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.