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Generation, amplification and titration of recombinant respiratory syncytial viruses to monitor viral multiplication and viral factories dynamics

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Paris, September the 26th 2018

Dear editor,

We submit a manuscript entitled "Generation, amplification and titration of recombinant respiratory syncytial viruses to monitor viral multiplication and viral factories dynamics." for publication in Jove responding to your kind invitation.

Reverse genetics is a powerful technology widely used in virology field. Rescue, amplification and titration of recombinant RSV have been long published. However, these "basic" methods remain difficult to handle and require a specific knowhow.

In this manuscript we describe in details the rescue and the amplification of recombinant RSV using 2 examples. We also detail an original titration assay method using microcrystalline cellulose (Avicel) overlay. We then illustrate how recombinant RSV expressing a reporter gene might be used to monitor viral multiplication to assess the impact on viral growth of drugs or cellular protein knock down. We also provide example of analysis of the dynamics of viral structure thanks to a recombinant RSV expressing a viral fluorescent protein.

RSV infection burden is major, leading to significant mortality in infants and elderly worldwide. There are still no vaccine neither efficient antivirals available yet. The tools and methods described here would facilitate the comprehension of RSV multiplication mechanisms and development of potential antivirals. These methods, especially the plaque assay protocol, may be adapted to other viruses.

We strongly believe that the present study will be of high interest to the readership of Jove and look forward to hearing back from you about this submission,

Yours sincerely,

Marie-Anne Rameix-Welti



TITLE:

Generation, Amplification, and Titration of Recombinant Respiratory Syncytial Viruses

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KEYWORDS:

Reverse genetics; respiratory syncytial virus; infection; recombinant virus; amplification; titration; tag; fluorescence; quantification, video microscopy

SUMMARY:

We describe a method for generating and amplifying genetically modified respiratory syncytial viruses (RSVs) and an optimized plaque assay for RSVs. We illustrate this protocol by creating two recombinant viruses that respectively allow quantification of RSV replication and live analysis of RSV inclusion bodies and inclusion bodies-associated granules dynamics.

ABSTRACT:

The use of recombinant viruses has become crucial in basic or applied virology. Reverse genetics has been proven to be an extremely powerful technology, both to decipher viral replication mechanisms and to study antivirals or provide development platform for vaccines. The construction and manipulation of a reverse genetic system for a negative-strand RNA virus such as a respiratory syncytial virus (RSV), however, remains delicate and requires special know-how. The RSV genome is a single-strand, negative-sense RNA of about 15 kb that serves as a template

for both viral RNA replication and transcription. Our reverse genetics system uses a cDNA copy of the human RSV long strain genome (HRSV). This cDNA, as well as cDNAs encoding viral proteins of the polymerase complex (L, P, N, and M2-1), are placed in individual expression vectors under T7 polymerase control sequences. The transfection of these elements in BSR-T7/5 cells, which stably express T7 polymerase, allows the cytoplasmic replication and transcription of the recombinant RSV, giving rise to genetically modified virions. A new RSV, which is present at the cell surface and in the culture supernatant of BSR-T7/5, is gathered to infect human HEp-2 cells for viral amplification. Two or three rounds of amplification are needed to obtain viral stocks containing 1×10^6 to 1×10^7 plaque-forming units (PFU)/mL. Methods for the optimal harvesting, freezing, and titration of viral stocks are described here in detail. We illustrate the protocol presented here by creating two recombinant viruses respectively expressing free green fluorescent protein (GFP) (RSV-GFP) or viral M2-1 fused to GFP (RSV-M2-1-GFP). We show how to use RSV-GFP to quantify RSV replication and the RSV-M2-1-GFP to visualize viral structures, as well as viral protein dynamics in live cells, by using video microscopy techniques.

INTRODUCTION:

Human RSV is the leading cause of hospitalization for acute respiratory tract infection in infants worldwide¹. In addition, RSV is associated with a substantial disease burden in adults comparable to influenza, with most of the hospitalization and mortality burden in the elderly². There are no vaccines or specific antivirals available yet against RSV, but promising new drugs are in development^{3,4}. The complexity and the heaviness of the techniques of quantification of RSV multiplication impede the search for antivirals or vaccines despite current considerable efforts. The quantification of RSV multiplication in vitro is generally based on laborious, time-consuming, and expensive methods, which consist mostly in the analysis of the cytopathic effect by microscopy, immunostaining, plaque reduction assays, quantitative reverse transcriptase (qRT)-polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay tests. Viruses with modified genomes and expressing reporter genes, such as those coding for the GFP, are more suitable for such screenings. Coupled to the use of automated plate readers, reporter gene-carrying recombinant viruses can make these assays more suitable for standardization and high-throughput purposes.

RSV is an enveloped, nonsegmented negative-sense RNA virus that belongs to the *Orthopneumovirus* genus of the *Pneumoviridae* family, order *Mononegavirales*⁵. The RSV genome is a single-strand, negative-sense RNA of about 15 kb, which contains a noncoding region at the 3' and 5' extremities called Leader and Trailer and 10 transcriptional units encoding 11 proteins. The genes are ordered as follows: 3'-NS1, NS2, N, P, M, SH, G, F, M2 (encoding for M2-1 and M2-2 proteins) and L-5'. The genomic RNA is tightly packaged by the nucleoprotein N. Using the encapsidated genomic RNA as a template, viral RNA-dependent RNA polymerase (RdRp) will ensure transcription and replication of the viral RNA. Viral RdRp is composed of the large protein L which carries the nucleotide polymerase activity per se, its mandatory cofactor the phosphoprotein P and the M2-1 protein which functions as a viral transcription factor⁶. In infected cells, RSV induces the formation of cytoplasmic inclusions called inclusion bodies (IBs). Morphologically similar cytoplasmic inclusions have been observed for several

Mononegavirales^{7–10}. Recent studies on rabies virus, vesicular stomatitis virus (VSV), Ebola virus, and RSV showed that viral RNA synthesis occurs in IBs, which can thus be regarded as viral factories^{8,9,11,12}. The virus factories concentrate the RNA and viral proteins required for viral RNA synthesis and also contain cellular proteins^{13–17}. IBs exhibit a functional subcompartment called IB-associated granules (IBAGs), which concentrate the newly synthesized nascent viral mRNA together with the M2-1 protein. The genomic RNA and the L, P, and N are not detected in IBAGs. IBAGs are small dynamic spherical structures inside IBs that exhibit the properties of liquid organelles¹². Despite the central role of IBs in viral multiplication, very little is known about the nature, internal structure, formation, and operation of these viral factories.

The expression of the genome of a poliovirus from a cDNA enabled the production of the first infectious viral clone in 1981¹⁸. For single-stranded negative RNA viruses, it was not until 1994 that the production of a first rabies virus following transfection of plasmids into cells¹⁹ took place. The first plasmid-based reverse genetic system for RSV was published in 1995²⁰. Reverse genetics have led to major advances in the field of virology. The possibility of introducing specific modifications into the viral genome has provided critical insights into the replication and pathogenesis of RNA viruses. This technology has also greatly facilitated the development of vaccines by allowing specific attenuation through targeted series of modifications. Genome modifications allowing a rapid quantification of viral multiplication greatly improved the antiviral screening and study of their mode of action.

Although previously described, obtaining genetically modified RSVs remains delicate. Here, we detail a protocol to create two types of recombinant HRSV, respectively expressing RSV-GFP or RSV-M2-1-GFP. In this protocol, we describe the transfection conditions needed to rescue the new recombinant viruses, as well as their amplification to obtain viral stocks with high titer, suitable for reproducible experimentations. The construction of the reverse genetics' vectors per se is not described here. We do describe methods for the optimal harvesting and freezing of viral stocks. The most accurate method to quantify viral infectious particles remains plaque assay. Cells are infected with serial dilutions of the analyzed suspension and incubated with an overlay that prohibits the diffusion of free viral particles in the supernatant. In such conditions, the virus will only infect contiguous cells forming a "plaque" for each initial infectious particle. In the conventional RSV titration assay, plaques are revealed by immunostaining and counted under microscopic observation. This method is expensive and time-consuming. Here we described a very simple protocol for an RSV plaque assay using microcrystalline cellulose overlay that enables the formation of plaques visible to the naked eye. We show how RSV-GFP can be used to measure RSV replication and, thus, to quantify the impact of antivirals. Combining reverse genetics and live imaging technology, we demonstrate how RSV-M2-1-GFP allows scientists to visualize M2-1 in live cells and to follow the dynamics of intracellular viral structures, such as IBs.

PROTOCOL:

1. Material preparation

1.1) Purchase cell media (reduced serum media, minimum essential media [MEM], 10x MEM, and Dulbecco's modified Eagle's medium [DMEM]), transfection reagent, and microcrystalline cellulose (see **Table of Materials**).

1.2) Obtain the following vectors for reverse genetics: the genomic vector(s) and the expression vectors encoding the N protein and the polymerase complex proteins. The genomic vectors contain the full cDNA genome of RSV-GFP (p-RSV-GFP) and of RSV-M2-1GFP (p-RSV-M2-1GFP) downstream from the bacteriophage T7 RNA polymerase (T7 pol) promoter. The expression vectors (designated as p-N, p-P, p-L, and p-M2-1) contain the coding sequence of N, P, L, or M2-1 downstream the T7 pol (see Rincheval et al.¹² and Rameix-Welti et al.²¹ for details regarding the plasmid constructs).

1.3) Prepare media for a cell culture in a sterile environment and for the transfection and infection. Use DMEM with 2 mM L-glutamine supplemented with 10% fetal calf serum (FCS), 1,000 units/mL penicillin, and 1 mg/mL streptomycin (or without antibiotics) and MEM with 2 mM L-glutamine supplemented with 0%, 2%, or 10% FCS, 1,000 units/mL penicillin, and 1 mg/mL streptomycin, designated as "complete medium" in the following protocol.

