

Submission ID #: 69628

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Title: Procedures for the Identification of SARS-CoV-2 Entry Inhibitors as Potential Antivirals using MLV-Based Pseudoviruses

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Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

✓ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No.**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**

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Current Protocol Length

Number of Steps: 25

Number of Shots: 57

Interviews

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. **Ricardo Leonart:** Our research focuses on identifying inhibitors that block SARS-CoV-2 entry using pseudovirus systems.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Ricardo Leonart:** Studying SARS-CoV-2 entry often requires high biosafety containment laboratories, typically BSL-3; this protocol enables safe antiviral screening using pseudoviruses in standard BSL-2 laboratories.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Andreina Ayala:** This protocol enables the production of high-titer pseudoviruses through optimized conditions. This allows researchers to measure pseudoviral entry and identify compounds that inhibit SARS-CoV-2 spike-mediated infection.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Andreina Ayala:** High transfection efficiency is critical to generate high-titer pseudoviruses and obtain reliable infection signals in downstream assays.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Andreina Ayala:** This system supports high-throughput antiviral screening and evaluation of entry inhibitors against emerging SARS-CoV-2 variants, and can be readily adapted to generate pseudoviruses displaying envelope proteins from other viruses.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. High Efficiency Transfection of HEK293T-Derived Cells Using Branched Polyethyleneimine (PEI)

Demonstrator: Andreina Ayala

- 2.1. To begin, place a 6-well tissue culture plate in the biosafety cabinet [1] and seed 7.5×10^5 HEK293 (*H-E-K-two ninety-three*)-derived cells per well in 2 milliliters of complete medium [2]. Incubate the plate at 37 degrees Celsius with 5 percent carbon dioxide overnight [3]. The following day, confirm that the cells have reached approximately 50 to 60 percent confluency [4].
 - 2.1.1. WIDE: Talent placing a 6-well plate in the biosafety hood.
 - 2.1.2. Talent seeding HEK293-derived cells into a 6-well tissue culture plate with complete medium.
 - 2.1.3. Talent placing the 6-well plate into a carbon dioxide incubator.
 - 2.1.4. Talent viewing the cells under a microscope.
- 2.2. On the next day, prepare the polyethyleneimine or PEI (*P-E-I*) solution by adding 5 microliters of 1 milligram per milliliter PEI stock to 50 microliters of reduced serum medium in a 1.5 milliliter microcentrifuge tube [1]. In a separate 1.5-milliliter microcentrifuge tube, prepare the DNA solution by adding 2.5 micrograms of GFP (*G-F-P*)-expressing plasmid to 50 microliters of the same medium [2].
 - 2.2.1. Talent pipetting polyethyleneimine stock and reduced serum medium into a 1.5 milliliter microcentrifuge tube.
 - 2.2.2. Talent pipetting the plasmid and reduced serum medium into a separate 1.5 milliliter microcentrifuge tube.
- 2.3. Mix each tube briefly by vortexing, then spin down for 5 seconds and incubate at room temperature [1].
 - 2.3.1. Talent vortexing each tube and immediately spinning them down.
- 2.4. During the 10-minute incubation period, carefully remove the medium from each well of the 6-well plate without disrupting the cell monolayer [1] and add 1 milliliter of complete transfection medium devoid of antibiotics to each well [2].

