1.25 U)
Sterile ddH <sub>2</sub> O	9.6 $\mu$ L
*dGTP was used for the vector, and dCTP was used for the insert DNA.	

**Table 4. T4 DNA polymerase treatment.**

Overlap PCR	
15 $\mu$ L	10 X Pfu polymerase reaction buffer
3 $\mu$ L	Forward primer (100 $\mu$ M)
3 $\mu$ L	Reverse primer (100 $\mu$ M)
15 $\mu$ L	dNTP Mix (2.5 mM)
3 $\mu$ L	DNA from 1 <sup>st</sup> round PCR which contain the gene N <sub>1-391</sub> (100 ng/ $\mu$ L )
3 $\mu$ L	DNA from 1 <sup>st</sup> round PCR which contain the gene P <sub>1-126</sub> (100 ng/ $\mu$ L )
7 $\mu$ L	DMSO
3 $\mu$ L	Pfu polymerase (2.5 U/ $\mu$ L)
Volume to fill to 150 $\mu$ L	Sterile ddH <sub>2</sub> O

**Table 5. Overlap PCR reagents.**

Overlap PCR			
Step	Time	Temperature	Cycles
Denaturation	4 min.	95 °C	1
Denaturation	45 sec.	95 °C	30
Annealing	30 sec.	62 °C	
Extension*	2 min.	72 °C	
Extension	10 min.	72 °C	1
Hold	$\infty$	4 °C	1
The 150 $\mu$ L mixture can be run in three separate PCR reactions (3 x 50 $\mu$ L).			
*For the Pfu DNA polymerase, 1 kb/min is the recommended speed for the extension phase. Here, the total length of the gene N <sub>1-391</sub> and P <sub>1-126</sub> is shorter than 2.0 Kb. Thus, 2 minutes were used for the extension step.			

**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<sup>15,16,17,18,19</sup>. 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<sup>28,29</sup>. 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<sup>28</sup>. 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<sup>30,31</sup>. 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<sup>31</sup>.

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<sup>0</sup>P complex could be obtained by coexpression of a K170A/R185A double N mutant with the N-terminus of P in bacteria<sup>32</sup>. 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<sub>1-50</sub>-TEV-N<sub>1-405</sub>-8xHis for MeV<sup>33,34</sup>. N<sup>0</sup>P can be obtained after the TEV protease cleavage. The purity of N<sup>0</sup>P 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<sup>0</sup>P 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<sup>20</sup>. Briefly, the overall procedure of the N<sup>0</sup>P purification is to purify His-tagged N<sup>0</sup>P 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<sup>0</sup>P complex with the SEC column. In the SEC step, the ratio of A<sub>260</sub>/A<sub>280</sub> can be monitored and double-checked with RNA extraction from the N<sup>0</sup>P peak fractions.

Collectively, in this protocol, the most critical steps are the strategy to design the construction of the coexpression of N<sup>0</sup>P complex and using a series affinity and ion-exchange column to separate the RNA free N<sup>0</sup>P complex from the other N-cellular RNA complexes. The efficiency of the coexpression strategy to get N<sup>0</sup>P 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<sup>0</sup>P and assembly with specific RNA with N to get N:RNA complex of other NNS viruses.

## Disclosures

The authors have nothing to disclose.

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