1.4) Obtain BSRT7/5²² cells and make stocks in complete medium supplemented with 10% dimethyl sulfoxide (DMSO) at 1 to 2 x 10⁶ cells/mL. Conserve the cell stocks in liquid nitrogen. Obtain HEp-2 cells. Culture BSRT7/5 cells in complete DMEM and HEp-2 cells in complete MEM at 37 °C and 5% CO₂ in a sterile environment.

1.5) Prepare a 10x RSV conservation solution (0.5 M HEPES and 1 M MgSO₄ [pH 7.5] in water) in a sterile environment.

1.6) Obtain an inverted fluorescence microscope compatible with GFP fluorescence measurements and compatible with live imaging if it is necessary to monitor an infection. Obtain a microplate reader compatible with GFP fluorescence measurements for the quantitation of RSV-GFP replication.

2. Rescue and first passage of recombinant virus

NOTE: Perform all the following steps in a sterile environment, using a class II safety cabinet.

2.1) The day before transfection, make a suspension of the BSRT7/5 cell line at 5 x 10⁵ cells/mL in complete medium. Distribute 2 mL of cell suspension per well in a 6-well plate. Prepare one well per virus that is going to be rescued and one additional well for negative control. Incubate the plate at 37 °C and 5% CO₂. Check that the cells are at a 80%–90% confluence the next day.

2.2) Unfreeze the reverse genetics vectors (from step 1.2) p-RSV-GFP and p-RSV-M2-1-GFP, as well as p-N, p-P, p-L, and p-M2-1. Mix, for each virus to rescue, 1 µg of p-N and p-P, 0.5 µg of p-L, 0.25 µg of p-M2-1, and 1.25 µg of p-RSV (GFP or M2-1 GFP) in a tube.

NOTE: Different expression vectors for N, P, L, and M2-1 may be used; however, the ratio between the proteins has to be maintained. Perform the negative control by replacing the p-RSV vector with an empty vector.

2.3) Proceed to transfection, following the transfection reagent manufacturer's protocol (see **Table of Materials**).

2.3.1) Add 250 μ L of reduced serum medium to the mixed vectors. In another tube, dilute 10 μ L of the transfection reagent in 250 μ L of reduced serum medium. Gently vortex both tubes and wait for 5 min. Mix the contents of both tubes and wait for 20 min at room temperature.

2.3.2) Rinse the BSRT7/5 cells with 1 mL of reduced serum medium and distribute 1.5 mL of MEM with 10% FCS without antibiotics per well. If necessary, incubate at 37 °C and 5% CO₂ until the incubation described in step 2.3.1 is completed.

2.3.3) Add 500 μ L of the transfection mix prepared in step 2.3.1 to a well when the 20 min incubation time is over. Place the cells in the incubator at 37 °C and 5% CO₂ for 3 days. Do not change the culture medium of the cells during the transfection.

2.4) Observe GFP fluorescence (excitation at 488 nm and emission at 515–535 nm) under an inverted fluorescence microscope at 20x magnification 1x per day to monitor the rescue efficiency, using the GFP filter.

2.5) On the second day after the transfection, seed the cells for the first passage of the rescued viruses. Prepare a suspension of HEp-2 cells at 5×10^5 cells/mL in complete medium. Distribute 2 mL of cell suspension per well in a 6-well plate (one well per virus to rescue and one negative control).

2.6) On the third day of transfection, scratch cells in each well of the transfected BSRT7/5 6-well plate, using a different scraper for each well. Transfer each well content (cells and supernatant) into a sterile 2 mL microcentrifuge tube. Vortex each tube vigorously for at least 30 s to release the rescued virus from the cell membranes.

NOTE: This corresponds to passage 0 (P0) of the rescued virus (**Figure 1**).

2.7) Use the fresh viral P0 suspension to perform the first amplification of the rescued viruses.

2.7.1) Remove the culture medium from the HEp-2 6-well plate seeded the day before (see step 2.5) and quickly add 500 μ L of the P0 suspension (from step 2.6) per well. Place the HEp-2 plate at 37 °C on a see-saw rocker for soft agitation for 2 h.

2.7.2) Remove and discard the 500 μ L of inoculum and add 2 mL of MEM with 2% FCS. Incubate

the plate at 37 °C and 5% CO₂ for 3 days. This will produce the first passage (P1) of the rescued viruses (**Figure 1**).

2.8) Add 1/10 of the volume of 10x RSV conservation solution (0.5 M HEPES and 1 M MgSO₄ [pH 7.5]) into the remaining P0 suspension (from step 2.6). Vortex the microtubes vigorously for 5 s and aliquot the contents in cryogenic tubes labeled with alcohol-resistant tags. Immerse the tubes for at least 1 h in alcohol precooled at -80 °C, and store them at -80 °C.

2.9) Titrate the P0 stock (see step 2.6) of each rescued virus (see section 4 for the microcrystalline cellulose titration).

2.10) Observe GFP fluorescence (excitation at 488 nm and emission at 515–535 nm) of the HEp-2 cells infected with the P0 suspension under an inverted fluorescence microscope at 20x magnification 1x per day to monitor the infection. Observe under a brightfield microscope the appearance of small syncytia and cell detachment which reflects the RSV cytopathogenic effect (CPE) (see **Figure 2**).

2.11) Note that the rescue has failed if neither fluorescence nor CPE is visible after 2–3 days.

2.12) Collect the first passage (P1) at day 3 or 4 as described in step 2.6. In brief, scrape the cells, collect the cells and supernatant together, vortex them, add the conservation solution as described in step 2.8, aliquot the sample, and freeze it.

2.13) Titrate (see section 4 for the titration assay) and amplify the first passage (see section 3 for the amplification).

3. Amplification of the rescued viruses

NOTE: The following protocol describes the amplification of the rescued viruses in a 75 cm² flask. Adapt the flask size to the volume needed and the required multiplicity of infection (MOI). **Table 1** indicates volumes for different flasks. Perform all the following steps in a sterile environment in a class II safety cabinet.

3.1) Prepare a suspension of HEp-2 cells at 5 x 10⁵ cells/mL in complete medium, the day prior to the amplification. Distribute 15 mL of the cell suspension per 75 cm² flask and incubate the flasks at 37 °C and 5% CO₂. Prepare one flask per virus to amplify.

3.2) The day after the start of the incubation, check that the cells are 80%-100% confluent.

3.3) Dilute the viral suspension from step 2.12 in MEM without FCS to obtain a 3 mL suspension at 50,000 PFU/mL (corresponding to an MOI of 0.01 PFU/cell).

3.4) Remove the medium and quickly add the 3 mL viral suspension. Place the flask at 37 °C on a

see-saw rocker for soft agitation for 2 h.

3.5) Remove and discard the inoculum and add 15 mL of MEM with 2% FCS. Incubate at 37 °C and 5% CO₂ for 2–4 days.

3.6) Check the cell morphology and GFP fluorescence (excitation at 488 nm and emission at 515–535 nm) under an inverted fluorescence microscope at 20x magnification in order to estimate the right time to harvest the viruses. Note that this is usually when 50%–80% of the HEp-2 cell layer is detached due to the RSV CPE that occurs between 48 and 72 h postinfection (p.i.) (see **Figure 3**).

3.7) Scrape all the cells using a cell scraper. Collect both the cells and the supernatant together and transfer them to a 50 mL centrifuge tube.

3.8) Add 1/10 of the volume of the 10x RSV conservation solution (0.5 M HEPES and 1 M MgSO₄ [pH 7.5]). Vortex the tubes vigorously for 5 s and clarify the suspension by a 5 min centrifugation at 200 x g.

3.9) Transfer the supernatant to a 50 mL tube. Vortex briefly and aliquot the suspension in cryogenic tubes labeled with alcohol-resistant tags. Immerse the tubes in precooled -80 °C alcohol for at least 1 h and store them at -80 °C.

3.10) Unfreeze one of the aliquots to titrate the viral suspension (see section 4).

4. Plaque titration assay

4.1) Prepare 12-well plates for titration the day before the titration assay is performed (six wells will be required to titrate one tube of virus). Seed the wells with 1 mL of HEp-2 cells at 5 x 10⁵ cells/mL in complete medium.

4.2) The next day, prepare a sterile microcrystalline cellulose suspension (2.4% [w/v] in water) (see **Table of Materials**).

4.2.1) Disperse 2.4 g of microcrystalline cellulose powder in 100 mL of distilled water, using a standard magnetic stirrer, until complete dissolution of the powder (usually 4–12 h). Autoclave the suspension at 121 °C for 20 min and store it at room temperature before use.

NOTE: Under such conditions, the suspension is stable for 1 year.

4.2.2) After opening the solution in a sterile environment, store it at 4 °C for 6 months. Always mix the suspension before use (by hand shaking or vortexing) to make sure it is homogeneous.

4.3) Prepare the 2x MEM in a sterile environment. Dilute commercial MEM 10x with sterile water

and add L-glutamine, 1,000 units/mL penicillin, and 1 mg/mL streptomycin. Shake the dilution vigorously and store it at 4 °C.

NOTE: Perform steps 4.4–4.10 in a sterile environment using a class II safety cabinet.

4.4) Prepare six tubes containing 900 µL of MEM without FCS per virus to be titrated (the titration tubes). Thaw the virus aliquots, vortex them vigorously for 5 s, and transfer 100 µL to the first titration tube.

4.5) Perform a tenfold dilution 6x, as follows. Add 100 µL of virus to 900 µL of medium in the first tube, put the cap on the tube, and mix its contents by vortexing for a few seconds. Change the tip on the pipette, add 100 µL of the first dilution to 900 µL of medium in the second tube, put the cap on the tube, and vortex. Repeat the procedure until the sixth tube.

NOTE: It is very important to change the tip for each dilution.

4.6) Write the virus name and the fold dilutions on the HEp-2 12-well plates. Add a mark to match the plate and its cover because they may be separated during staining (step 4.9). Remove the medium from the plates and distribute 400 µL of one dilution per well. Incubate the plates at 37 °C for 2 h, for virus adsorption.