- 2.4.1. Talent aspirating medium from a well while maintaining the cell monolayer intact.
- 2.4.2. Talent gently adding complete transfection medium to each well of the plate.
- 2.5. After the incubation, add 50 microliters of the DNA solution to the tube containing PEI solution [1]. Mix quickly by pipetting 6 times and incubate at room temperature for exactly 3 minutes [2]. Then, quickly add the PEI-DNA (*P-E-I-D-N-A*) complex dropwise to the corresponding well [3].
 - 2.5.1. Talent transferring the deoxyribonucleic acid solution into the polyethyleneimine solution tube.
 - 2.5.2. Talent pipetting the mixture up and down 6 times to mix.
 - 2.5.3. Talent adding the transfection complex dropwise to the cells in a well.
- 2.6. Now, seal the borders of the plate with parafilm [1] and centrifuge at 1,000 *g* for 30 minutes at room temperature without brake using a swing rotor and a plate bucket [2].
 - 2.6.1. Talent sealing the edges of the 6-well plate with parafilm.
 - 2.6.2. Talent placing the sealed plate into the centrifuge.
- 2.7. After centrifugation, gently remove the parafilm [1] and incubate the plate at 37 degrees Celsius with 5 percent carbon dioxide for 3 hours [2].
 - 2.7.1. Talent removing parafilm from the 6-well plate.
 - 2.7.2. Talent placing the plate into a carbon dioxide incubator.
- 2.8. Then, gently replace the transfection medium in each well with 2 milliliters of pre-warmed complete DMEM medium [1].
 - 2.8.1. Talent slowly adding pre-warmed DMEM-C to each well.
- 2.9. Incubate the transfected cells at 37 degrees Celsius with 5 percent carbon dioxide for 48 hours [1] and assess the transfection efficiency by evaluating GFP expression using fluorescence microscopy [2].
 - 2.9.1. Talent placing the 6-well plate into the carbon dioxide incubator for the final incubation.

Added shot: LAB MEDIA: Figure 1

3. Generation of SARS-CoV-2 Spike- and VSV-G Pseudotyped Pseudoviral Particles

3.1. Seed HEK293T-derived cells into two 6-well plates as demonstrated earlier [1]. Designate one plate for SARS-CoV-2 (*sars-kov-2*) spike pseudovirus generation and the other for VSV-G pseudoviruses [2-TXT].

3.1.1. Talent adding HEK293T-derived cells into two 6-well plates.

3.1.2. Talent labeling one plate as spike pseudoviruses and the other as vesicular stomatitis virus glycoprotein pseudoviruses. **TXT: SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2; VSV-G: Vesicular Stomatitis Virus Glycoprotein**

3.2. Prepare the transfection mix and PEI-DNA complexes [1] on the day of the experiment and incubate for 10 minutes at room temperature [2]. During the incubation, carefully remove the medium from each well of the 6-well plates without disrupting the cell monolayer [3]. Slowly add 1 milliliter of pre-warmed complete transfection medium without antibiotics to each well, allowing the medium to run down the wall of the well [4]. Add the prepared transfection complexes to the cells to transfect them as demonstrated previously [5].

Added shot: *Reuse 2.2.2.*

Added shot: LAB MEDIA: Table 1

3.2.1. Talent aspirating medium from a well while maintaining the cell monolayer intact.

3.2.2. Talent slowly adding pre-warmed DMEM without antibiotics along the wall of the well.

Added shot: *Reuse 2.5.3.*

3.3. After 48 hours of incubation post-transfection, collect the supernatants from each well into a 50-milliliter sterile conical centrifuge tube [1] and centrifuge at 290 *g* for 7 minutes at 4 degrees Celsius [2]. Collect the cell-free supernatant and filter it through a sterile 0.45-micrometer polyvinylidene fluoride top bottle filter unit [3]. Prepare 1 milliliter aliquots of the filtered supernatant [4] and store them at minus 80 degrees Celsius [5].

3.3.1. Talent collecting supernatant from the wells into a sterile container.

3.3.2. Talent placing the 50 milliliter conical centrifuge tubes into a refrigerated

centrifuge.

3.3.3. Talent filtering the clarified supernatant through a 0.45 micrometer polyvinylidene fluoride top bottle filter unit.

3.3.4. Talent aliquoting 1 milliliter volumes into sterile tubes.

Added shot: Talent placing them into a minus 80 degrees Celsius freezer.

4. Titration of SARS-CoV-2 and VSV-G Pseudotyped Particles

4.1. Seed 2.5×10^4 HSI (*H-S-I*) cells, a highly SARS-CoV-2-susceptible cell line, in 50 microliters of complete DMEM per well in a 96-well tissue culture plate [1]. Prepare and label triplicate wells for each condition to be tested, including spike-less pseudovirus infection and non-infected controls [2]. Incubate the plate at 37 degrees Celsius with 5 percent carbon dioxide overnight [3].

