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RSV Pseudovirus Neutralization Assay V.1

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Abstract

Here, we develop an experimental system for measuring neutralization titers against RSV F using pseudotyped lentiviral particles. This system is easily adaptable to evaluate neutralization of relevant strains. This method is first described in the manuscript: Simonich CAL, McMahon TE, Ju X, Yu TC, Brunette N, Stevens-Ayers T, Boeckh MJ, King NP, Greninger AL, Bloom JD. 2025. RSV F evolution escapes some monoclonal antibodies but does not strongly erode neutralization by human polyclonal sera. J Virol 99:e00531-25.

<https://doi.org/10.1128/jvi.00531-25>**Materials****Cell Lines:**

- **293T** (ATCC CRL-3216)
- **293T-TIM1** (request from the Bloom Lab <https://doi.org/10.1128/jvi.00531-25>)

D10 Media Recipe:

- 440 mL DMEM (High glucose, Fisher MT10013CV)
- 50 mL Heat-inactivated Fetal Bovine Serum (FBS, Gemini 100-106)
- 5 mL Penicillin Streptomycin Solution (10000 U/mL, Fisher MT30002CI)
- 5 mL L-Glutamine (200 mM, Fisher MT25005CI)
→sterile filter into a 500mL bottle

For transfection/pseudovirus production:

- 6 well tissue culture treated sterile plate (Fisher 720083)
- DMEM (Fisher MT10013CV)
- BioT transfection reagent (Fisher NC0765006)
- CoolRack® CF45 Cooling Block (Fisher UX-04392-51)
- DWK Life Sciences Wheaton™ CryoELITETM Cryogenic Storage Vials (Fisher 02-912-728)
- Syringe Filter 0.45 micron membrane; 50/case (Corning 431220)

Plasmids:

- lentivirus helper plasmids (**HDM-tat1b** AddGene product ID 204154, **pRC-CMV-Rev1b** AddGene product ID 20413, **HDM-Hgpm2** AddGene product ID 204152)
- lentiviral backbone plasmid that uses a CMV promoter to express luciferase followed by an IRES and ZsGreen (**pHAGE-CMV-Luc2-IRES-ZsGreen-W** AddGene product ID 164432)
- VSV-G expression plasmid used as a positive control (**HDM_VSV_G** AddGene product ID 204156)
- Carrier DNA (Promega E4881)
- RSV F and G expression plasmids (Various strains available at <https://www.addgene.org/browse/article/28253256/>)

Note: For RSV G's, we deleted 31 amino acids from the N-terminal cytoplasmic tail as shown in **Fig. 1** RSV F and G sequences were human codon optimized using a tool by GenScript found at <https://www.genscript.com/tools/gensmart-codon-optimization>. Codon-optimized sequences were then modified to remove homopolymers (>5 nucleotides) and premature poly A signals (AATAAA), which have previously been shown to impact RSV F protein synthesis from transfection. All plasmid maps are available at https://github.com/jbloomylab/RSV-evolution-neut/tree/main/04_plasmid_maps

For titering/neutralization assays:

- 96 white plate with clear bottom (VWR 655098)
- white sticker backing (REvvity 6005199)
- BrightGlo (Promega E2620)
- 96 well clear plates (for set up, Fisher 720089)
- monoclonal antibody or sera of interest

Producing RSV Pseudovirus

1 Production

Day 0:

- Trypsinize, spin down and plate 3e5 HEK293T cells/mL onto a 6 well TC coated dish (2mL D10 per well 6e5 cells per well)
- Make sure cells are in single cell suspension, we are aiming for ~50-70% confluence at the time of transfection.

Day 1:

- Prep DNA (this can also be done on day 0)
- We follow this [protocol](#) except we do not swap media before transfecting. We add 2ug of DNA per well of a 6 well dish.
- Half of this (1ug) is backbone, 200 ng is RSV F, 100 ng is RSV G 31AA CT del, and the rest is split between the 3 helper plasmids. See an example of this here:

A	B	C	D	E	F	G	H	I	J	K
Plate and Well(s)	Treatment	RSV F (uL to add at 100 ng/uL)	RSV Long G 31AA CT del (uL to add at 100 ng/uL)	VSV-G (uL to add at 100 ng/uL)	Carrier DNA (uL to add at 100 ng/uL)	Backbone ex. 2728 (uL to add at 100 ng/uL)	HDM - tat1b (uL to add at 100 ng/uL)	pRC-CMV - Rev1b (uL to add at 100 ng/uL)	HDM - Hgpm2 (uL to add at 100 ng/uL)	Total ng DNA
1-1	RSV psv 1	2	1	-	-	10	2.33	2.33	2.33	2000
1-2	RSV psv 2	2	1	-	-	10	2.33	2.33	2.33	2000
1-3	RSV psv 3	2	1	-	-	10	2.33	2.33	2.33	2000
1-4	RSV psv 4	2	1	-	-	10	2.33	2.33	2.33	2000
1-5	Bald/Negative control	-	-	-	3	10	2.33	2.33	2.33	2000
1-6	VSV-G/Positive control	-	-	2	-	10	2.33	2.33	2.33	2000

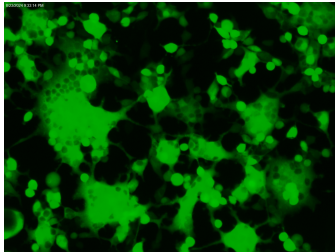
Example of set up for transfections for making pseudotyped lentiviral particles. Relevant expression plasmids for the RSV F and G sequences can be found on AddGene at <https://www.addgene.org/browse/article/28253256/>. All plasmid maps are available at https://github.com/jbloomlab/RSV-evolution-neut/tree/main/04_plasmid_maps. The lentivirus helper plasmids are available from AddGene: HDM-tat1b product ID 204154, pRC-CMV-Rev1b product ID 20413, HDM-Hgpm2 product ID 204152. The VSV-G expression plasmid used as a positive control is also available from AddGene: HDM_VSV_G product ID 204156. A lentiviral backbone plasmid that uses a CMV promoter to express luciferase followed by an IRES and ZsGreen is available from AddGene pHAGE-CMV-Luc2-IRES-ZsGreen-W product ID 164432.

- We typically mix the DNA for one treatment/condition in each centrifuge tube. After mixing these on our bench, we bring them into the tissue culture room.
- Check cells to make sure they are appropriate density
- Vortex and spin down DNA mix, then add appropriate amount of DMEM (100 uL per well of 6 well dish). Vortex and spin down DMEM+DNA mix. Add appropriate amount of BioT (3 uL per well of 6 well dish). Vortex and spin down DMEM+DNA+BioT mix.
- Let rest in the hood for ~15 minutes (aim for 10-20 minutes).

- Add total volume dropwise to cells, mark wells with condition and place them back in the incubator.

Day 3:

- Around 48 hrs post transfection (we have noticed between 40 and 48hrs has worked fine) its time to 'harvest' the virus. At this point we consider all disposables to be treated as virus waste and are bleached/disposed of accordingly.
- Image producer cells. They should be green (from backbone expression) and with RSV pseudovirus there are often lots of dead/floating cells and syncytia (example below is RSV Long strain, this can get much more impressive with clinical strains, sometimes no cells are left attached to the plate by 48 hrs but titers are still fine).



An example of 293T cells 48 hrs post transfection to produce RSV lentiviral particles pseudotyped with the lab-adapted, subtype A strain of RSV commonly referred to as the "Long strain."

- Syringe filter supernatant from well into labeled externally threaded cryotube (Fisher 02-912-728 DWK Life Sciences Wheaton™ CryoELITETM Cryogenic Storage Vials). Draw supernatant into a 3 mL syringe with luer lock tip. Then attach a .45 syringe filter (Corning 431220 Syringe Filter 0.45 micron membrane; 50/case) and filter into the externally threaded cryotube or another vial of choice, aliquot according to your needs (a volume that will minimize freeze thaw cycles). Note: we have tested other methods and none outperform syringe filtering for us.
- In our hands it is crucial that RSV pseudovirus is flash frozen after harvest. We have noticed some drop in titer when RSV pseudovirus is stored at 4C/on ice but little to no drop when RSV pseudovirus is left at room temperature (for hours). In order to keep titers as high as 'fresh' unfrozen virus it is necessary to flash freeze RSV pseudovirus before storing long term at -80C.