NOTE: Change the pipette tip between each inoculum or proceed from lowest to highest concentration with the same tip. Inoculate a limited series of plates (1 to 2) simultaneously to avoid the cells drying.

4.7) Prepare the microcrystalline cellulose overlay during the virus adsorption (extemporaneous preparation). To obtain 100 mL of overlay, mix 10 mL of 2.4% microcrystalline cellulose suspension, 10 mL of 2x MEM, and 80 mL of MEM with 2% FCS.

4.7.1) Adjust the pH of the 2x MEM to around 7.2 with a sterile sodium bicarbonate solution at 7.5%, following the color indicator. Add the microcrystalline cellulose suspension and the MEM and mix vigorously.

4.8) At the end of the 2 h incubation, add 2 to 3 mL of overlay to each well of the 12-well plates without removing the inoculum. Be careful to avoid the contamination of the adjacent wells with high viral titer inoculums. Incubate the plate at 37 °C and 5% CO₂ for 6 days. Do not move the plate and do not move the incubator during incubation.

4.9) Proceed to stain the cells, using crystal violet solution (8% crystal violet [v/v], 2% formaldehyde [v/v], and 20% ethanol [v/v] in water).

4.9.1) Protect the work surface of the biosafety cabinet with a sheet (the crystal violet strongly colors surfaces).

4.9.2) Gently shake the plates to take off the microcrystalline cellulose overlay. Remove the supernatants and wash the cells 2x with 1x phosphate-buffered saline (PBS). Handle the plates one by one to avoid the cells drying. Add 1–2 mL of the crystal violet solution and wait 10–15 min. Remove the solution, which can be reused for subsequent plate staining.

4.9.3) Immerse the plates and lids in fresh bleach for a few seconds and, then, wash them thoroughly with tap water. Note that the plates and covers are decontaminated by the bleach.

4.9.4) Put the plates and lids on paper towels and let them dry. Dry the plates at an ambient temperature after the water rinsing and store them at room temperature. For long storage periods (months), keep the plates protected from light to protect the color. Note that if the cells lose their coloration, they can be stained again with crystal violet.

4.10) Calculate virus titers. Count the plaques in the wells of the dry plates, which are visible to the naked eye. Check that the number of plaques of the different dilutions is coherent (factor 10 between each dilution). Choose the well on which the plaques are the easiest to count. Assess the number of plaques versus the inoculum volume and the dilution.

NOTE: On the example provided in **Figure 4**, 21 plaques are counted at the 10^{-5} dilution. These correspond to a titer of

$$\begin{aligned} \text{Titer} &= \frac{\text{plaque number}}{\text{dilution} \times \text{inoculum volume}} \\ &= \frac{21}{0.00001 \times 0.4} = 5,250,000 \text{ PFU/mL} \end{aligned}$$

5. The use of HRSV-GFP recombinant virus to monitor viral replication in cells treated with small interfering RNA or antivirals

NOTE: Perform all steps except 5.1 and 5.2.5 in a sterile environment using a class II safety cabinet.

5.1) Monitoring the effect of cellular gene silencing on RSV multiplication

NOTE: The transfection protocol depends on the reagent (see **Table of Materials**).

5.1.1) Prepare 96-well plates for the GFP measurement. Two days before the assay, for given small interfering RNA (siRNA), prepare a solution of reduced serum media containing siRNA at a concentration of 100 nM and an siRNA transfection reagent diluted at 1/500. Incubate the solution for 30 min at room temperature.

5.1.2) Add 25 μL of the solution to the wells of the plate prepared in 5.1.1 (in triplicate). Seed the wells with 75 μL of a suspension of A549 cells at 4×10^5 cells/mL in complete medium without

antibiotics to obtain a final cell concentration of 3×10^5 cells/mL. Incubate the plate for 48 h at 37 °C and 5% CO₂.

NOTE: The final siRNA concentration is 25 nM and the final transfection reagent volume is 0.5 µL/well).

5.1.3) Infect the cells as follows. Remove the medium from the wells. Add 100 µL of RSV-GFP suspension at 50,000 PFU/mL and incubate for 2 h at 37 °C and 5% CO₂. Remove the viral suspension and add 100 µL of DMEM with 2% FCS and without phenol red. Incubate the plate at 37 °C and 5% CO₂.

5.1.4) At 24 h and 48 h p.i., measure the fluorescence, using a spectrofluorometer set to excitation and emission wavelengths of 488 and 520 nm, respectively (fluorescence is expressed in relative fluorescence units). Use noninfected A549 cells as standards for fluorescence and background levels.

NOTE: The cells need to be fixed with 4% paraformaldehyde (PFA) before measuring them without the plate cover.

5.2) Assessment of the drug inhibition using RSV-GFP

5.2.1) Prepare 96-well plates for the GFP measurement. The day before the assay, seed the wells with 100 µL of a suspension of HEp-2 cells at 5×10^5 cells/mL in complete medium without phenol red.

5.2.2) Prepare a serial dilution of the tested drugs (AZ4316 in this example) in MEM complemented with 2% FCS and antibiotics (50 µL per well). Prepare a viral suspension at 10,000 PFU/mL in MEM without stromal vascular factor (SVF) and without phenol red (50 µL per well).

5.2.3) Remove the medium from the 96-well HEp-2 plate and add 50 µL of the drug suspension and 50 µL of the viral suspension (in triplicate). Perform a mock infection in parallel as a control.

NOTE: The drug dilution and viral suspension may be mixed before adding them on the cells, or they can be added sequentially.

5.2.4) Incubate the plate for 48 h at 37 °C and 5% CO₂.

5.2.5) Measure the fluorescence, using a spectrofluorometer as described in step 5.1.4. Use mock-infected HEp-2 cells as standards for fluorescence background levels.

6. Characterization of M2-1 localization in vivo with the RSV-M2-1-GFP recombinant virus

NOTE: Perform steps 6.1 and 6.2 in a sterile environment, using a class II safety cabinet.

6.1) Prepare a suspension of HEp-2 cells at 5×10^5 cells/mL in complete medium. Seed 1.5 mL of the cell suspension in a 35 mm Petri dish permeant to CO₂ and adapted for live imaging.

6.2) Perform the infection the day after seeding with the RSV-M2-1-GFP virus at MOI 1, as described in steps 3.3–3.5 (remove the medium, add 500 µL to 1 mL of inoculum, and incubate the sample at 37 °C while gently shaking for 2 h; remove the inoculum and add 1.5 mL of MEM with 2% FCS). Incubate the cells at 37 °C and 5% CO₂ for the desired time (IBs will start to appear from 10 h p.i.).

6.3) Preheat the incubation chamber of an inverted microscope equipped with 40x to 100x objectives at 37 °C, prior to placing the Petri dish containing the infected cells on the stage. Open the CO₂ supply and wait for focus stabilization.

6.4) Perform imaging with GFP-compatible filters, under a low excitation intensity and image frequency (from 1 to 0.1 image per minute) to minimize phototoxicity.

REPRESENTATIVE RESULTS:

In this work, we described a detailed protocol to produce recombinant RSV viruses expressing a fluorescent protein (**Figure 2**). In pRSV-GFP, the GFP gene was introduced between the P and M genes, as described for the Cherry gene in previously published work²¹. In the pRSV-M2-1-GFP, the M2 gene was left untouched and an additional gene coding for M2-1-GFP was inserted between SH and G genes¹². The first step, corresponding to the rescue of the virus in BSRT7/5 cells, is shown in **Figure 2A**. Small clusters of green fluorescent cells were visible 72 h posttransfection in wells corresponding to RSV-GFP and RSV-M2-1-GFP rescue. The fluorescent signal could be observed in both cytoplasm and nuclei in RSV-GFP-infected cells, corresponding to the expression of the free GFP. In contrast, in the RSV-M2-1-GFP rescue, small fluorescent cytoplasmic dots could be observed, corresponding to M2-1-GFP accumulation in IBs. Usually no CPE (syncytia, detached cells) is observed at this step. Conversely, during the second step, corresponding to virus amplification (first passage) on HEp-2 cells, the CPE was visible in infected cells at 72 h p.i. (**Figure 2B**). **Figure 3A,B** shows the strong CPE of the RSV infection, characterized by large syncytia, detached or not, and many floating cells. Syncytia and cells both exhibited bright green fluorescence. **Figure 3A** shows the evolution of the cytopathic effect between 24 and 72 h p.i. in cells infected with the RSV-M2-1-GFP virus. A few scattered fluorescent cells were visible at 24 h p.i. without a detectable CPE. Small syncytia (cluster of fluorescent cells) and a few detached cells/syncytia started to appear at 48 h p.i. Large fluorescent syncytia and floating cells were clearly visible at 72 h p.i.

Pictures of plaque titration assay and viral titers corresponding to the whole process of RSV production are shown in **Figure 5**. Performing the plaque assay on the negative control, transfected with only the expression plasmids of N, P, L, and M2-1, revealed no plaque at the lowest dilution. The titers obtained from the transfected cells were expected to be above 100 PFU/mL if the rescue was efficient, as shown in **Figure 5**. Then, the titers increased over the

passages, to reach 10^6 – 10^7 PFU/mL at passage 1 or 2. Note that the viral titers were similar between the two recombinant viruses.