4.1.1. Talent seeding HSI cells into a 96-well tissue culture plate with complete DMEM.

4.1.2. Talent labeling wells for different pseudovirus amounts, spike-less controls, and non-infected controls.

4.1.3. Talent placing the 96-well plate into a carbon dioxide incubator.

4.2. On the day of transduction, thaw one aliquot of each pseudovirus batch on ice and gently mix by inversion, including one aliquot of the spike-less control [1]. Add the required volumes of pseudovirus preparation directly to the designated wells [2]. Adjust the volume of each well to 150 microliters with complete DMEM [3].

4.2.1. Talent thawing pseudovirus aliquots on ice and gently inverting the tubes to mix.

4.2.2. Talent adding specified volumes of pseudovirus to the appropriate wells of the 96-well plate.

4.2.3. Talent adding complete DMEM to each well to reach a final volume of 150 microliters.

4.3. Now, seal the plate with parafilm [1] and centrifuge at 2,500 *g* for 45 minutes at room temperature without brake [2].

4.3.1. Talent sealing the 96-well plate with parafilm.

4.3.2. Talent placing the sealed plate into the centrifuge.

4.4. Remove the parafilm seal [1] and incubate the plate for 72 hours at 37 degrees Celsius with 5 percent carbon dioxide [2].

4.4.1. Talent removing parafilm from the plate.

4.4.2. Talent placing the plate into a carbon dioxide incubator.

4.5. After incubation, prepare the luciferase assay reagents according to the manufacturer's instructions [1]. Thaw the luciferase substrate and the 5x luciferase assay lysis buffer at room temperature for 10 to 15 minutes [2]. Dilute the 5x luciferase assay lysis buffer to 1x with sterile water [3].

4.5.1. Talent arranging luciferase assay reagents on the bench.

4.5.2. Talent placing luciferase substrate and 5 times luciferase assay lysis buffer at room temperature.

4.5.3. Talent diluting the 5 times lysis buffer with sterile water.

4.6. Carefully, aspirate and discard the supernatant [1]. Next, add 30 microliters of 1x lysis buffer to each well [2]. Place the plate on a plate shaker and shake for 10 minutes at 150 revolutions per minute at room temperature [3]. Verify complete cell lysis under an inverted microscope to confirm that cells are fully lysed and not distinguishable [4]. Then, transfer the lysates to an opaque white 96-well plate [5].

4.6.1. Talent slowly aspirates and discards the supernatant.

4.6.2. Talent adding 30 microliters of 1X lysis buffer to each well with a multichannel pipette.

4.6.3. Talent placing the plate on a plate shaker for incubation.

4.6.4. Talent placing the sample under an inverted microscope.

4.6.5. Talent transferring lysates into an opaque white 96-well plate.

4.7. Add 50 microliters of luciferase substrate to each well [1]. Mix briefly by gently moving the plate in a circular motion [2] and place the plate into the luminometer and measure luminescence [3].

4.7.1. Talent adding luciferase substrate to the wells of the opaque white plate.

4.7.2. Talent gently swirling the plate to mix contents.

4.7.3. Talent placing the plate into a luminometer for luminescence measurement.

5. Screening of Compounds for Pseudoviral Entry Inhibition Using Spike-Pseudoviruses (PVs)

5.1. Seed 2.5×10^4 HSI cells in 50 microliters of complete DMEM per well in a 96-well plate

as previously described [1]. Incubate overnight at 37 degrees Celsius with 5 percent carbon dioxide [2].

5.1.1. Talent seeding HSI cells into a 96-well plate with complete DMEM.

5.1.2. Talent placing the 96-well plate into a carbon dioxide incubator.

5.2. The next day, prepare 1 to 3 serial dilutions of the compounds to be tested, such as arbidol, in complete DMEM containing 0.5 percent dimethyl sulfoxide [1-TXT]. Next, transfer 50 microliters of each dilution to the plate containing the cells [2].

5.2.1. Talent performing 1 to 3 serial dilutions of compounds in DMEM with 0.5 percent dimethyl sulfoxide. **TXT: Final compound concentrations: 50, 16.6, 5.5, 1.8, 0.6, 0.2, and 0.06 μ M**

5.2.2. Talent ~~preparing compound dilutions in a separate 96-well plate and~~ transferring 50 microliters to the cell plate.