To flash freeze:

- We use a metal cooling rack (Fisher UX-04392-51 CoolRack® CF45 Cooling Block) placed in a rectangular ice bucket with dry ice beneath and surrounding the sides of the metal block. We allow this to cool on our bench for at least 15 minutes or until the metal block is visibly cold/frosty. **It is best to prepare the block before filtering.**
- Once the virus is filtered, aliquoted and ready to freeze. Place in the freezing block until visibly frozen (usually the liquid will turn from D10 pink to a yellow color when fully frozen). Bring the block out of tissue culture and to the -80 to transfer vials into -80 box/rack.

2 Titer

Day 0:

- Trypsinize, spin down and plate 60,000 HEK293T **TIM1 overexpressing** cells/well (in 100 uL per well) onto a 96 well TC coated dish (clear if running flow or transferring for luciferase or ideally clear bottomed white plate *Fisher 655098 Greiner Bio-One CELLSTAR 96-well, Cell Culture-Treated, Flat-Bottom Microplate* if running luciferase). Make sure cells are in single cell suspension, we are aiming for ~90% confluence at the time of transduction/infection.

Day 1:

- Get together a plan for your titer set up, here is a minimal example (more details in the linked spreadsheet):



	A	B	C	D	E	F	G	H	uL virus
		RSV psv 1	RSV psv 2	RSV psv 3	RSV psv 4	Negati ve contro l (Bald)	Positiv e contro l (VSV- G)		
		120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10		50
		120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10		25
		120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10		12.5
		120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10		6.25
		120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10		3.125
		120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10	120uL virus + 120uL D10		1.5625

perform dilution series (usually 1:2) in a set up plate
add 100 uL of virus dilution to HEK 293T TIM1 over expressing cells plated 24 hrs earlier @
60,000 cells/well

- Check the health and density of your cells.
- Get your pseudovirus from the -80 and thaw your virus at 37C in the water bath. We use a floating rack and swirl the virus until fully thawed (its okay to leave them at 37C past when they are fully thawed, we have left them in the bath accidentally for an hour with no noticeable change in titer).
- Add D10 to the set up plate.
- Add virus to the set up plate, mix and perform serial dilutions.
- Add 100 uL of the serially diluted virus to the cells

Day 3:

- Around 48 hrs post transduction/infection (we have also done 72hrs and it has worked fine) its time to 'harvest' the titer.
- Image infected cells. This can be helpful as reference since brightglo will lyse cells and if running flow you will take the whole well.

For BrightGlo (RLU/uL):

- We use brightglo (Promega E2620 Bright-Glo™ Luciferase Assay System, 10×100 mL). This reagent is aliquoted to 3.5mL in 5 mL amber tubes and stored at -80. Remove one aliquot per plate to thaw ~1 hr before harvesting/reading the plate.
- Remove 150 uL of media from the 96 well plate (all wells/whole plate). We remove this amount to aim for an equal amount of media volume to bright glo. We will be adding 30uL of brightglow so we assume some evaporation and remove only 150uL of media to leave 30 uL of media in the plate. We remove from the 'bottom' (least expected positive cells/highest virus concentration) and hover over our bleach to dispel liquid. This allows us to use one row of p200 tips per plate. If reading many plates it is fine for these to sit in the hood with their media removed. We typically remove media from all plates before starting to add bright glo.
- Turn off the lights in the hood (since bright glo is light sensitive) and put the bright glo into a reagent reservoir. Get out a full box of SureOne 100uL tips per plate you will be

reading. We have found that you can add bright glo and read around 4 plates at a time without disrupting the timing of the reagent, so we typically do this in round of 4.

- Hover to add 30 uL of bright glo per well. Then use this same row of tips to mix and scrape the cells only row. We mix ~10x and then vigorously scrape the bottom of the well to remove/lyse all cells, then mix 5-10x again.
- If you plated on a clear bottom white plate then you just have to grab a new row of tips and mix/scrape for each row of the plate (this should end up being one box of tips per plate). Continue with the rest of the plates in your set.
- If you plated on a fully clear 96 well plate then you will need to transfer the media/cell/bright glo mix to an opaque white plate. We typically do this by directly transferring over the full volume during the mixing process, using the same tips per row.
- Cover the plates with tin foil and place in a secondary container. Bring plates to the plate reader, add white sticker to the bottom of the plate and read luminescence values.
- Calculate RLU/uL, check to ensure you are calculating from within the linear range.