Cells were transfected with siRNA targeting the mRNA of a viral protein (N) or of two cellular proteins (inosine-5'-monophosphate dehydrogenase [IMPDH] and glyceraldehyde 3'-phosphate dehydrogenase [GAPDH]). Cells were also transfected with nontargeting siRNA. **Figure 6** shows the monitoring of RSV multiplication using RSV-GFP virus on siRNA-treated cells. A strong GFP signal was observed (**Figure 6A**) and measured (**Figure 6B**) on control cells transfected with nontargeting siRNA or cells transfected with siRNA against GAPDH mRNA. In contrast, the GFP expression was decreased in infected cells expressing siRNA targeting N or IMPDH. Note that we verified that the GFP fluorescent signal in RSV-GFP-infected cells at 48h p.i. was correlated with the viral dose as previously demonstrated for a similar recombinant RSV expressing Cherry (RSV-Cherry)²¹. To assess drug efficiency on RSV multiplication, HEp-2 cells were infected with RSV-GFP for 48 h in the presence of various drug concentrations. We observed a strong decrease of the GFP signal, which reached the background noise (there was a signal observed on uninfected cells), in the presence of an increased drug concentration, as shown in **Figure 7**. The observed IC₅₀ for AZD4316 was about 4 nM, similar to the published EC₅₀ of about 2–40 nM against different HRSV strains²³. The analysis of the dynamics of IBs and IBAGs in living cells, thanks to RSV-M2-1-GFP, are shown in **Figure 8** and **Figure 9** (and **Movie 1** and **Movie 2**). IBs appear as mobile spherical structures able to fuse, forming a larger spherical inclusion. IBAGs are very dynamic. They undergo continuous assembly-disassembly cycles with the formation of small IBAGs that grow, fuse into large IBAGs, and then disappear.

FIGURE AND TABLE LEGENDS:

Table 1: The number of cells and inoculum volume to use in different flasks.

Figure 1: Schematic representation of the rescue and amplification steps. Transfection of the expression vector of N, P, L, M2-1, and RSV antigenomic RNA into BSRT5/7 cells (Rescue). Expression of the antigenomic RSV RNA and of the mRNA of N, P, L and M2-1, by the T7 RNA polymerase. The N, P, L, and M2-1 proteins replicate and transcribe the genomic RNA, initiating a viral multiplication cycle. New viral particles are produced and multiply, giving rise to the P0. The virus harvested from the rescue (P0) is then amplified on HEp-2 cells to produce a higher titer viral suspension (P1) (Amplification). This is then amplified to obtain viral stocks.

Figure 2: CPE and the pattern of fluorescence observed during the rescue of RSV-GFP and RSV-M2-1-GFP. (A) BSRT5/7 cells were transfected with the reverse genetics' vectors as indicated, and phase-contrast and fluorescence images were taken at 72 h posttransfection. The negative control (Neg Ctrl) corresponds to cells transfected with the expression vectors of N, P, L, and M2-1 without the reverse genetic vector. (B) HEp-2 cells were infected with the virus harvested from the transfected BSRT5/7 cells (the zero passage, 72 h posttransfection) and images were taken 72 h postinfection. The images shown are of representative fields; scale bar = 100 μ m. The boxed area encloses cells shown in magnification; scale bar = 20 μ m.

Figure 3: Evolution of the CPE and the fluorescence observed during the amplification of recombinant RSV. HEp-2 cells were infected at a MOI of 0.01 PFU/cells for 72 h with the first passage of (A) RSV-M2-1-GFP or (B) RSV-GFP. Phase-contrast and fluorescence images were taken at 24 h, 48 h, and 72 h postinfection. The images shown are of a representative field; scale bar = 100 μ m. The boxed area encloses cells shown in magnification; scale bar = 20 μ m.

Figure 4: Determination of RSV titer using the plaque assay. (A) Results of the plaque titration assay in a 12-well plate. The six wells infected with serial dilutions of one viral stock are shown. Dilutions are indicated in a base-10 logarithm. Cells infected with the three first dilutions are all detached. The plaque numbers observed with the 10^{-4} , 10^{-5} , and 10^{-6} dilutions are consistent. (B) Illustration of the plaque enumeration (yellow numbers). The green star indicates scratches on the cell layer.

Figure 5: Titration of a rescued and amplified virus. Plaque phenotypes of the RSV-GFP and RSV-M2-1-GFP at the different passages assayed on HEp-2 cells in a 12-well plate (the images show the entirety of the wells). The titers of the subsequent passages are shown in the table. Representative data are shown. Dilutions of the viral stocks are indicated in a base-10 logarithm.

Figure 6: Inhibition of the RSV-GFP expression by siRNA targeting RSV N or IMPDH. A549 cells were treated with control nontargeting siRNA (NT) (light blue bar) or siRNA targeting GAPDH (blue bar), RSV N (orange bar), or IMPDH2 (green bar) for 48 h and then infected with RSV-GFP at an MOI of 0.05 PFU/cell. The green fluorescence was read at 48 h postinfection. (A) Representative images of the RSV-GFP-infected cells at 48h p.i., treated with siRNA as seen on the pictures. The scale bar = 100 μ m. (B) Data are the mean \pm SD of two independent experiments performed in triplicate.

Figure 7: Inhibition of RSV-GFP multiplication by AZD4136 compound. HEp-2 cells in 96-well plates were infected with RSV-GFP at an MOI of 0.05 PFU/cell in the presence of serial dilutions of AZD4136 compound or control DMSO. The green fluorescence was read at 48 h p.i. Data are the mean \pm SD of two independent experiments performed in triplicate.

Figure 8: Analysis of the dynamics of IBs by tracking the fluorescent protein M2-1-GFP in HEp-2-infected cells by time lapse microscopy. At 18 h p.i., cells were imaged every 5 min for 5 h with a fluorescence microscope, in a chamber heated at 37 $^{\circ}$ C. The IBs are fluorescent (green) because they host M2-1-GFP, and nuclei are stained with Hoechst (blue). The white arrows indicate IBs undergoing fusion. The scale bar = 10 μ m.

Figure 9: Analysis of the dynamics of IBAGs in RSV-M2-1-GFP-infected cells by time lapse microscopy. At 18 h p.i., cells were imaged with a fluorescence microscope, in a chamber heated at 37 $^{\circ}$ C. The M2-1-GFP protein was visualized by green fluorescence. The white arrows indicate IBs undergoing a fusion of IBAGs. The scale bar = 5 μ m.

Movie 1: In vivo analysis of the dynamics of IBs in RSV-M2-1-GFP in HEp-2-infected cells. At 18 h p.i., cells were imaged every 5 min for 5 h with fluorescence microscope, in a chamber heated at 37 °C. The scale bar = 10 µm. The resulting movie shows 7 frames per second (fps).

Movie 2: In vivo analysis of the dynamics of IBAGs in RSV-M2-1-GFP-infected cells. At 18 h p.i., cells were imaged every 5 min for 3 h and 40 min with fluorescence microscope, in a chamber heated at 37 °C. The scale bar = 2 µm. The resulting movie shows 4 fps.

DISCUSSION:

Here we present a method of rescue of recombinant RSVs from five plasmids, and their amplification. The ability to manipulate the genome of viruses has revolutionized virology research to test mutations and express an additional gene or a tagged viral protein. The RSV we have described and used as an example in this article is a virus expressing a reporter gene, the RSV-GFP (unpublished), and expresses an M2-1 protein fused to a GFP tag¹². RSV rescue is challenging and requires practice. The transfection efficiency is critical, depending on a wise selection of the transfection reagent and a prior optimization of the transfection protocol. The use of cells expressing the bacteriophage T7 pol is mandatory because the viral cDNA is placed downstream the T7 pol promoter in most of the reverse genetic vector. An alternative is to express the T7 pol from a vaccinia helper virus. However, the use of cells stably expressing the T7 pol avoids the necessity of separating the two viruses and prevents possible interference of vaccinia with the rescue. It is important to perform the first passage of the reverse genetic (P0 to P1) without freezing the inoculum to ensure maximal rescue efficiency. This implies that the MOI is not controlled. However, at this step, the titers remain very low, resulting in a low MOI for the first passage. To obtain RSV stocks with high infectious titers (10^6 – 10^7 PFU/mL), it is important to wait for a strong CPE and to scrape the cells to gather the viral particles attached to the cells. In this study, titers did not increase after 96 h p.i. The fast cooling of the viral suspension is important to maintain high titers. Instead of by an immersion in alcohol precooled at -80 °C, this may also be achieved by immersion in a dry ice/ethanol mix or in liquid nitrogen. The addition of the conservation solution will ensure a longer stability of the virus suspension at -80 °C. The storage at -80 °C is critical, since the virus will quickly loss its infectivity when stored at -20 °C or in liquid nitrogen. We described the RSV amplification on HEp-2 cells, which is the most popular cell line to grow RSV, but it is also able to grow efficiently on numerous other cell lines in vitro. Note, however, that growth on Vero cells may result in an alteration in G expression²⁴.

We described a very simple protocol of plaque titration of RSV using a microcrystalline cellulose overlay. As for all titration assays, it is sensitive to contamination with high titer suspensions, requiring careful manipulation. Conventional RSV titration assays use agarose or carboxymethyl cellulose (CMC) overlays and require immunostaining and microscopic observations for titer determination. A protocol using immunodiffusion grade agarose has been described enabling the direct visualization of plaques without immunostaining²⁵. However, HEp-2 cells are very sensitive to a heated agar overlay, which makes this protocol difficult to use for multiple virus titrations (e.g., a too hot overlay destroys the cell layer); on the other hand, when it is not hot enough, it solidifies after the first plate distribution. The use of microcrystalline cellulose for a plaque assay

has been first described by Matrosovich et al. for an influenza A virus titration assay²⁶. Thanks to its low viscosity, the microcrystalline cellulose overlay is easy to dispense and to remove from plate wells, making it compatible with 96-well plates. It is, thus, particularly adaptable to serological studies and drug sensitivity analyses. Note that since microcrystalline cellulose does not need to be heated, the drugs may be easily incorporated in the overlay. It is, however, important that the plates remain perfectly still during the incubation; otherwise, large comet-shaped foci will form instead of round plaques. Plaque revelation using crystal violet is cheap and simple, but this solution is toxic and has to be properly disposed of. The recycling of the solution limits waste production. Moreover, this method is sensitive to cell monolayer damage that would appear as false white spots that are not viral plaques. One example is shown in **Figure 4** (green star). To prevent this bias, 1) cells have to be handled with caution to avoid scratching or flushing the cell monolayer, 2) aspiration and dispensing always have to be made at the same spot to circumscribe the damage to one known area, and 3) false white spots may be identified thanks to their shape (not spherical), their sharp edges, and their position. We first used Avicel RC 581, as previously published^{21,26}. However, the RC 581 is no longer available, and we successfully replaced it by RC 591. To adapt this assay to other cell/virus couples, the concentration of microcrystalline cellulose has to be determined depending on the virus and the cells. Too much microcrystalline cellulose may be toxic for cells and lead to small plaques, too little will lead to the diffusion of the virus in the medium.