5.3. Using a multichannel pipette, gently aspirate the medium from each well [1]. Add 50 microliters of the corresponding compound dilutions to each well, while adding complete DMEM alone to the column 12 control wells [2]. Incubate for 1 hour at 37 degrees Celsius with 5 percent carbon dioxide [3].

5.3.1. Talent aspirating medium from the 96-well plate with a multichannel pipette.

5.3.2. Talent adding compound dilutions to the corresponding wells.

5.3.3. Talent placing the plate into the incubator.

5.4. Without removing the compounds, directly add 50 microliters of SARS-CoV-2 spike pseudovirus to each well, diluted in complete medium containing the same compound concentrations [1]. Seal the plate with parafilm [2].

5.4.1. Talent adding pseudotyped severe acute respiratory syndrome coronavirus 2 pseudovirus to each well.

5.4.2. Talent sealing the 96-well plate with parafilm.

5.5. Centrifuge at 2,500 *g* for 45 minutes at room temperature without brake [1]. After removing the parafilm, incubate the plate at 37 degrees Celsius with 5% carbon dioxide for 72 hours [2]. Finally, quantify infectivity using a luciferase assay as demonstrated previously [3].

5.5.1. Talent placing the sealed plate into the centrifuge.

5.5.2. Talent placing the plate without parafilm in CO₂ incubator.

5.5.3. Talent handling the 96-well plate and placing the plate in a reader.

Results

6. Results

6.1. Transfection of HEK293T-derived cells with branched PEI resulted in high transfection efficiency, resulting in roughly 70% GFP-positive cells 48 hours after transfection [1].

6.1.1. LAB MEDIA: Figure 1.

6.2. Infection of susceptible HSI cells with decreasing amounts of SARS-CoV-2-spike pseudoviruses or VSV-G pseudoviruses showed a dose-dependent increase in luminescence with increasing pseudovirus input [1].

6.2.1. LAB MEDIA: Figure 2. *Video editor: Highlight the series of bars under “Added spike-PVs (μL)”*

6.3. Spike-less pseudoviruses and uninfected cells showed very low levels of luminescence [1].

6.3.1. LAB MEDIA: Figure 2. *Video editor: Highlight the bars labeled “NE” and “NI”.*

6.4. The compound arbidol was tested for its ability to inhibit pseudoviral entry [1] and it produced clear dose-response inhibition of luciferase activity generated by spike pseudoviruses [2], with less inhibition observed for VSV-G pseudoviruses [3].

6.4.1. LAB MEDIA: Figure 3B

6.4.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the bars under “Added Arbidol (μM) to spike-PVs” showing decreasing luminescence with increasing concentration.*

6.4.3. LAB MEDIA: Figure 3B. *Video editor: Highlight the bars under “Added Arbidol (μM) to VSV-G-PVs” showing comparatively higher bars than those for spike-PVs at similar concentrations.*

6.5. The assay showed a Z prime-factor of 0.54, indicating suitability for screening purposes [1].

6.5.1. LAB MEDIA: Figure 4. *Video editor: Highlight the text “Z’-factor: 0.54”*

6.6. Treatment with arbidol at concentrations starting at 30 micromolar did not significantly

affect cell viability compared to [1] the cells treated with 0.5% dimethyl sulfoxide vehicle alone [2].

6.6.1. LAB MEDIA: Figure 5. *Video editor: Highlight the bars corresponding to the different “ARB concentrations”*

6.6.2. LAB MEDIA: Figure 5. *Video editor: Highlight the bar labeled “NTD”*

Pronunciation guide:

1. HEK293T
Pronunciation link: <https://www.merriam-webster.com/dictionary/HEK>
IPA: /ˌeɪtʃ.iː.keɪ.tuːˈnaɪnti.θriː/
Phonetic Spelling: aych-ee-kay-too-nine-tee-three
2. Transfection
Pronunciation link: <https://www.merriam-webster.com/dictionary/transfection>
IPA: /trænˈsfekʃən/
Phonetic Spelling: trans-fek-shuhn
3. Polyethyleneimine
Pronunciation link: <https://www.howtopronounce.com/polyethyleneimine>
IPA: /ˌpɑːli.ɛθəˈliːn.iˌmaɪn/
Phonetic Spelling: paa-lee-eth-uh-leen-ih-mine
4. Microcentrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>
IPA: /ˌmaɪkroʊˈsentrɪˌfjuːdʒ/
Phonetic Spelling: my-kroh-sen-truh-fyooj
5. Plasmid
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /ˈplæzmɪd/
Phonetic Spelling: plaz-mid
6. Fluorescent
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
IPA: /flɒˈresənt/
Phonetic Spelling: flor-ess-uhnt
7. Vortexing
Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
IPA: /ˈvɔːrˌtɛks/
Phonetic Spelling: vor-tek