Running Neutralization Assay

3 Day 0:

- Trypsinize, spin down and plate 60,000 HEK293T **TIM1 overexpressing** cells/well (in 100 uL per well) onto a 96 well TC coated dish (clear if running flow or transferring for luciferase or ideally clear bottomed white plate Fisher 655098 Greiner Bio-One CELLSTAR 96-well, Cell Culture-Treated, Flat-Bottom Microplate if running luciferase). Make sure cells are in single cell suspension, we are aiming for ~90% confluence at the time of transduction/infection.

Day 1:

- Make sure you have a plan for your neutralization assay set up. Also make sure all reagents are appropriately prepared (sera is heat inactivated, mAbs are diluted+nano dropped etc.) Here is an example (more details in the linked spreadsheet):

	A	Antibody/sera 1 (replicate 1)	Antibody/sera 1 (replicate 2)	Antibody/sera 2 (replicate 1)	Antibody/sera 2 (replicate 2)	Antibody/sera 3 (replicate 1)	Antibody/sera 3 (replicate 2)
cells		cells	cells	cells	cells	cells	cells
cells		Nirsevimab (most antibody)	Nirsevimab (most antibody)	Clesrovimab (most antibody)	Clesrovimab (most antibody)	Suptavumab (most antibody)	Suptavumab (most antibody)
cells		Nirsevimab	Nirsevimab	Clesrovimab	Clesrovimab	Suptavumab	Suptavumab
cells		Nirsevimab	Nirsevimab	Clesrovimab	Clesrovimab	Suptavumab	Suptavumab
cells		Nirsevimab	Nirsevimab	Clesrovimab	Clesrovimab	Suptavumab	Suptavumab
cells		Nirsevimab	Nirsevimab	Clesrovimab	Clesrovimab	Suptavumab	Suptavumab
cells		Nirsevimab	Nirsevimab	Clesrovimab	Clesrovimab	Suptavumab	Suptavumab
cells		Nirsevimab	Nirsevimab	Clesrovimab	Clesrovimab	Suptavumab	Suptavumab
cells		Nirsevimab	Nirsevimab	Clesrovimab	Clesrovimab	Suptavumab	Suptavumab



	A	Antibody/sera 1 (replicate 1)	Antibody/sera 1 (replicate 2)	Antibody/sera 2 (replicate 1)	Antibody/sera 2 (replicate 2)	Antibody/sera 3 (replicate 1)	Antibody/sera 3 (replicate 2)
cells	cells	Nirsevimab (least antibody)	Nirsevimab (least antibody)	Clesrovimab (least antibody)	Clesrovimab (least antibody)	Suptavumab (least antibody)	Suptavumab (least antibody)
cells	cells	Virus + Cells	Virus + Cells	Virus + Cells	Virus + Cells	Virus + Cells	Virus + Cells
cells	cells	cells	cells	cells	cells	cells	cells

White-walled clear bottom TC plates were plated with 60000 cells (293T-TIM1) per well (100uL per well)
 For each virus, aim for 600000 RLU/well, more is fine as long as you arent in ligand depletion range
 Each well will ultimately have 50uL virus + 50uL sera/mAb or 100uL total D10 for cells only
 Add 50uL appropriate virus as detailed in the plate maps
 Incubate at 37C 5% CO2 (TC incubator) for 1 hour
 Then transfer 100 uL from each well of the plate to the plate that was seeded with indicated target cells

- Check the health and density of your cells, we have gotten fine results with cells 80-95% confluent on day of set up. Note TIM1 overexpressing 293Ts usually look clumpier than normal 293Ts.
- To do these neutralization assays we make 'set up' plates. We have one set up plate per cell plate (if that makes sense). In the end our set up plate (usually just a normal TC coated clear 96 well plate) will have 50uL of mAb/sera dilution and 50uL of virus per well. These are then incubated together for ~an hour before the full 100 uL volume is added to the cells.
- To start out we get together the appropriate amount of 96 well 'set up' plates and label them with the virus and mAb/sera that will be added according to our plan. Being organized on this plate lid will help down the line when 'stamping' the mAb/sera and also when reading the plate as we transfer the same lid for use throughout.
- Set up your plate of mAb/sera dilutions. We make one plate of this at the total volume we will need for the experiment and then 'stamp' the appropriate volume (50 uL) in the appropriate place on our set up plate.
- For the stamping we use just one row of tips per mAb/sera. We get out all the plates getting this mAb/sera and add 50 uL of the serial dilution to two rows next to each other (2 within experiment replicates).
- Get your pseudovirus from the -80 and thaw your virus at 37C in the water bath. We use a floating rack and swirl the virus until fully thawed (its okay to leave them at 37C past when they are fully thawed, we have left them in the bath accidentally for an hour with no noticeable change in titer).
- Make the appropriate volume of D10 + virus mixture to add to your plates. We aim for adding around 600,000 to 2,000,000 RLU per well. This will be different for your own plate reader so you will have to figure out the range that is best for your system.
- Add 50 uL of virus to the set up plate (stamp this as well), add to all conditions with mAb/sera as well as a row for 'virus only'. Obviously avoid adding virus to the 'cells only' wells.
- Place the set up plates in the incubator for around an hour.
- Add 100 uL/full volume from the set up plate to the appropriate place on the cell plate. You can hover to add and reuse tips for the same plate as long as you move from least amount of mAb/sera to most amount of mAb/sera (bottom to top).
- Make sure outer wells/cell only wells also get 100 uL of D10.