We described two examples of use of the RSV-GFP virus to monitor RSV multiplication: in the presence of an antiviral drug or when silencing a cellular protein. We demonstrated that the GFP signal is correlated with viral multiplication. The method presented here allows the effortless evaluation of viral multiplication in real-time. It enables scientists to easily determine the IC50 of an antiviral drug, as shown in **Figure 7**. Importantly, this measurement is adaptable for use in medium or broadband, especially for the screening of chemical libraries. This reporter-expressing virus may also be useful to assess the effect on virus multiplication of a modulation of cellular protein expression. RNA interference is a biological process whereby a specific mRNA is degraded following its specific recognition by siRNA, thus reducing or, ideally, abolishing the expression of the corresponding protein²⁷. In the example given here, by monitoring GFP signal intensity in RSV-GFP-infected cells, we assessed the impact of the silencing of the viral nucleocapsid (N) mRNA or the host IMPDH mRNA on RSV multiplication. IMPDH2 is a purine biosynthetic enzyme that catalyzes a rate-limiting step towards the de novo biosynthesis of guanine nucleotides from IMP²⁸. It is thus a regulator of the intracellular guanine nucleotide pool. IMPDH inhibitors, such as ribavirin, exert inhibitory effects on RNA viruses, including RSV infection^{29–31}. As shown in **Figure 6**, the inhibition of IMPDH expression impairs viral multiplication as indicated by the reduction of the GFP signal, mimicking the effects of ribavirin on RSV growth. Likewise, the viral multiplication is almost abolished in the presence of siRNA targeting the viral N protein. This result was expected since siRNA targeting the N protein was expected to prevent viral nucleocapsid assembly and has proven to strongly impair viral replication³². The administration of these siRNA by nebulization inhibited the subsequent infection by RSV in healthy adults³³. The effect of N or IMPDH siRNA is specific since the inhibition of the GAPDH expression, chosen as a control gene, does not impair viral multiplication as compared to the nontargeting siRNA. Note

that no cell toxicity was detected in any conditions. Taken together, these results validate the strategy presented here, which could be up scaled to high-throughput screening using siRNA libraries or other knockout methods, such as CRISPR-Cas9 technology³⁴.

Recombinant viruses expressing a fusion fluorescent protein represent powerful tools to study viral proteins and viral structures dynamics. RSV expressing a fluorescent M2-1 protein enables the observation of the dynamics of IBs and of IBAGs. IBs, which may be considered as RSV viral factories, appear as spherical dynamic structures. They are able to fuse together to form larger spherical structures (**Figure 8** and **Movie 1**). These data suggest that RSV IBs are liquid organelles, similar to what has been described for rabies virus³⁵. IBAGs represent a subcompartment inside IBs, in which viral mRNA and M2-1 protein concentrate, such as the genomic RNA, the nucleocapsid, and the polymerase, are only present in the rest of the IBs¹². Video microscopy experiments reveal that IBAGs are very dynamic structures exhibiting liquid properties (**Figure 9** and **Movie 2**). They may be considered as liquid compartments resulting from liquid-liquid phase transition.

The possibility of genetically manipulating viruses remains a tool of choice to study both the mechanisms of their multiplication and their sensitivity to drugs. Reverse genetics might be considered now as part of the “classical” techniques of virology. However, it remains arduous for some viruses, like RSV. This is why this protocol describes in detail the steps to successfully rescue and amplify recombinant RSVs.

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DISCLOSURES:

The authors have nothing to disclose.

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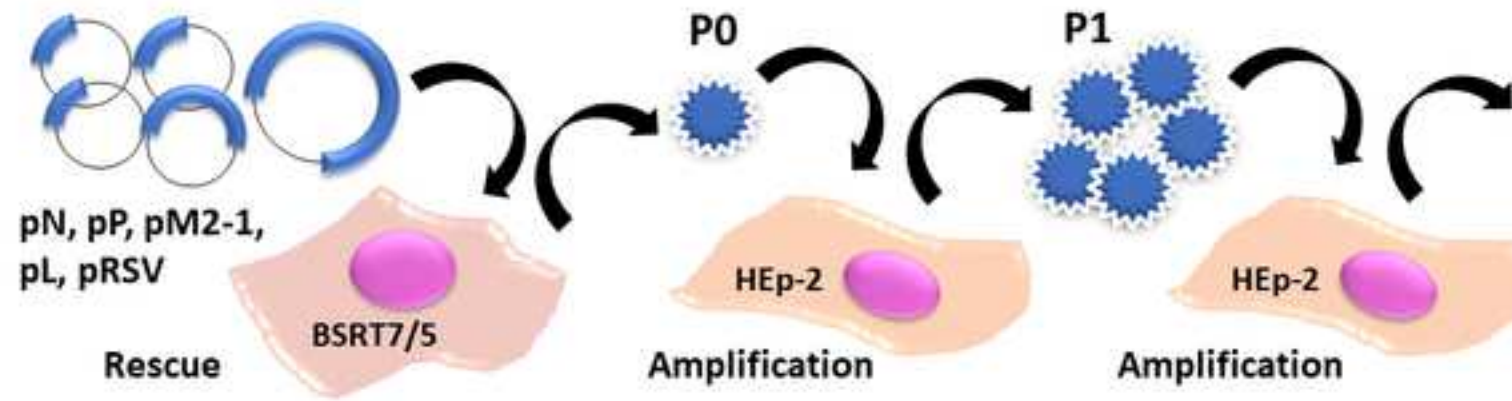
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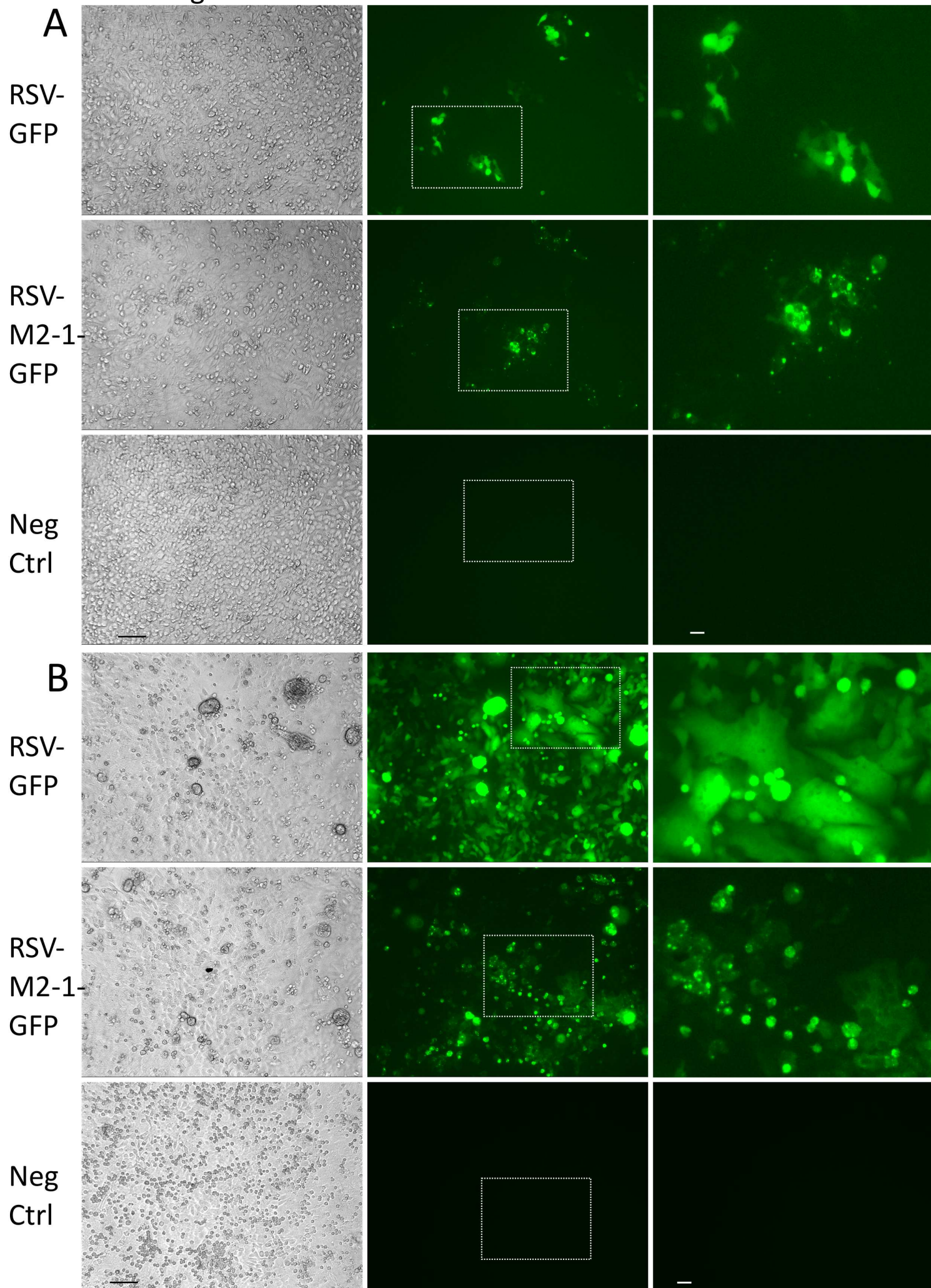