8. Confluency
Pronunciation link: <https://www.howtopronounce.com/confluency>
IPA: /kən'flu:ənsi/
Phonetic Spelling: kuhn·floo·uhn·see
9. Monolayer
Pronunciation link: <https://www.merriam-webster.com/dictionary/monolayer>
IPA: /'mɑ:nos,leɪər/
Phonetic Spelling: mon·oh·lay·ur
10. Centrifugation
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifugation>
IPA: /,sentrəfə'geɪʃən/
Phonetic Spelling: sen·truh·fuh·gay·shuhn
11. Rotor
Pronunciation link: <https://www.merriam-webster.com/dictionary/rotor>
IPA: /'roʊtər/
Phonetic Spelling: roh·tur
12. Pseudotyped
Pronunciation link: <https://www.howtopronounce.com/pseudotyped>
IPA: /'su:dɒstaɪpt/
Phonetic Spelling: soo·doh·typed
13. Pseudoviral
Pronunciation link: <https://www.howtopronounce.com/pseudoviral>
IPA: /,su:dɒs'vaɪrəl/
Phonetic Spelling: soo·doh·vy·ruhl
14. SARS-CoV-2
Pronunciation link: <https://www.merriam-webster.com/medical/SARS-CoV-2>
IPA: /,sɑrʒ,kɒv,vi:'tu:/
Phonetic Spelling: sarz·koh·vee·too
15. Vesicular
Pronunciation link: <https://www.merriam-webster.com/dictionary/vesicular>
IPA: /və'sɪkjələr/
Phonetic Spelling: vuh·sik·yuh·ler
16. Stomatitis
Pronunciation link: <https://www.merriam-webster.com/dictionary/stomatitis>
IPA: /,stɒmə'taɪtəs/
Phonetic Spelling: stoh·muh·tie·tuhs
17. Glycoprotein
Pronunciation link: <https://www.merriam-webster.com/dictionary/glycoprotein>
IPA: /,glʌɪkəʊ'prəʊti:n/

Phonetic Spelling: gly·koh·proh·teen

18. Supernatant

Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>

IPA: /ˌsuːpərˈneɪtənt/

Phonetic Spelling: soo·per·nay·tuhnt

19. Polyvinylidene

Pronunciation link: <https://www.howtopronounce.com/polyvinylidene>

IPA: /ˌpɑːliˌvaɪnəˈliːdiːn/

Phonetic Spelling: paa·lee·vy·nuh·lee·deen

20. Luciferase

Pronunciation link: <https://www.merriam-webster.com/dictionary/luciferase>

IPA: /luːˈsɪfəˌreɪs/

Phonetic Spelling: loo·sif·uh·rays

21. Luminescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/luminescence>

IPA: /ˌluːməˈnɛsəns/

Phonetic Spelling: loo·muh·ness·uhns

22. Luminometer

Pronunciation link: <https://www.howtopronounce.com/luminometer>

IPA: /ˌluːməˈnɑːmɛtər/

Phonetic Spelling: loo·muh·nom·uh·tur

23. Dimethyl Sulfoxide

Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl%20sulfoxide>

IPA: /daɪˈmɛθəl sʌlˈfɑːksaɪd/

Phonetic Spelling: dye·meth·uhl sul·fok·side

24. Micromolar

Pronunciation link: <https://www.merriam-webster.com/medical/micromolar>

IPA: /ˌmaɪkroʊˈmoʊlər/

Phonetic Spelling: my·kroh·moh·ler

25. Infectivity

Pronunciation link: <https://www.merriam-webster.com/dictionary/infectivity>

IPA: /ˌɪnfɛkˈtɪvəti/

Phonetic Spelling: in·fek·tiv·uh·tee