Day 3:

- Around 48 hrs post transduction/infection (we have also done 72hrs and it has worked fine) its time to 'harvest' the neut.
- Image infected cells. This can be helpful as reference since brightglo will lyse cells.

For BrightGlo/plate reader:

- We use brightglo (Promega E2620 Bright-Glo™ Luciferase Assay System, 10×100 mL). This reagent is aliquoted to 3.5mL in 5 mL amber tubes and stored at -80. Remove one aliquot per plate to thaw ~1 hr before harvesting/reading the plate.



- Remove 150 uL of media from the 96 well plate (all wells/whole plate). We remove this amount to aim for an equal amount of media volume to bright glo. We will be adding 30uL of brightglo so we assume some evaporation and remove only 150uL of media to leave 30 uL of media in the plate. We remove from the 'top' (least expected positive cells/highest virus concentration/lowest mAb/sera concentration) and hover over our bleach to dispel liquid. This allows us to use one row of p200 tips per plate. If reading many plates it is fine for these to sit in the hood with their media removed. We typically remove media from all plates before starting to add bright glo.
- Turn off the lights in the hood (since bright glo is light sensitive) and put the bright glo into a reagent reservoir. Get out a full box of SureOne 100uL tips per plate you will be reading. We have found that you can add bright glo and read around 4 plates at a time without disrupting the timing of the reagent, so we typically do this in round of 4.
- Hover to add 30 uL of bright glo per well. Then use this same row of tips to mix and scrape the cells only row. We mix ~10x and then vigorously scrape the bottom of the well to remove/lyse all cells, then mix 5-10x again.
- If you plated on a clear bottom white plate then you just have to grab a new row of tips and mix/scrape for each row of the plate (this should end up being one box of 100uL tips per plate). Continue with the rest of the plates in your set.
- If you plated on a fully clear 96 well plate then you will need to transfer the media/cell/bright glo mix to an opaque white plate. We typically do this by directly transferring over the full volume during the mixing process, using the same tips per row.
- Cover the plates with tin foil and place in a secondary container. Bring plates to the plate reader, add white sticker to the bottom of the plate and read luminescence values.
- Calculate IC50s and plot data using [neutcurve](#).

Other advice

- 4
 - For the helper plasmids we order large preps from GenScript or we grow up large preps and send for whole plasmid sequencing (Plasmidsaurus)
 - What was our success rate on pseudotyping different F sequences? we would make mutations in a 'good' codon optimization or from a strain we knew had high titers and we had no failures
 - For 293T TIM1 cells we do not regularly stain for TIM1 expression. TIM1 is marked with BFP so cells appear slightly blue under the microscope and when running flow we have seen minimal BFP silencing after passaging for around a month. We do not think this is a toxic protein so cells are happy and we do not worry much about passaging.
 - We did not have a specific aim for a cells only control (RLU)
 - For lab adapted sequences we have used the ultracentrifuge to concentrate. We have not done this for neuts but we have also seen spinfection increase titers for RSV pseudovirus
 - For spinfection, after adding virus to cells we spin at 900 g and 30°C for 1-3hrs before transferring to the 37°C CO2 incubator we have seen this increase infection around 4 fold and could be useful for lower titer RSV F sequences although we have not tried this in a neutralization assay context yet
 - Do we supplement media before freezing RSV pseudovirus? no, we tested this and it was worse
 - We have also tried Takara's Xfect reagent (631318) with good results