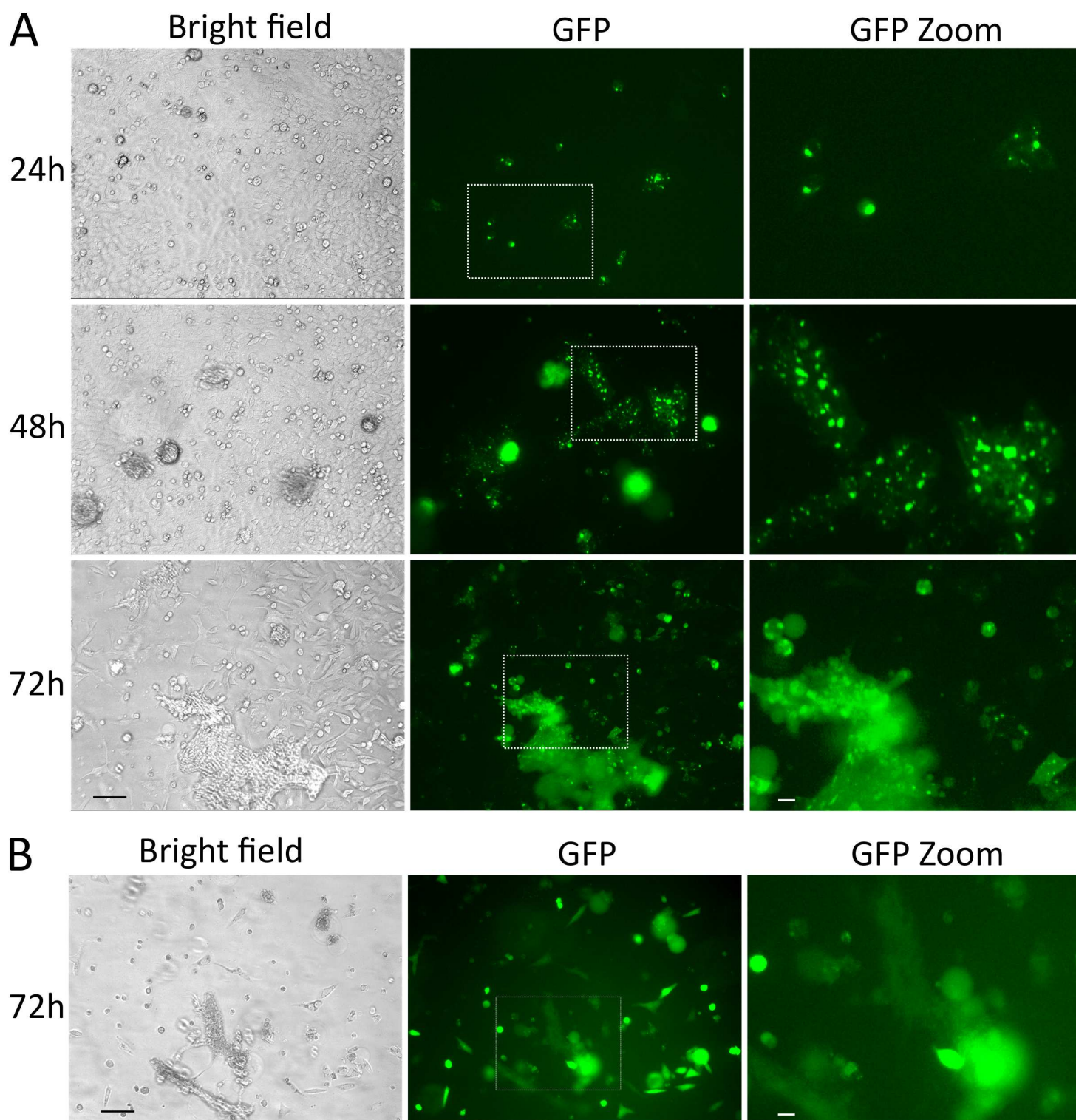
Bright field

GFP

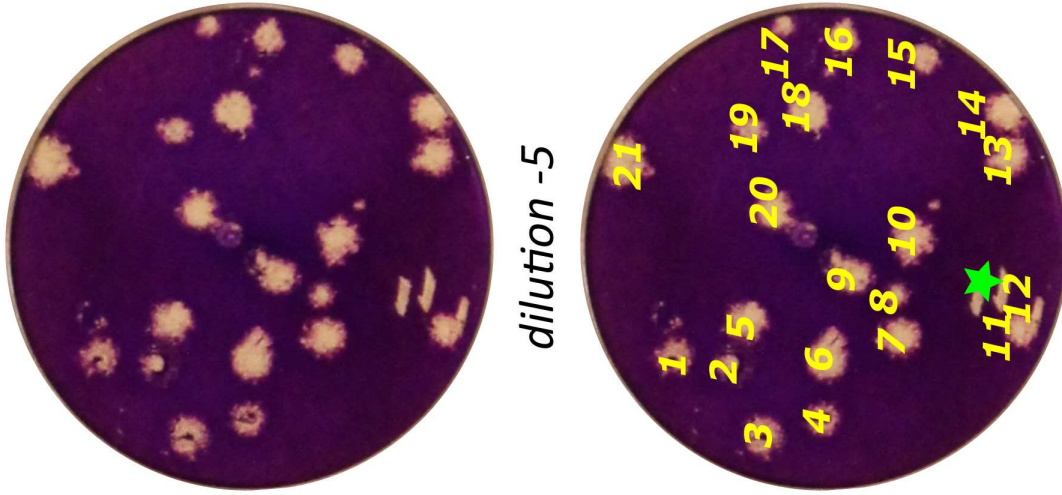
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GFP Zoom



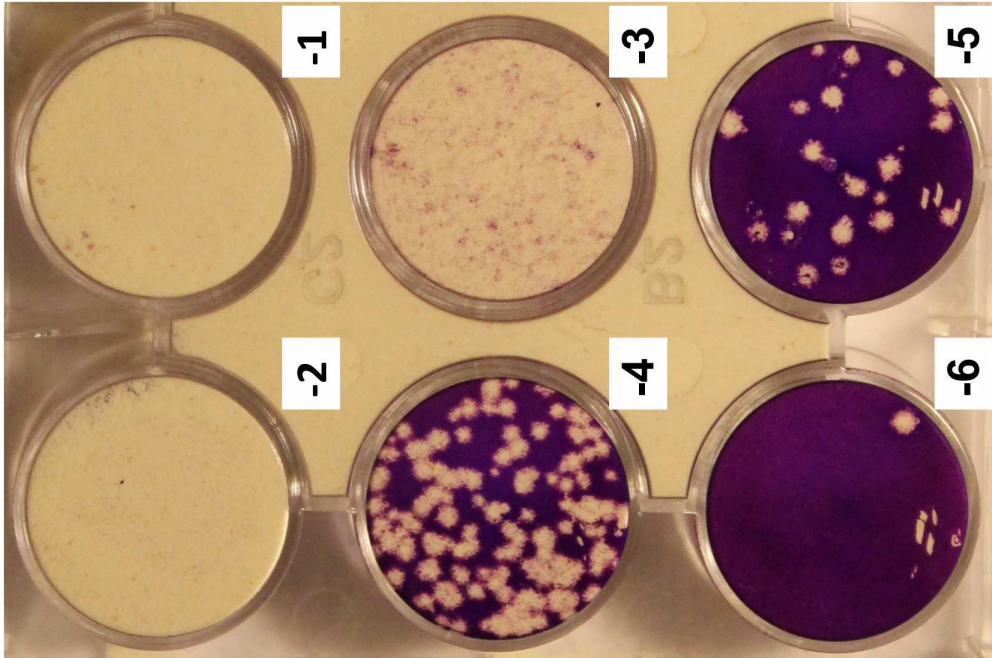


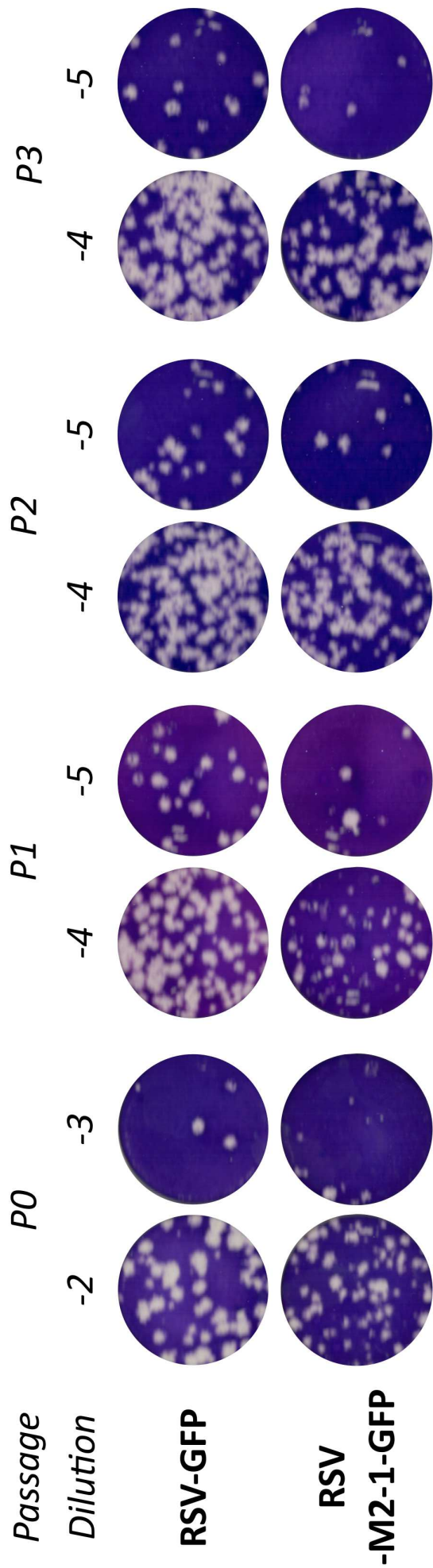
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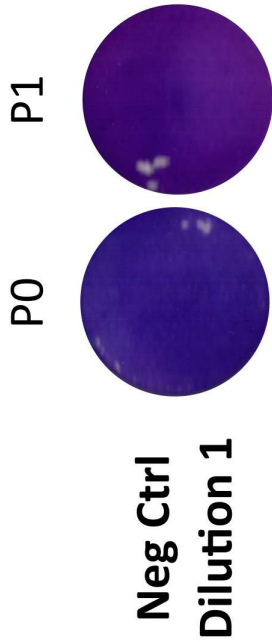
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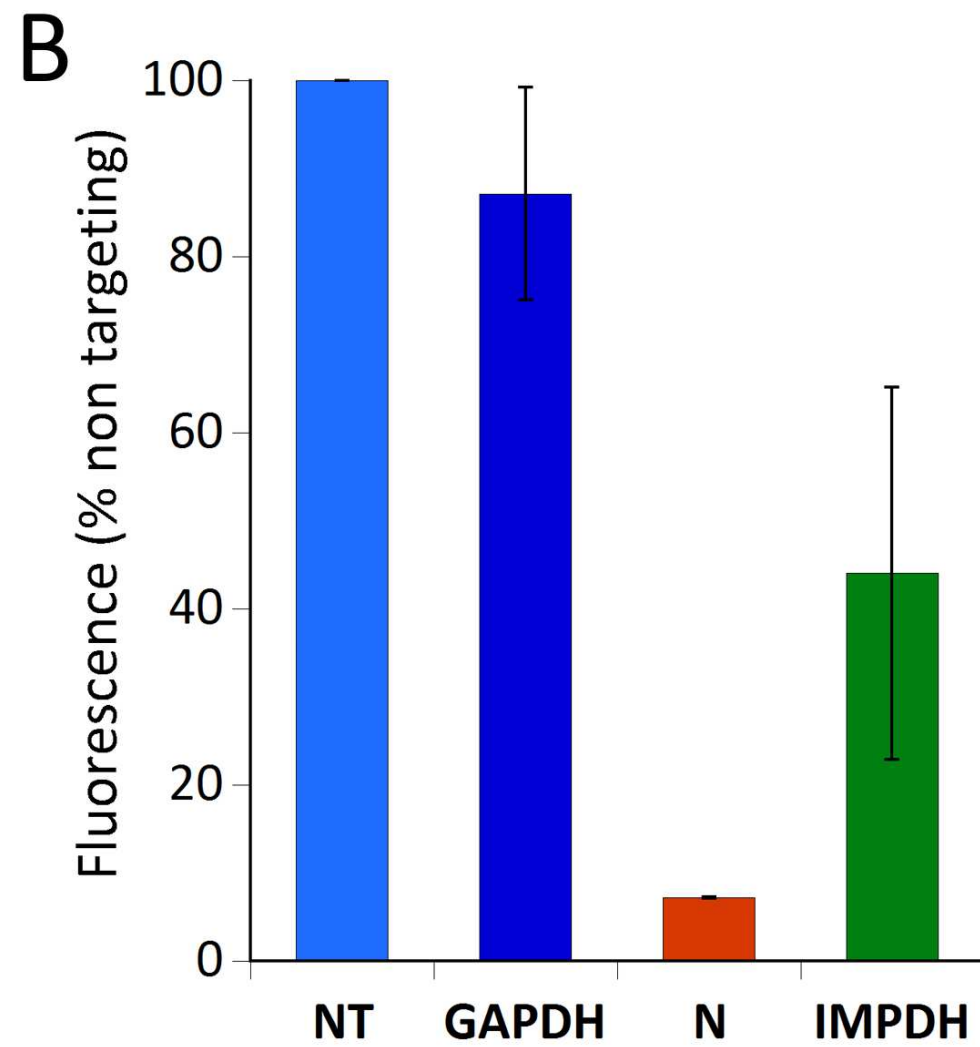
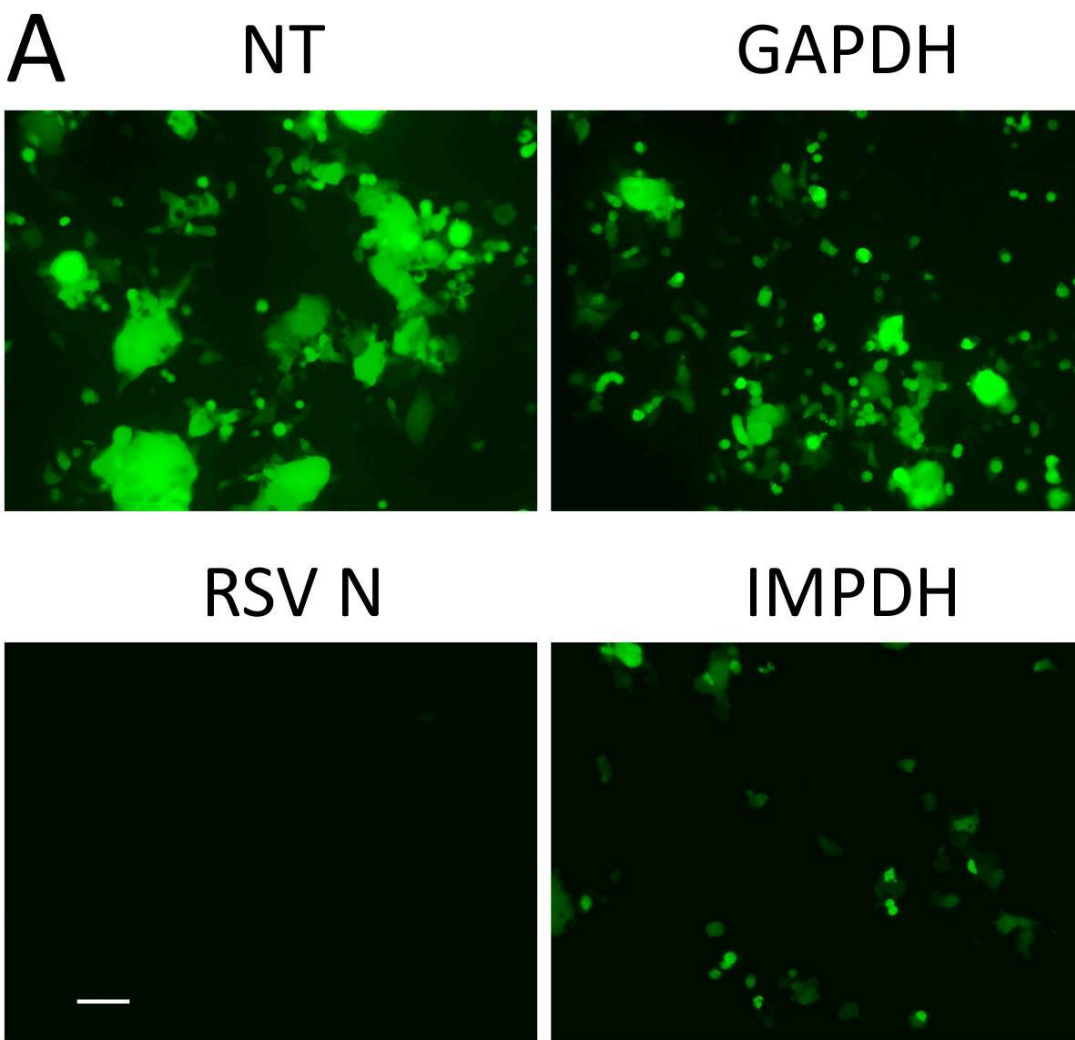
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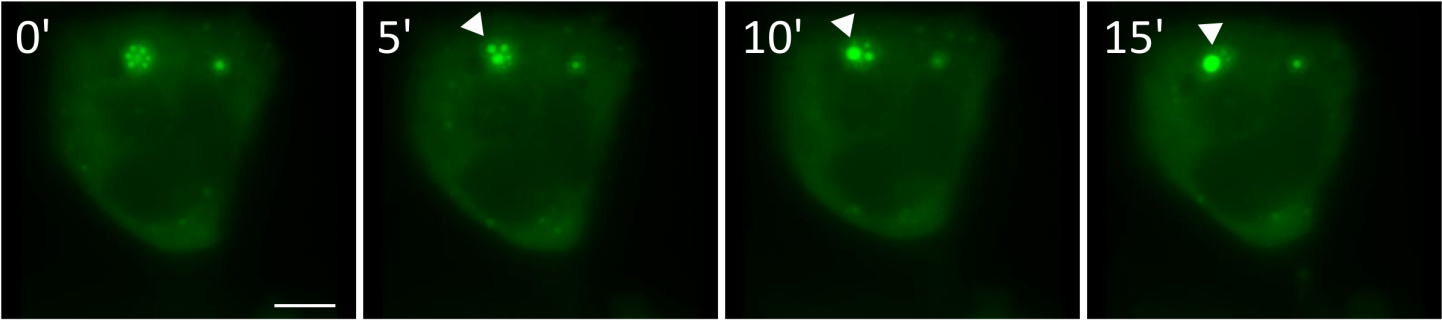


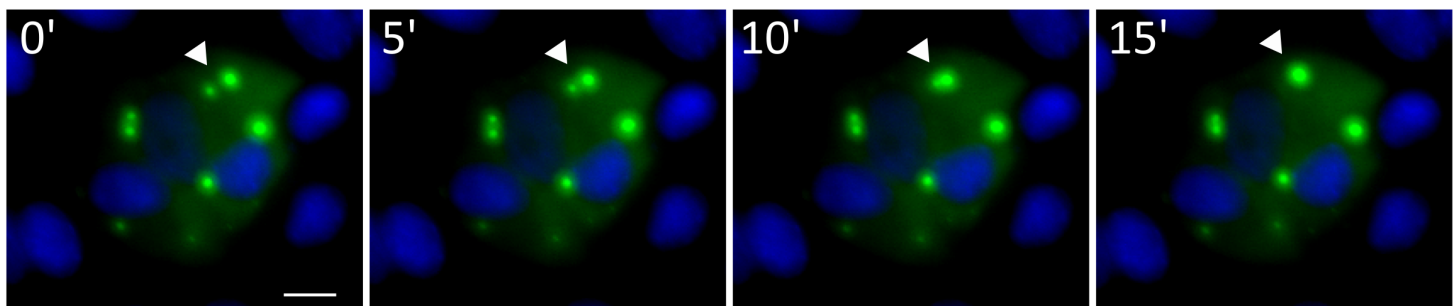


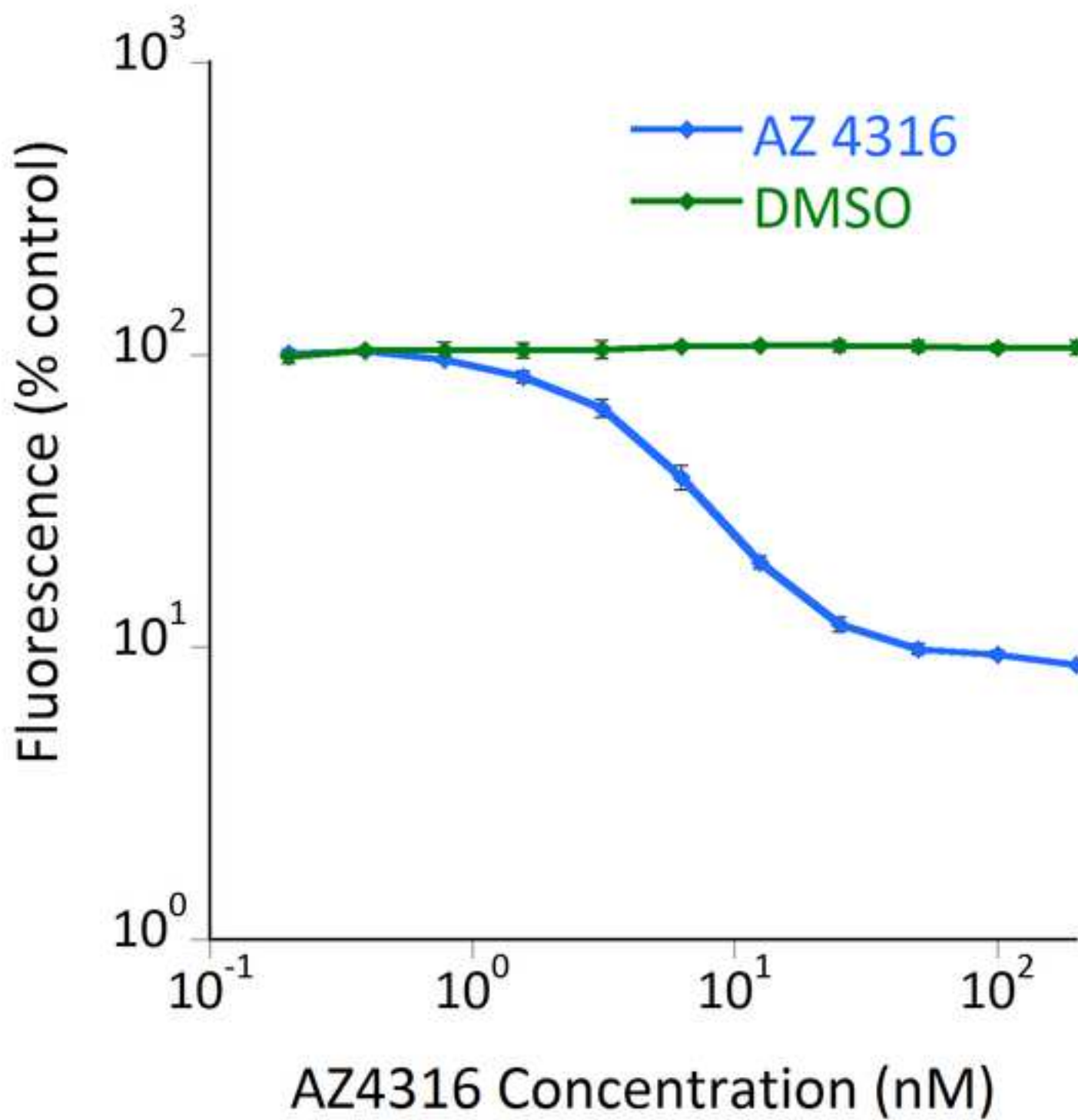
Virus	Titers (PFU/mL)			
	P0	P1	P2	P3
RSV-GFP	7.5×10^3	5.0×10^6	3.2×10^6	3.5×10^6
RSV-M2-1-GFP	1.5×10^4	1.0×10^6	2.2×10^6	1.2×10^6
Neg Ctrl	< 25	< 25	ND	ND

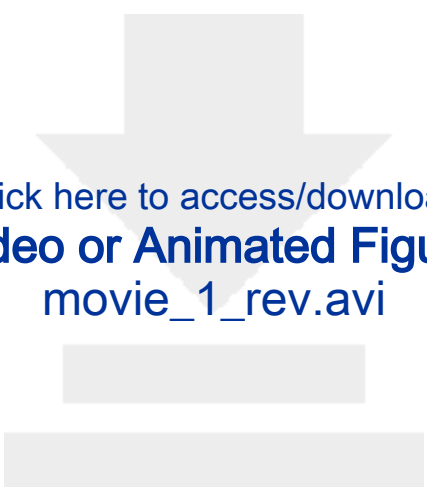













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Video or Animated Figure
movie_2_rev.avi

Plate or flask	HEp-2 cells to be seeded the day before	Medium volume (mL)	Virus Inoculum volume (mL)
150 cm ² flask	15 x 10 ⁶	30	5
75 cm ² flask	7.5 x 10 ⁶	15	3
25 cm ² flask	2.5 x 10 ⁶	5	1
6-well plate	1 x 10 ⁶	2	0.5

Name of Material/ Equipment	Company
35mm μ dish for live cell imaging	Ibidi
A549	ATCC
Avicel RC-591	FMC BioPolymer
BSRT7/5	
Crystal violet solution	Sigma
Fluorescence microscope for observations	Olympus
Fluorescence microscope for videomicroscopy	Olympus
HEp-2	ATCC
HEPES $\geq 99.5\%$	Sigma
L-Glutamine (200 mM)	ThermoFisher Scientific
LIPOFECTAMINE 2000 REAGENT	ThermoFisher Scientific
MEM (10X), no glutamine	ThermoFisher Scientific
MEM, GlutaMAX Supplement	ThermoFisher Scientific
MgSO4 ReagentPlus, $\geq 99.5\%$	Sigma
Opti-MEM I Reduced Serum Medium	ThermoFisher Scientific
Paraformaldehyde Aqueous Solution, 32%, EM Grade	Electron Microscopy Sciences
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher Scientific
Plasmids	
See Saw Rocker	VWR
Si RNA GAPDH	Dharmacon
Si RNA IMPDH2	Dharmacon
Si RNA RSV N	Dharmacon
SiRNA NT	Dharmacon
SiRNA transfection reagent	Dharmacon

Sodium Bicarbonate 7.5% solution	ThermoFisher Scientific
Spectrofluorometer	Tecan

Catalog Number
81156
ATCC CCL-185
Avicel RC-591
not commercially available
HT90132
IX73 Olympus microscope
ScanR Olympus microscope
ATCC CCL-23
H3375
25030024
11668019
11430030
41090-028
M7506
51985-026
15714
15140122
not commercially available
444-0341
ON-TARGETplus siRNA D-001810-10-05
ON-TARGETplus siRNA IMPDH2 Pool- Human L-004330-00-0005
ON-TARGETplus custom siRNA
ON-TARGETplus Non-targeting Pool
DharmaFECT 1 Ref: T-2001-03

25080094
Tecan infinite M200PRO

Comments/Description
Technical and other information on Avicels is available at http://www.fmcbiopolymer.com . Store at room temperature.
Protocol in step 4 is optimized for this reagent.
See ref 22. Buchholz et al, 1999
Protocol in step 2.3. is optimized for this reagent.
see ref 21. Rameix-Welti <i>et al</i> , 2014
SMARTpool and 3 of 4 individual siRNAs designed by Dharmacon.
SMARTpool of 4 individual siRNAs designed by Dharmacon. Individual references and sequences J-004330-06: GGAAAGUUGCCCAUUGUAA; J-004330-07: GCACGGCGCUUUGGUGUUC; J-004330-08: AAGGGUCAAUCCACAAAUU; J-004330-09: GGUAUGGGUUCUCUCGAUG;
UUCAGAAGAACUAGAGGCUAU and UUUCAUAAAUUCACUGGGUUA
Protocol in steps 5.1.and 5.1.2 are optimized for this reagent.



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Title of Article: *Generation, amplification and titration of recombinant respiratory syncytial viruses to monitor viral multiplication and viral factories dynamics*

Author(s): *C Bouillier, V Kienhues, D Sitterlin, S Bloquet-Lange, A Desquesnes, JF Eleaume, E Gault, MA Kameir Widi*

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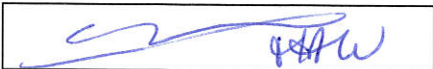
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Paris, November the 6th 2018

Revised manuscript JoVE59218

Dear Editor,

We are extremely grateful for the constructive comments and criticisms from the two expert reviewers regarding our recent submission to *Jove* by Bouillier et al.

All the editorials comments have been addressed. We have responded to all the comment of the reviewers, and we have brought additional data and clarifications. We describe the modifications of text and figures in detail in our point-by-point response below and also highlighted the modifications of the text in the revised version of the manuscript (change tracking versions of the manuscript).

The figures original format was .svg (Inkscape software). I converted them in .eps but I had no software to check them. Let me know if you need the original files or if the conversion has led to poor results.

We strongly believe that the present protocols have been improved and will be of high interest to the readership of *Jove*.

We look forward to your response.

Yours sincerely,

Dr Rameix-Welti Marie-Anne

A handwritten signature in blue ink, reading 'MA Rameix-Welti', with a long horizontal flourish underneath.